3 Biotech (2019) 9:446
https://doi.org/10.1007/s13205-019-1981-4

ORIGINAL ARTICLE

Enhancement of glycerol production by UV-mutagenesis of the marine yeast *Wickerhamomyces anomalus* HH16: kinetics and optimization of the fermentation process

Heba Hawary[1] · Abdel-Hamied M. Rasmey[1] · Akram A. Aboseidah[1] · El-Shahat El-Morsi[1] · Mohamed Hafez[1]

Received: 23 August 2019 / Accepted: 29 October 2019 / Published online: 11 November 2019

Abstract
The current study aims to enhance glycerol production using UV-mutagenesis of the marine yeast *Wickerhamomyces anomalus* HH16 isolated from marine sediment collected from South Sinai Governorate, Egypt. Besides optimization of the culture conditions and analyzing the kinetic parameters of growth and glycerol biosynthesis by the mutant strain were studied. The marine yeast isolate HH16 was selected as the front runner glycerol-producer among all tested isolates, with glycerol yield recorded as 66.55 gl^{-1}. The isolate was identified based on the phenotypic and genotypic characteristics of *W. anomalus*. The genotypic characterization based on the internal transcribed spacer (ITS) sequence was deposited in the GenBank database with the accession number MK182824. UV-mutagenesis of *W. anomalus* HH16 by its exposure to UV radiation (254 nm, 200 mW cm^{-2}) for 5 min; increased its capability in the glycerol production rate with 16.97% (80.15 g l^{-1}). Based on the kinetic and Monod equations, the maximum specific growth rate (μ_{max}) and maximum specific glycerol production rate (v_{max}) by the mutant strain *W. anomalus* HH16MU5 were 0.21 h^{-1} and 0.103 g g^{-1}, respectively. Optimization of the fermentation parameters such as nitrogen source, salinity and pH has been achieved. The maximum glycerol production 86.55 g l^{-1} has been attained in a fermentation medium composed of 200 g l^{-1} glucose, 1 g l^{-1} peptone, 3 g l^{-1} yeast extract, and 58.44 g l^{-1} NaCl, this medium was adjusted at pH 8 and incubated for 3 days at 30° C. Moreover, results indicated the ability of this yeast to produce glycerol (73.33 g l^{-1}) using a seawater based medium. These findings suggest the applicability of using the yeast isolate *W. anomalus* HH16MU5 as a potential producer of glycerol for industrial purposes.

Keywords Fermentation · Marine yeast · Glycerol · Optimization · Kinetic study · Genotyping · *Wickerhamomyces anomalus*

Introduction

Glycerol (glycerin) is a simple sugar alcohol (1,2,3-propanetriol) with various industrial uses in pharmacy, cosmetics, paints, food, paper, dynamite and leather (Zhang et al. 2002; Sivasankaran et al. 2014). Glycerol is also a feedstock for numerous valuable chemicals such as lactic acid, citric acid, 1,3-dihydroxyacetone (DHA), 1,3-propanediol (1,3-PD), dichloro-2- propanol (DCP), acrolein, hydrogen and ethanol (Fan et al. 2010). Glycerol can be synthesized either by chemical or microbial routes. However, the chemical route of glycerol synthesis from petrochemical feedstock or soap manufacture has been declined because of the gradual increase in cost and replacement of soaps by detergents (Wang et al. 2001). In contrast, the fermentative route of glycerol production seems more applicable nowadays due to the simplicity of the method and using low-cost substrates in a short time (Grembecka, 2015). Yeasts have been considered the most widely used microorganisms for the microbial synthesis of glycerol (Yaçlin and Özbas 2005). Production of polyols such as glycerol is an integral part of the normal growth processes of the various yeast strains, which form them according to the requirement of growth (Butinar et al. 2005). Among yeasts, the brewing and baking yeast, *Saccharomyces cerevisiae* is the most widely glycerol-producing (Taherzadeh et al. 2002). However, technical problems for commercial applications have limited glycerol production by *S. cerevisiae*, and the other non osmophilic yeasts, these

✉ Abdel-Hamied M. Rasmey
am_rasmey@yahoo.com

[1] Botany and Microbiology Department, Faculty of Science, Suez University, Suez, Egypt

include: (1) a relatively low glycerol yield due to the production of other by-products such as ethanol, acetic acid, acetaldehyde and 2,3-butanediol in significant amounts; (2) using of high amounts of steering agents such as sulfite during the fermentation; and (3) a low productivity rate with a low final concentration in the fermentation medium make the recovery of glycerol expensive and insufficient (Sahoo and Agarwal 2001; Agarwal 1990; Vijaikishore and Karanth 1986). Therefore, the production of glycerol by osmotolerant and marine yeasts is a promising research area for many researchers; this is due to the high potentiality of these yeasts to produce high amounts of glycerol during the growth in its natural habitat to overcome the high external osmotic pressure of marine environment (Connell et al. 2008). The adaptation to an environment with a low water activity (a_w), i.e. high osmotic pressure, can vary from a species to another but is generally based on the accumulation of osmolytes as glycerol to recompense the increased external osmotic pressure (Bubnová et al. 2014). Marine yeasts have many advantages for glycerol production compared to conventional yeasts; they do not require anaerobic conditions or steering agents for cell growth and fermentation. Moreover, most marine yeasts can tolerate very high sugar concentrations which necessary for the enhancement of glycerol production yield and rate (Petrovska et al. 1999). Various osmotolerant and marine yeast species were reported as highly glycerol producers including *Candida magnolia*, *Debaryomyces hansenii, Pichia anomala, Candida glycerinogenes* and *Zygosaccharomyces rouxii* (Klein et al. 2017). Obtaining yeast strains with high glycerol fermentation rate considered an extremely challenging task, newly characterized yeasts that are highly efficient in glycerol production can be reached by exploring extreme saline habitats like marine water or by altering important genes in glycerol production pathway through ultraviolet (UV)-mutagenesis (Martinez et al. 2008; Shahsavarani et al. 2012). This study aims to enhance the glycerol production by selected marine yeast *W.anomalus* HH16 and to investigate the impact of carbon, nitrogen, salinity, and pH of the fermentation medium on glycerol production by this selected mutant.

Materials and methods

Yeast cultures

Seaweed and marine sediment samples were collected from different locations along Suez Canal region in Suez governorate and Suez Gulf area in South Sinai governorate, Egypt. The yeast extract–malt extract (YM) medium was used to isolate potential halotolerant/halophilic yeasts. The YM agar medium consisting of (g l^{-1} seawater): yeast extract, 3.0; malt extract, 3.0; peptone, 5.0; dextrose, 10.0 and supplemented with 20.0 g agar–agar. The medium pH was adjusted to 5.5 ± 0.2 using 0.1 N HCl and 0.1 N NaOH. Among the isolated yeasts, five isolates were selected for the present study based on their growth rate on YM medium and their strong capability to produce glycerol.

Screening of glycerol production

Preparation of yeast inocula

Yeast inoculum was prepared by inoculating a loopfull of 48 h old culture into 50 ml sterilized YM broth and incubated at 30° C for 48 h on a rotary shaker (150 rpm).

Fermentation process

The used glycerol fermentation medium is composed of (g l^{-1} distilled water): glucose, 180; yeast extract, 1; peptone, 1; KH_2PO_4, 1; $MgSO_4$, 0.5 and NaCl, 58.44 (1 M). The medium pH was adjusted to 6 ± 0.2 using 0.1 N HCl and 0.1 N NaOH. The fermentation process was conducted in glass bottles of 100 ml volume each containing 50 ml medium inoculated with 20% of the selected yeasts (HH1, HH16, HH56, HH60, HH91) inocula (10^6 cells ml^{-1}) and incubated at aerobic conditions for 96 h on a rotary shaker (150 rpm) at 30° C.

Glycerol extraction

The concentrations of both intracellular and extracellular glycerol were measured to determine the total glycerol produced and accumulated by the used yeast strains. Yeast cultures were centrifuged at 4000*g* for 10 min. The cell pellets were used for the intracellular glycerol measurement while the supernatant was used to determine the extracellular glycerol (Aslankoohi et al. 2015). The cell pellets were washed twice with PBS (Phosphate Buffered Saline); 1× contains 0.137 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4, 0.0018 M KH_2PO_4. After washing, the cell pellets were added into 3 ml 0.1 mol l^{-1} TRIS/HCl buffer (pH 7.7) containing 2 mmol l^{-1} EDTA for and boiled for 5 min. After centrifugation at 11,000*g* for 10 min to remove the cell debris, the glycerol concentration was measured in the supernatant using sodium periodate method.

Glycerol determination

Glycerol was quantitatively determined using sodium periodate method according to Kuhn et al. (2015). http://www.sciencedirect.com/science/article/pii/S0717345815000226—af0005. For each experiment, the following parameters were calculated according to the following equations: intracellular glycerol concentrations (P_{in}), extracellular glycerol

concentrations (P_{ex}), total glycerol concentrations (P_m), biomass (X_m), glycerol coefficient yield ($Y_{P/S}$), biomass coefficient yield ($Y_{X/S}$), volumetric glycerol productivity (Q_p) and average substrate uptake (Q_s). Glycerol and biomass coefficient yields were based on initial sugar concentration (S_{Go}) and expressed in g g^{-1}:

$$Y_{p/s}\left[g\ g^{-1}\right] = X_m/S_{G0} \quad (1)$$

$$Y_{x/s}\left[g\ g^{-1}\right] = P_m/S_{G0}. \quad (2)$$

Volumetric glycerol productivity (Q_p) was calculated as grams of glycerol formed per liter per hour (g l^{-1} h^{-1}):

$$Q_p = dP/dt. \quad (3)$$

Whereas average substrate uptake (Q_s) was calculated as grams of substrate consumed per liter per hour (g l^{-1} h^{1}):

$$Q_s = -dS/dt. \quad (4)$$

To determine the microbial kinetic growth, the specific glycerol production rates (v_g) and the specific growth rate (μ_x) values were calculated. Specific glycerol production rates (v_g) were expressed as g g^{-1} h^{-1} and calculated from the following relationship by using the changes in glycerol and dry biomass concentrations with time:

$$v_g = dP/Xdt. \quad (5)$$

The specific growth rate values were calculated from the logarithmic plots of the dry weight data with the fermentation time.

$$\mu_x = dX/dt. \quad (6)$$

Furthermore, the maximum values of specific growth rates (μ_{max}), maximum specific glycerol production rates (v_{max}), the maximum dry weight (X_{max}) and the maximum glycerol concentrations (p_{max}) were also calculated.

Total residual sugars (TRS) determination

The residual sugars were determined by dinitrosalicylic acid (DNS) method as described by Miller (1959). Three ml of DNS reagent was added to3 ml of diluted fermentation medium and the mixture was boiled for 5 min. After cooling down to room temperature, 1 ml of Rochell salt (40% potassium sodium tartarate) was added and absorbance was measured at 510 nm by UV-9200 VIS spectrophotometer. DNS reagent was prepared by dissolving 10.0 g of sodium hydroxide, 10 g of 3, 5-dinitrosalicylic acid, 2.0 g of phenol and 50 mg of sodium sulfite in one liter distilled water.

Biomass determination

The biomass was determined as dry weight (g l^{-1}) by drying the collected cells from fermentation medium in an oven at 85° C for 24 h and then was weighted (Zhang et al. 2018).

Phenotypic characterization

Based on the glycerol production capability, *W. anomalus* HH16 was found to be the yeast isolate that showed the highest glycerol yield. *W. anomalus* HH16 was selected to characterize phenotypically and genotypically as well. *W. anomalus* HH16 isolate was morphologically characterized on four different types of culture media; the colony characteristics including color, texture, appearance, elevation, and margin are examined in cultures streaked on YM agar medium. The cell morphology, budding, formation of pellicle and ascospores were examined on 5% malt broth medium (Ali and Khan 2014) and sodium acetate agar medium (Sulieman et al. 2015). The formation of pseudohyphae or true hyphae was studied on cornmeal agar using the coverslip method (Lodder and Kreger-van Rij 1952). Traditional biochemical and physiological tests were used for isolate identification. The isolate was tested for the production of enzymes; amylase, cellulase, and urease. Other biochemical criteria were tested as diazonium Blue B (DBB) test, citrate, indole, methyl red (MR), voges-proskauer (VP), growth in vitamin free and osmotic medium, assimilation and fermentation of various carbon compounds. Furthermore, the temperature and halo-tolerance profiles of the isolate were performed.

Genotypic identification

DNA extraction and PCR amplification

Genomic DNA (gDNA) was extracted according to Kumar et al. (2010) using chloroform-extraction and ethanol-precipitation method. The primers ITS1:5′-TCCGTAGGT GAACCTGCGG-3′and ITS4 5′ TCCTCCGCTTATTGA TATGC-3′ (White et al. 1990) were used to amplify ~ 750 bp from the internal transcribed spacer (ITS) Region. PCR reactions were performed in a final volume of 100 µl with the following reagent concentrations: Taq buffer (1×); dNTP mixture (200 µM each); Forward and reverse primers (0.2 µM each); Taq DNA polymerase (2.5 U/100 µl); ~ 50:100 ng of gDNA template and the total volume of the PCR reaction adjusted to 100 µl with nuclease-free H_2O. The complete reaction mixture was incubated at an automated MJ research thermal cycler (USA). PCR amplification included an initial denaturation step at 95° C for 3 min, followed by 35 cycles: 95° C for 30 s, annealing at 55° C for 30 s, extension 72° C for 60 s, and a final extension at 72° C for 5 min. All PCR amplicons were analyzed by gel electrophoresis

through 1% agarose gels in 1×Tris-acetate-EDTA (TAE) buffer (40 mMTris, 20mM Acetate and 1 mM EDTA, pH 8.0). The sizes of the PCR amplicons were estimated against a DNA ladder (Applied Biotechnology Co, Egypt) and visualized under UV light after staining with ethidium bromide (5 µg ml^{-1}).

Nucleotide sequence analysis

The amplified PCR product was sent to Solgent Co Ltd (South Korea) for sequencing. The resulted sequences were trimmed and assembled in Geneious software (Biomatters). Consequently, the trimmed sequences were identified by search in basic local alignment tool (BLAST) in GenBank. The full-length sequences obtained were matched with previously published sequences available in NCBI using BLAST at NCBI website: http://www.ncbi.nlm.nih.gov/BLAST/ to assess the degree of DNA similarity.

UV-mutagenesis of the selected yeast isolate

Wickerhamomyces anomalus HH16 culture was inoculated in 10 ml of YM medium and incubated overnight at 30 °C until cell density reached 2×10^8 cell ml^{-1}. The cells were diluted to obtain a final density of 10–100 cell ml^{-1}. A 100 µl of the diluted suspension was taken and inoculated on YM agar plate. Exposure was conducted at UV irradiation intensity (wavelength 254 nm) of 200 mW cm^{-2} for 5, 10, 15 and 20 min. To stop photoreactions, plates were kept in dark for 24 h then incubated for 3 days at 30 °C. The surviving mutant colonies were picked and streaked on YM medium.

Optimizations of glycerol production by the selected mutant isolate *W. anomalus* HH16 MU5

Parameters such as carbon, nitrogen sources, salinity, and pH were investigated to obtain the maximum yield of glycerol by the UV mutant isolate. The fermentation medium was supplemented individually with organic different carbon sources (glucose, fructose, sucrose, maltose, and lactose) at a concentration of 18% of each carbon source separately. Furthermore, determination of growth and glycerol production kinetics at different glucose concentrations was achieved. Effect of individual addition of various organic and inorganic nitrogen substances (peptone, yeast extract, beef extract, malt extract, urea, ammonium phosphate and ammonium sulfate) at 0.3% level on glycerol production was examined. Also, the influence of the combination of different nitrogen sources (yeast extract- peptone- urea) at different ratios were investigated. The effect of different salinity concentrations (0.5 M, 1 M, 2 M, 3 M, 4 M, and seawater) on glycerol production was studied. To determine optimum pH for glycerol production, the pH of fermentation was controlled using 2.5 N Na_2CO_3. The effect of pH was tested at uncontrolled pH, pH 4.0, pH 5.0, pH 6.0, pH 7.0 and pH 8.0.

Results and discussion

Screening of glycerol and biomass production by the tested yeast isolates

Results in Table 1 showed that all the tested marine yeast isolates (HH1, HH6, HH56, HH60 and HH91) could produce and accumulate glycerol at variable concentrations. The highest amount of total glycerol (66.55 g l^{-1}) was produced by the isolate HH16 with volumetric glycerol productivity 0.92 g $l^{-1}h^{-1}$.On the other hand, the isolate HH60 produced the lowest amount of total glycerol (52.30 g l^{-1}), with volumetric glycerol productivity 0.73 g l^{-1} h^{-1}. The potentiality of marine yeasts to survive the external high osmotic pressure with a low water activity (a_w) can vary from species to a species but in general based on the amount of osmolytes that accumulated in the cells (Hohmann 2002; Thome 2007). Passoth et al. (2006) reported that the ascomycetous yeast

Table 1 Glycerol and biomass production by five yeast isolates

Parameters	HH1	HH16	HH56	HH60	HH91
Intracellular glycerol, P_{in} (g l^{-1})	$31.83^a \pm 0.2$	$37.65^b \pm 0.1$	$35.83^c \pm 0.09$	$28.88^d \pm 0.17$	$38.04^a \pm 0.1$
Extracellular glycerol, P_{ex} (g l^{-1})	$25.11^a \pm 0.1$	$28.90^b \pm 0.02$	$25.61^a \pm 0.16$	$23.42^c \pm 0.09$	$25.17^a \pm 0.1$
Total glycerol production, P_m (g l^{-1})	$56.94^a \pm 0.3$	$66.55^b \pm 0.12$	$61.44^c \pm 0.25$	$52.30^d \pm 0.26$	$63.21^e \pm 0.2$
Biomass, X_m (g l^{-1})	$16.50^a \pm 0.22$	$14.49^b \pm 0.14$	$20.34^c \pm 0.21$	$19.90^c \pm 0.31$	$13.50^d \pm 0.25$
Volumetric glycerol productivity, Q_p (g l^{-1} h^{-1})	0.79	0.92	0.85	0.73	0.88
Specific glycerol production rates, v_g (g g^{-1} h^{-1})	0.05	0.06	0.04	0.04	0.07
Glycerol coefficient yield, $Y_{P/S}$ (g g^{-1})	0.32	0.37	0.34	0.29	0.35
Biomass coefficient yield, $Y_{X/S}$ (g g^{-1})	0.09	0.08	0.11	0.11	0.08
Average glucose uptake, Q_s (g l^{-1} h^{-1})	2.20	2.30	2.31	2.06	2.18

Values are means of three replicates, values followed by same letters on the same row are not significantly different ($P < 0.005$) in Tukey's test

can adapt to surrounding osmotic stress by the production of excess glycerol as an intracellularly osmolyte. The intracellular glycerol can be adjusted by exporting some of the synthesized glycerol extracellular to balance the osmotic pressure across plasma membrane.

Characterization and identification of the isolate *W. anomalus* HH16

The morphological characteristics of the isolate HH16 were shown in Table 2. The colony on YM agar was tannish-white colored butryous, smooth, glistening and umbonate. Cells' morphological characters were measured on 5% Malt extract broth and sodium acetate agar media; it was obvious that the cell shape was sub-globose to ovoidal with monoploar, bipolar and multilateral budding. Diploid budding cells are transformed into asci containing 2–8 hat-shaped ascospores. Pseudohyphae formation was studied on cornmeal agar using the coverslip method which indicated that pseudohyphae are formed and branching out bearing clusters of ovoidal blastoconidia. Pseudohyphae also have ramified chains of cells bearing dense groups of ovoidal blastoconidia in verticils, the cells are transformed into asci with ascospores (Fig. 1). The morphological characters obtained in this study were as described by Kurtzman and Fell (1998) who characterized the yeast *W. anomalus* as heterothallic, ascomycetous yeast, forming one to four hat-shaped ascospores.

The biochemical and physiological characteristics (Table 3) revealed that the isolate HH16 is ascomycetous yeast relying on its negative reaction to diazonium blue B test (DBB). This test is employed to determine whether asexual yeast belongs to a basidiomycetous (+ve reaction) or an ascomycetous genus (−ve reaction) (Ghindea et al. 2009; Kurtzman and Fell 1998). The isolate was positive to voges-proskauer (VP) and negative to both of methyl red (MR) and indole tests. This isolate showed the ability to utilize citrate, glucose, sucrose, maltose, lactose, galactose, L- arabinose, erythritol, cellobiose, cellulose, starch and, urea, but not D-xylose. The results also indicate that *W.anomalus*HH16 is unable to ferment lactose and galactose but can assimilate these two sugars. Results showed that this non-conventional isolate could grow under extreme environmental stress conditions such as high osmotic pressure (50% glucose) and vitamin free medium. It could tolerate sodium chloride concentrations up to 4.0 M and grow well at temperature range 8–42 °C.

The ITS region sequence was used for genotyping and confirm the identity of some selected yeast isolates during the current study. ITS sequences were deposited into GenBank and assigned the following accession numbers: MK156297–MK156309, MK182823, and MK182824. Using BLASTN search tool (Altschul et al. 1990), the ITS sequence of *W.anomalus* HH16 (GenBank accession MK182824) matched the type sequence of *W. anomalus* 1033L1 strain 1033L1 (Accession KP638741) with identities = 562/562 (100%) and gaps = 0/546 (0%). *Wickerhamomyces anomalus*, formerly known as *Pichia anomala* and *Hansenula anomala* was recently assigned to the genus *Wickerhamomyces* based on phylogenetic analysis of a dataset of D1/D2 LSU rRNA gene sequences, which has caused great changes in the yeasts classification analysis (Kurtzman et al. 2011). *W. anomalus* is a biotechnologically important yeast species isolated from diverse natural habitats including marine environments. This organism exhibits unique and

Table 2 Morphological characteristics of the isolate HH16

Medium	Character	Observation
Yeast malt agar	Colony color	Tannish-white
	Nature	Butryous
	Appearance	Smooth, glistening
	Elevation	Umbonate
	Margin	Entire
5% malt broth	Cell shape	Subglobose to ovoidal
	Budding	Monobloar, bibolar and multilateral
	Sporulation	Diploid budding cells are transformed into asci containing two to eight hat shaped ascospores
	Pellicle formation	Formed and thick folded
Sodium acetate agar	Cell shape	Subglobose to ovoidal
	Budding	Monobloar, bibolar and multilateral
	Sporulation	Asci containing two to eight hat shaped ascospores
	Conjugation	Cell with cell
Cornmeal agar	Pseudohyphae formation	Pseudohyphae are branching out bearing clusters of ovoidalblastoconidia. Pseudohyphae also have ramified chains of cells bearing dense groups of ovoidalblastoconidia in verticils, the cells are transformed into asci with ascospores

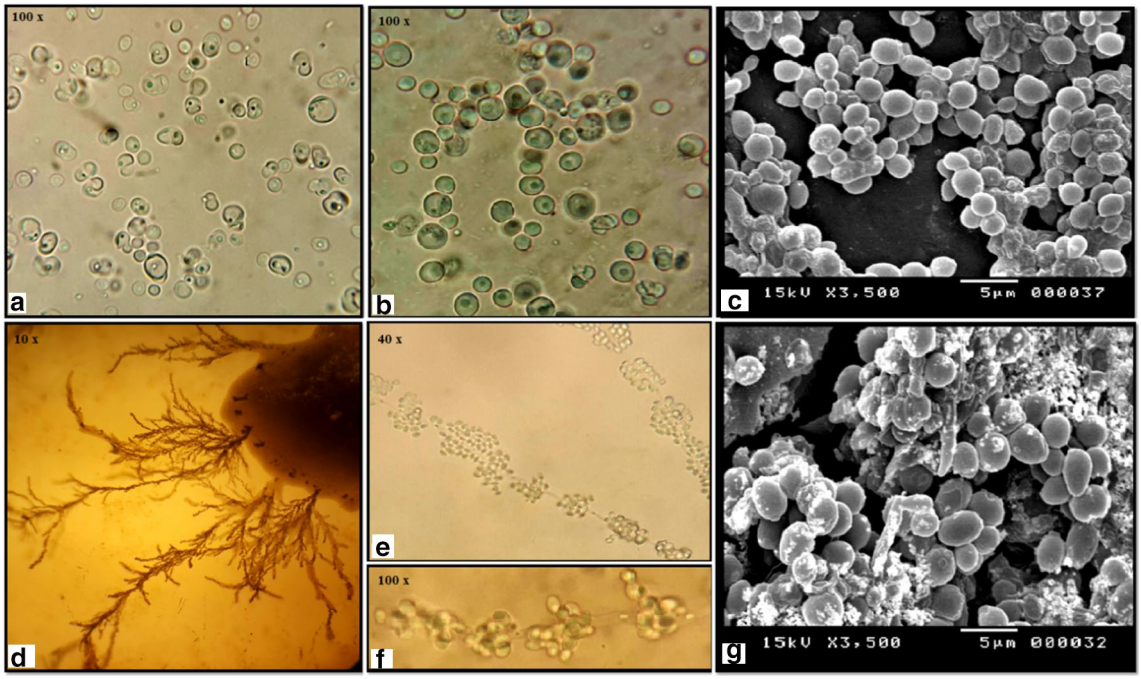

Fig. 1 Morphological characteristics of the yeast isolate HH16. **a** Conjugation of yeast cells on sodium acetate agar (100×), **b** Cells are subglobose, asci containing two to eight hat shaped ascospores on 5% malt broth(100×), **c** Scanning electron microscope (SEM) of subglobose budding yeast cells on 5% malt broth (3.500×), **d** Pseudohyphae formation on cornmeal agar (10x), **e**, **f** Long and branched pseudohyphae with numerous ovoid blastospores occur in clusters on cornmeal agar (40×, 100×), **g** SEM of pseudohyphae formation on cornmeal agar (3.500×)

potentially exploitable physiological and metabolic characteristics (Padilla et al. 2018); this is due to the efficient capability to tolerate extreme environmental conditions like oxidative, salt, and osmotic stresses (Walker 2011).

UV-mutagenesis of *W. anomalus* HH16

The influence of (UV) irradiation on the marine isolate *W. anomalus* HH16 was studied to enhance its glycerol production. The results revealed that there was a dramatic reduction in the percentage of the surviving yeast colonies number as the UV exposure time increased (Fig. 2). Data showed that only 12% of the yeast colonies survive after 5 min UV irradiation, 5% surviving after 10 min and only one yeast colony survive after 15 min, whereas at UV exposure time (20 min), no colonies were reported. Two surviving mutants were selected for glycerol production, the selection based on the resistance to exogenous ethanol in contrast to the wild isolate at medium containing 10–30% (v/v) ethanol. The alcohol resistance of the selected irradiated strains, possibly due to yeast mutation during UV irradiation (Revin et al. 2018). UV irradiation can induce mutagenic and cytotoxic DNA lesions such as cyclobutane–pyrimidine dimers (CPDs) and 6–4 photoproducts (6–4 PPs); however, yeast cells have developed a wide range of DNA-damage repair mechanisms to counteract the UV effect. Photoreactivation with photolyase enzyme considered one of the most effective repair mechanisms developed by yeasts (Sancar and Smith 1989). Zhang et al. (2015a, b) found that several genes were highly expressed only in the ethanol-tolerant mutant but not in the parent strain; the ethanol-tolerant mutant also exhibited resistance to other stresses including high osmosis (Zhao and Bai 2009). Random mutagenesis in yeast by mutagens such as mutagenic agents and UV light is a promising strategy for improving lipid production (Tapia et al. 2012), ethanol production (Watanabe et al. 2011) and sugar alcohols production (Zhang et al. 2015a, b; Savergave et al. 2013). UV exposure causes various types of DNA damage, the intensity and time exposure of UV irradiation can highly affect both the activation of yeast (as a protective mechanism against oxidative stress) and mutation that can occur (Kumari and Pramanik 2012; Ikehata and Tetsuya 2011). At high UV doses, most recombination events reflect the repair of two sister chromatids broken at the same position whereas, at lower UV doses, most events involve the repair of a single broken chromatid which could cause mutagenesis in different regions (Yin and Petes 2013), this might explain why the mutant HH16 MU5 which exposed to UV irradiation for 5 min gives high glycerol production (80.15 g l^{-1}) compared to the mutant HH16 MU15 which irradiated for 15 min (Table 4).

Table 3 Biochemical and physiological characteristics of the isolate HH16

Characteristic	Result
DBB	–
Citrate	+
MR	–
VP	+
Indole	–
Urease	+
Starch hydroysis	+
Celluolse hydrolysis	+
Vitamin free	+
50% glucose	+
Fermentation	
Glucose	+
Sucrose	+
Maltose	+
Lactose	–
Galactose	+
Assimilation	
Glucose	+
Sucrose	+
Maltose	+
Lactose	+
Galactose	+
D-Xylose	–
L-Arabinose	+
Erythritol	+
Cellobiose	+
Nitrate	+
Glycerol	+
Temperature profile:	
4 °C	–
8 °C	+
12 °C	+
25 °C	+
30 °C	+
42 °C	+
Halo-tolerance profile	
0.5 M NaCl	+
1 M NaCl	+
2 M NaCl	+
3 M NaCl	+
4 M NaCl	+

Optimizations of glycerol production by the selected mutant isolate *W. anomalus* HH16MU5

Glycerol yield by yeasts can be affected by various growth and environmental factors; substrate type, initial substrate concentration, nitrogen source, pH and salinity are among these factors (Zhao et al. 2015).

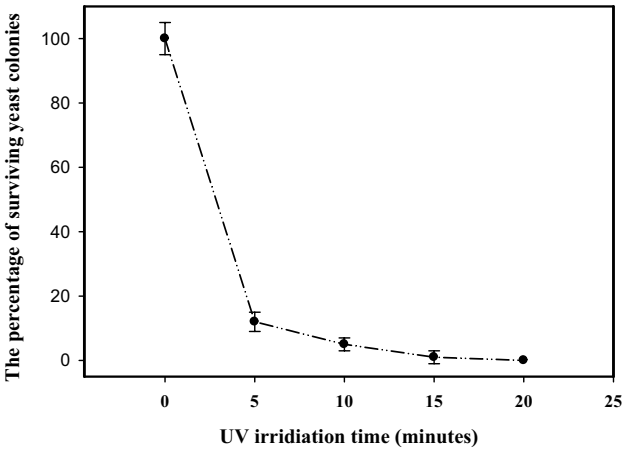

Fig. 2 The percentage of surviving yeast colonies at different exposure time to UV

Effect of different carbon sources

Data in Table 5 show that the wild and mutant isolates could assimilate different sugars and produce glycerol at variable concentrations. The yeast osmotic stress and glycerol production is carbon source dependent (Babazadeh et al. 2017). According to Leandro et al. (2011), marine yeasts could utilize a variety of substrates, the various sugars and carbon sources used in fermentation might exhibit osmotic pressure of different degrees on the yeast cell. Moreover, our results indicated that the highest glycerol yields were achieved by both the wild and mutant isolate *W. anomalus* HH16MU5 using a growth medium supplemented with monosaccharides such as glucose or fructose. The maximum glycerol yields were recorded as 0.3697 and 0.4452 for the wild and the mutant isolates, respectively, using glucose as a carbon source. Fructose was the second-best substrate with glycerol and biomass yields recorded as 0.4268, 0.0881 g g^{-1} respectively, and average fructose uptake of 2.1289 g l^{-1} h^{-1} for the mutant isolate. The minimum glycerol production was achieved using medium supplemented with lactose and maltose with glycerol yields 0.2766, 0.3077 g g^{-1} for the mutant and 0.3083, 0.3005 for the wild isolate, respectively. These results were consistent with Piddocke et al. (2009) who revealed that glycerol production was higher in the fermentation of glucose-rich medium than in medium supplemented with maltose. The reason could be that at high monosaccharide medium more DHAP and NADH were generated (Orlic et al. 2010).

Effect of different initial glucose concentrations

The integration of experimental studies with mathematical modeling makes it is possible to obtain quantitative and meaningful interpretation of the experimental results and

Table 4 Glycerol and biomass production by the wild and UV-mutant isolates

Parameters	*W. anomalus* HH16	*W. anomalus* HH16 MU5	*W. anomalus* HH16 MU15
Total glycerol production (P_m) g l^{-1}	$66.55^{a} \pm 0.12$	$80.15^{b} \pm 0.09$	$75.16^{c} \pm 0.17$
Biomass (X_m) g l^{-1}	$14.490^{a} \pm 0.1$	$16.146^{b} \pm 0.11$	$15.192^{a} \pm 0.2$
Volumetric glycerol productivity (Q_p) g l^{-1} h^{-1}	0.9243	1.1132	0.9384
Specific glycerol production rates (v_g) g g^{-1} h^{-1}	0.0638	0.0689	0.06176
Glycerol coefficient yield ($Y_{P/S}$) g g^{-1}	0.3697	0.4452	0.4176
Biomass coefficient yield ($Y_{X/S}$) g g^{-1}	0.0805	0.0897	0.0844
Average glucose uptake (Q_s) g l^{-1} h^{-1}	2.3019	2.2840	2.4123

Values are means of three replicates, values followed by same letters on the same row are not significantly different ($P < 0.005$) in Tukey's test

Table 5 Effect of different carbon sources on glycerol and biomass yields by the selected mutant isolate

Parameters	Sucrose	Glucose	Fructose	Lactose	Maltose
Total glycerol production (P_m) g l^{-1}	$63.50^{a} \pm 0.16$	$80.15^{b} \pm 0.09$	$76.83^{c} \pm 0.11$	$49.78^{c} \pm 0.21$	$55.39^{d} \pm 0.35$
Biomass (X_m) g l^{-1}	$15.282^{a} \pm 0.05$	$16.146^{b} \pm 0.1$	$15.858^{ab} \pm 0.12$	$11.176^{c} \pm 0.07$	$13.698^{d} \pm 0.01$
Volumetric glycerol productivity (Q_p) g l^{-1} h^{-1}	0.8819	1.1132	1.0671	0.6914	0.7693
Specific glycerol production rates (v_g) g g^{-1} h^{-1}	0.05771	0.0689	0.06729	0.05247	0.05616
Glycerol coefficient yield ($Y_{P/S}$) g g	0.3528	0.4452	0.4268	0.2766	0.3077
Biomass coefficient yield ($Y_{X/S}$) g g	0.0849	0.0897	0.0881	0.0732	0.0761
Average glucose uptake (Q_s) g l^{-1} h^{-1}	2.4195	2.2840	2.1289	2.1871	2.1939
Control* glycerol production g l^{-1}	$59.63^{a} \pm 0.39$	$66.55^{b} \pm 0.22$	$62.77^{a} \pm 0.14$	$55.50^{c} \pm 0.19$	$54.09^{c} \pm 0.35$

Values are means of three replicates, values followed by same letters on the same row are not significantly different ($P < 0.005$) in Tukey's test

*Control: glycerol production by the wild isolate

provide new aspects of microbial physiology (Snoep et al. 2009). In shake-flask experiments, the effects of different initial concentrations of glucose (50–250 g l^{-1}) at specific time intervals were examined. For each time interval, the changes in glycerol production concentration and dry biomass were determined. Variations in glycerol concentration and biomass of the isolate *W. anomalus* HH16 MU5during fermentation time were shown in Fig. 3. The initial glucose concentration of the medium was chosen as independent variable for the specific growth rate and glycerol production rate of the culture. The growth data was represented by the following Monod model:

$$\mu = \frac{\mu_{\max} + S_{\circ}}{ks + S_{\circ}}, \tag{7}$$

where μ_{max} is the maximum specific growth rate, S_o is initial glucose concentration, K_s is substrate utilization constant of the Monod model.

It was noted that the maximum specific growth rate obtained at an initial glucose concentration of 175 g l^{-1} whereas the maximum specific glycerol production rate was obtained at high initial concentration of glucose (200 g l^{-1}). High glucose concentrations in the growth medium increase the extracellular osmotic pressure, and as a result, osmotolerant yeasts retain more glycerol in the fermentation medium (Petrovska et al. 1999). The role of high sugar is to create a high osmotic pressure thus force the organism to produce the compatible solutes as glycerol to overcome the surrounding environment. According to Nissen et al. (2000), a high concentration of sugar is necessary to obtain maximum yield of glycerol, where respiration is repressed, reducing the capability of the organism to reoxidize surplus amounts of NADH formed in biosynthetic reactions. Furthermore, under intracellular conditions, an increase towards glycerol 3-phosphate dehydrogenase was reported and glycerol accumulated inside the cell during growth under osmotic stress as an efficient osmolyte protecting the cell against lysis. However, our data revealed that both the specific growth and glycerol production rate were declined above the optimum glucose concentration; this may be due to the inhibitory effect of glucose. The maximum specific growth and glycerol production rates for the mutant isolate were correlated by using the non-linear regression method as follows:

$$\mu = \frac{0.21 + S_{o}}{43.15 + S_{o}}, \quad R^2 = 0.860,$$

$$v = \frac{0.103 + S_{o}}{89.03 + S_{o}}, \quad R^2 = 0.840.$$

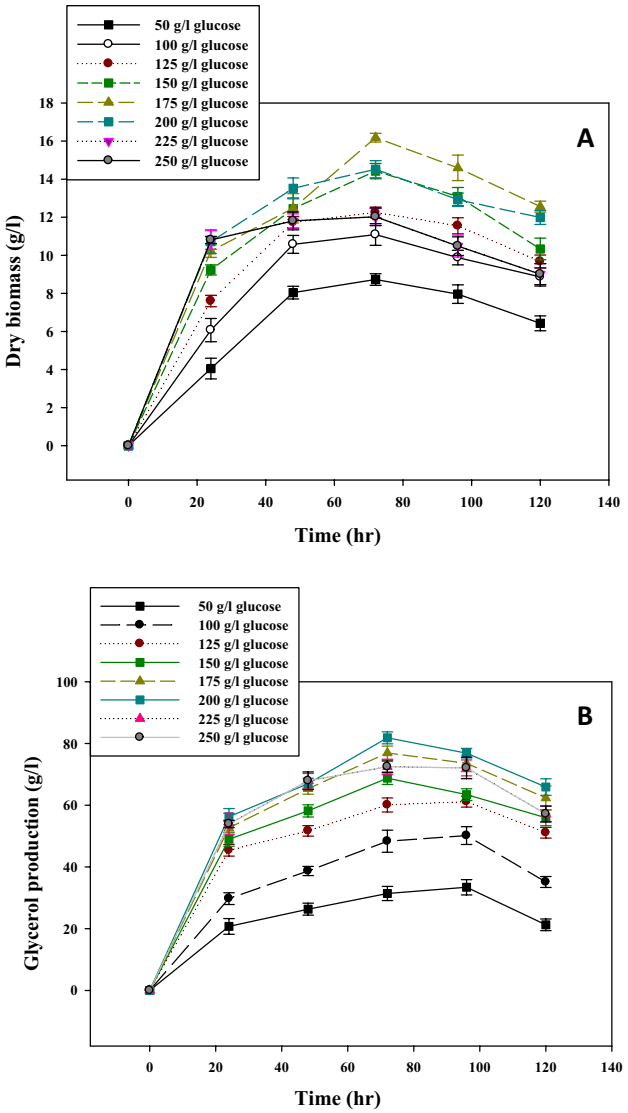

Fig. 3 Variation in **a** biomass and **b** glycerol production of *W. anomalus* HH16MU5 on glucose during different fermentation periods

It was estimated that the maximum specific growth rate (μ_{max}) and maximum specific glycerol production rates (ν_{max}) were calculated as 0.21 h^{-1} and 0.103 g gly g^{-1} yeast cells (Fig. 4). These results were inconsistent with Yaçlin and Özbas (2005) who studied the growth and glycerol kinetics of *S.cerevisiae* Kalecik 1 at different sugar concentrations and estimated that an inhibition effect was noted at initial sugar concentrations were above the optimum substrate concentrations necessary for yeast growth.

Effect of different nitrogen sources on glycerol production

The type of nitrogen in the fermentation medium greatly affects the glycerol and biomass yields. Effect of different organic (peptone, yeast extract, beef extract and, malt extract) and inorganic (urea, ammonium phosphate and ammonium sulfate) nitrogen sources on glycerol production was investigated (Table 6).The maximum glycerol production rate was achieved with peptone as the sole nitrogen source in the fermentation medium with recorded glycerol and biomass yields 0.3725 g g^{-1} and 0.0481 respectively. Junior et al. (2008) found that peptone supplementation enhanced the fermentation performance of the yeast. Both yeast and beef extracts had no significant difference for glycerol production, these extracts provide convenient growth factors for yeast growth (Nancib et al. 2001). Ammonium sulphate is a cheap and efficient nitrogen source for yeast growth; it does not produce a toxic effect towards the microbial enzymes (Bamforth 2005). However, the minimum glycerol yields were recorded from the addition of ammonium sulphates and ammonium phosphate individually with no significant difference, this may be due to that these inorganic nitrogen sources probably contain only the nutrients that satisfy no more than the minimum requirement for growth (Costa et al. 2002). Nitrogen deficiency dramatically declines the yeast growth and possibly the

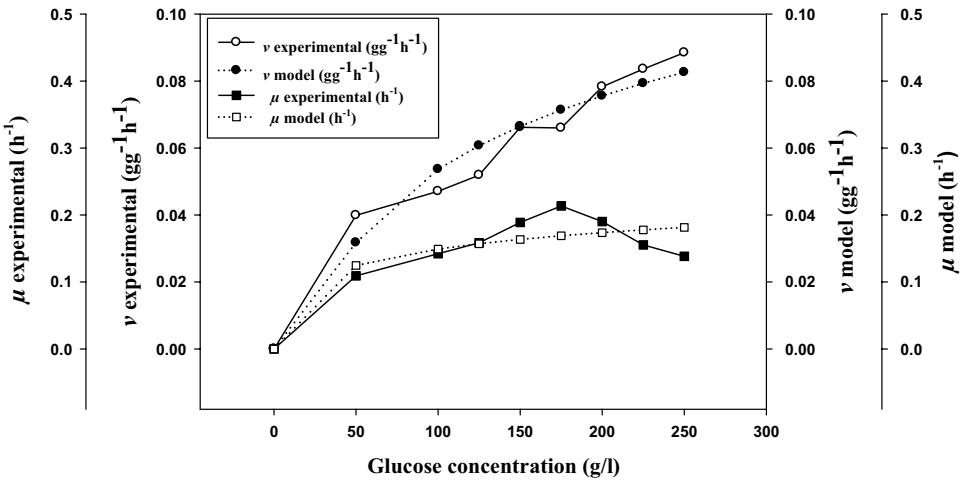

Fig. 4 The experimental and model predicted specific growth and glycerol production rates by *W. anomalus* HH16MU5 at different glucose concentrations

Table 6 Effect of different nitrogen sources on glycerol and biomass yields by the selected mutant isolate

Parameters	Yeast extract	Peptone	Beef extract	Malt extract	Urea	Amm. phosphate	Amm. sulphate
P_m (g l^{-1})	$68.584^{a} \pm 0.2$	$74.499^{b} \pm 0.2$	$68.137^{a} \pm 0.23$	$64.42^{c} \pm 0.11$	$62.08^{c} \pm 0.1$	$58.50^{d} \pm 0.22$	$58.33^{d} \pm 0.11$
X_m (g l^{-1})	$13.54^{a} \pm 0.09$	$9.620^{b} \pm 0.08$	$11.76^{c} \pm 0.11$	$8.38^{d} \pm 0.12$	$12.16^{c} \pm 0.06$	$13.80^{a} \pm 0.11$	$10.70^{e} \pm 0.09$
Q_p (g l^{-1} h^{-1})	0.9526	1.0347	0.9463	0.8947	0.8622	0.8125	0.8101
v_g (g g^{-1} h^{-1})	0.0703	0.1076	0.0805	0.1068	0.0709	0.0589	0.0757
$Y_{P/S}$ (g g^{-1})	0.3429	0.3725	0.3407	0.3221	0.3104	0.2925	0.2917
$Y_{X/S}$ (g g^{-1})	0.0677	0.0481	0.0588	0.0419	0.0608	0.0690	0.0535
Q_s (g l^{-1} h^{-1})	2.7252	2.6439	2.7278	2.6364	2.595	2.5525	2.5494
Control* production g l^{-1}	$62.511^{a} \pm 0.29$	$65.07^{a} \pm 0.31$	$58.857^{b} \pm 0.33$	$61.08^{ab} \pm 0.49$	$59.08^{b} \pm 0.1$	$56.92^{b} \pm 0.34$	$53.15^{c} \pm 0.18$

Values are means of three replicates, values followed by same letters on the same row are not significantly different ($P < 0.005$) in Tukey's test

*Control: glycerol production by the wild isolate

metabolic reactions, this decline results from the inhibition of the synthesis of protein transporting sugars through the cell membrane to the interior of the cells (Varela et al. 2004). The combination of different organic nitrogen sources has a marked effect on the metabolic pathways of the microorganism which regulates the production of numerous metabolites (Bhasin and Modi 2012). Therefore, four different fermentation media supplemented with different combinations of the three organic nitrogen sources (yeast extract, peptone, and urea) were investigated (Fig. 5). The maximum glycerol productivity 1.146 g l^{-1} $h^{-1}$was obtained with medium supplemented with peptone and yeast extract at the ratio of 1:3% (w/v) with glycerol and biomass yields recorded as 0.4120 and 0.0886 g g^{-1} respectively. This increase in glycerol productivity might be due to the availability of different amino acids, peptides, water-soluble vitamins and carbohydrates in both of peptone and yeast extract (Costa et al. 2002). However, our results indicated that the supplementation of urea to other nitrogen combinations has no significant impact on glycerol production, which is consistent with Zhuge et al. (2001) who observed that the addition of commercial urea to other nitrogen sources has a little effect on glycerol production by *C. glycerinogenes*.

Effect of salinity stress on glycerol production

The effect of different salt concentrations on glycerol production was investigated. Data in Fig. 6 revealed that intracellular glycerol accumulation was enhanced by increasing sodium chloride concentrations, whereas extracellular glycerol production showed a marked decline. Abdel Nasser and El-Moghaz (2010) studied the salt tolerance of *Pichia pastoris* and reported that salt stress was accompanied by an increase in the intracellular level of glycerol to decrease the intracellular water potential, restore water influx and consequently restore cell volume and turgor pressure. Shen et al. (1999) revealed that yeast cells grown under salt stress could accumulate much higher amounts of glycerol than cells grown without sodium chloride, suggesting that the metabolic flux may be changed to favor glycerol biosynthesis and the membrane permeability to glycerol may be reduced under salt-stress conditions. Our data explained

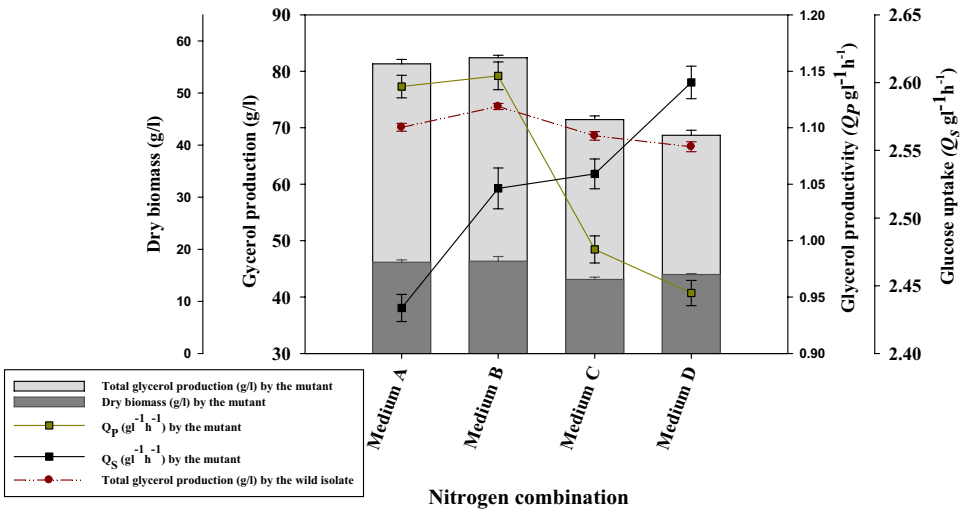

Fig. 5 Effect of combination of different nitrogen sources: medium A, peptone + yeast extract (1:1); medium B, peptone + yeast extract (1:3); medium C, peptone + yeast extract (3:1); medium D, peptone + yeast extract + urea (1:1:3) on glycerol and biomass production parameters

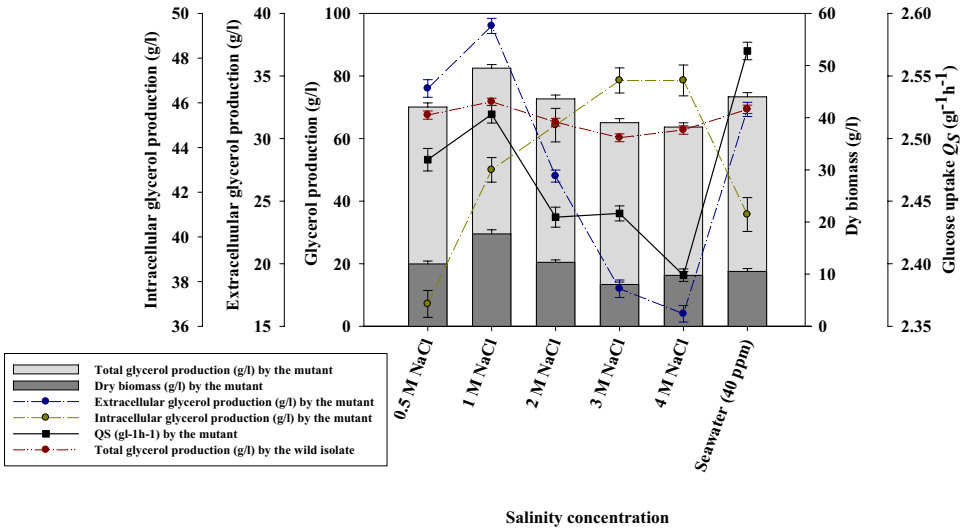

Fig. 6 Effect of seawater and different salinity concentrations (0.5 M, 1 M, 2 M, 3 M and 4 M) on glycerol and biomass production parameters

that the accumulation of glycerol as a compatible solute is a strategy for the marine yeasts at high salt concentrations and plays a central role in the osmotic adaptation of the yeast cells (Silva-Graça and Lucas 2003). Moreover, our results revealed that both the wild and mutant isolates could tolerate high salt concentrations up to 4 M; however, the total glycerol and biomass yields showed a marked decrease at very high salt concentrations. This reduction of growth might be due to the disproportional deviation of carbon and energy into production of the cellular osmolytes (Fernanda et al. 1999). Seawater was a good substitute for distilled water supplemented with NaCl; our mutant achieved glycerol production equal to 73.33 g l^{-1} and volumetric glycerol productivity 1.0185 g l^{-1} h^{-1}. In contrast, the wild isolate could achieve glycerol production equal to 67.334 g l^{-1} using seawater based medium. This may be due to the wide spectrum of minerals content of seawater that may avoid the addition of essential nutrients currently required for commercial fermentation medium (Lin and Tanka 2006). Thus, using marine yeasts in industrial fermentations could potentially enhance the overall economics of the process.

Effect of hydrogen ion concentration

The effect of different hydrogen ion concentrations (pH) on glycerol and biomass production was investigated. The resulted data in Fig. 7 revealed that glycerol production rate gradually increased along with the increase in pH values with a sharp increase in the glycerol yield of at the pH values 7 and 8 for both the two tested isolates. The maximum glycerol production 86.55 g l^{-1} was achieved by the mutant isolate at slightly alkaline pH 8.00 with glycerol productivity 1.2021 g l^{-1} h^{-1} and the substrate uptake rate achieved 2.5833 g l^{-1} h^{-1}.The positive effect of increasing pH on glycerol production may explained in relation with the activity of the enzyme aldehyde dehydrogenase which increased at high pH values and cause the fixation of acetaldehyde, eliminating the ethanol formation and diverting the excess

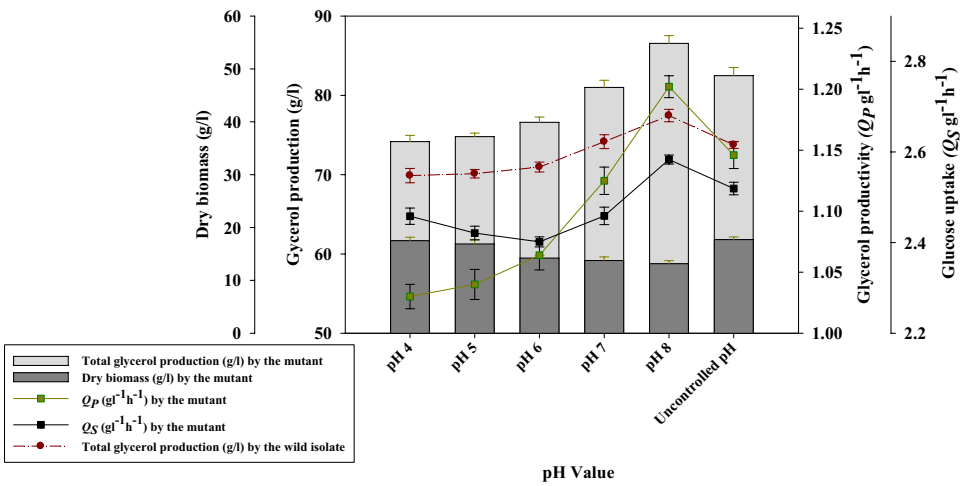

Fig. 7 Effect of different pH values (uncontrolled pH, pH 4.0, pH 5.0, pH 6.0, pH 7.0 and pH 8.0) on glycerol and biomass production parameters

NADH towards glycerol formation by reduction of dihydroxyacetone phosphate to glycerol-3-phosphate in order to maintain the redox balance of the cell (Wang et al. 2001). Thus, commercially industrial glycerol may be enhanced by alkali-stressed (Taherzadeh et al. 2002). Patil (2013) also indicated that pH 8.0 is optimum for glycerol production by thermotolerant yeast *H. anomala*. This was in agreement with Yaçlin and Özbas (2008) who studied the effect of pH on growth and glycerol production kinetics of two indigenous wine yeast strains *S. Cerevisiae* Kalecik 1 and Narince 3. The maximum biomass yield 0.0886 g g^{-1} was achieved by the mutant isolate at uncontrolled pH with glycerol production 82.49 g l^{-1} and glucose uptake rate 2.5194 g l^{-1} h^{-1}. Walker (1998) stated that yeast grow very well between pH 4.5–6.5, but nearly all species can be able to grow in more acidic or alkaline media that low or high pH values may cause chemical stress on yeast cell, which was in consistence with the results presented in the current study.

Comparison of glycerol production in this study with previously reported studies

Data in Table 7 showed a comparison of the glycerol production in the current study with previously reported studies. Overproduction of glycerol by *S. cerevisiae* can be achieved by forming a complex of acetaldehyde with the bisulfite ion (known as the steering agent) that limits ethanol production and promotes reoxidation of glycolytically formed NADH by glycerol synthesis (Wang et al. 2001). Several other efforts such as mutagenesis, strain breeding or overexpression of the genes associated with glycerol formation have been attempted to improve glycerol synthesis (Remize et al. 2001; Overkamp et al. 2002). Glycerol production via osmotolerant yeasts is an attractive approach that can produce significant amounts without a need for a steering agent (Zhang et al. 2002; Zhuge et al. 2001). However, the selection of yeast strains, optimization of fermentation conditions and yeast genetic modifications are critical parameters for glycerol overproduction (Mirończuk et al. 2016; Ehsani et al. 2009). Based on the comparison table, it was revealed that our mutant isolate has promising features for glycerol production especially using seawater based medium.

Conclusions

Marine yeasts are an interesting group with great potentials for large scale production of worthy industrial chemicals such as glycerol. UV-mutagenesis is a significant tool to improve the glycerol productivity. It was found that the substrate type, initial concentration and fermentation periods significantly affects the yeast growth and glycerol production. Glucose is the best substrate for glycerol production by *W. anomalus* HH16 MU5. The maximum specific growth rate was obtained at an initial glucose concentration of 175 g l^{-1}, whereas the maximum specific glycerol production rate was achieved at a high initial concentration of glucose (200 g l^{-1}). Other fermentation parameters as the nitrogen source, salinity and pH are critical factors influence glycerol accumulation and production. It was revealed that the combination of different nitrogen sources (peptone + yeast extract) could enhance glycerol productivity. Although the isolate *W. anomalus* HH16 MU5 could tolerate up to 4 M, the highest total glycerol production was achieved at 1 M. Furthermore, the results indicated that low or high pH values may cause chemical stress on yeast cell and the highest glycerol production was achieved at pH 8.

Table 7 Comparison of glycerol production in this study with previously reported studies

Organism and conditions	Glycerol production P_m (g l^{-1})	Glycerol coefficient yield $Y_{P/S}$ (g g^{-1})	Glycerol productivity Q_p (g l^{-1} day^{-1})	References
S. cerevisiae				
Sulfite batch	45	0.23	9	Freeman and Donald (1957)
Sulfite, Shake flasks	34.4	0.35	34.4	Petrovska et al. (1999)
Sulfite; fed batch under vacuum	82	0.25	32.5	Kalle et al. (1985)
GPD1 overproduction; batch	25	0.12	4.3	Remize et al. (2001)
Shake-flask, no steering agent	10.4	0.029	2.6	Yaçlin and Özbas (2005)
Osmotolerant yeasts				
Candida glycerinogenes; batch	127	0.64	40.6	Zhuge et al. (2001)
Candida magnoliae I2B; batch	80	0.32	15.6	Sahoo and Agarwal (2001)
Candida krusei, shake-flask	37.1	0.19	10.2	Chen and Yao (2013)
W. anomalus HH16, shake-flask	86.55	0.43	28.85	The current study

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

References

Abdel Nasser A, El-Moghaz (2010) Comparative study of salt tolerance in *Saccharomyces cerevisiae* and *Pichia pastoris* yeast strains. Adv Bioresour 1:169–176

Agarwal GP (1990) Glycerol. Adv Biochem Eng Biotechnol 41:95–128

Ali N, Khan MN (2014) Screening, identification and characterization of alcohol tolerant potential bioethanol producing yeasts. Curr Res Microbiol Biotechnol 2(1):316–324

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215(3):403–410

Aslankoohi E, Rezaei MN, Vervoort Y, Courtin CM, Verstrepen KJ (2015) Glycerol production by fermenting yeast cells is essential for optimal bread dough fermentation. PLoS One 10(3):e0119364

Babazadeh R, Lahtvee PJ, Adiels CB, Goksör M, Nielsen JB, Hohmann S (2017) The yeast osmostress response iscarbon source dependent. Sci Rep 7(1):990

Bamforth CW (2005) The science underpinning food fermentations. Fermentation and micro-organisms, vol 1. Blackwell Science Ltd Publishing, Oxford, pp 78–84

Bhasin S, Modi HA (2012) Optimization of fermentation medium for the production of glucose isomerase using *Streptomyces* sp. SB-P1. Biotechnol Res Int Article ID 874152:1–10

Bubnová M, Zemančíková J, Sychrová H (2014) Osmotolerant yeast species differ in basic physiological parameters and in tolerance of non-osmotic stresses. Yeast 31:309–321

Butinar L, Santos S, Spencer-Marrtins I, Gunde-Cimerman N (2005) Yeast diversity in hypersaline habitats. FEMS Microbiol Lett 244:229–234

Chen G, Yao S (2013) The effect of created local hyperosmotic microenvironment in microcapsule for the growth and metabolism of Osmotolerant Yeast Candida krusei. BioMed Res Inter 2013(4):467263

Connell L, Redman R, Craig S, Scorzetti G, Iszard M, Rodriguez R (2008) Diversity of soil yeasts isolated from South Victoria Land, Antarctica. Microbiol Ecol 56:448–459

Costa E, Teixidó N, Usall J, Atarés E, Viñas I (2002) The effect of nitrogen and carbon sources on growth of the biocontrol agent *Pantoeaagglomerans* strain CPA-2. Lett Appl Microbiol 35:117–120

Ehsani M, Fernández MR, Biosca JA, Julien A, Dequin S (2009) Engineering of 2,3-butanediol dehydrogenase to reduce acetoin formation by glycerol-overproducing, low-alcohol *Saccharomyces cerevisiae*. Appl Environ Microbiol 75:3196–3205

Fan X, Burton R, Zhou Y (2010) Glycerol (byproduct of biodiesel production) as a source for fuels and chemicals—mini review. Open Fuel Energy Sci 3:17–22

Fernanda L, Magda S, Candida L (1999) Active glycerol uptake is a mechanism underlying halotolerance in yeasts: a study of 42 species. Microbiol 45:2577–2585

Freeman GG, Donald GMS (1957) Fermentation processes leading to glycerol: II. Studies on the effect of sulfites on viability, growth and fermentation of *Saccharomyces cerevisiae*. Appl Microbiol 5:211–215

Ghindea R, Vassu T, Stoica I, Tanase AM, Csutak O (2009) Preliminary taxonomic studies on yeast strains isolated from dairy products. Rom Biotechnol Lett 14(1):4170–4179

Grembecka M (2015) Sugar alcohols-their role in the modern world of sweeteners: a review. Eur Food Res Technol 241:1–14

Hohmann S (2002) Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev 66:300–372

Ikehata H, Tetsuya O (2011) The mechanisms of UV mutagenesis. J Radiat Res 52:115–125

Junior M, Batistote M, Ernandes J (2008) Glucose and fructose fermentation by wine yeasts in media containing structurally complex nitrogen sources. J Inst Brew 114:199–204

Kalle GP, Naik SC, Lashkari BZ (1985) Improved glycerol production from cane molasses by the sulfite process with vacuum or continuous carbon dioxide sparging during fermentation. J Ferment Technol 63:231–237

Klein M, Swinnen S, Thevelein JM, Nevoigt E (2017) Glycerol metabolism and transport in yeast and fungi: established knowledge and ambiguities. Environ Microbiol 19(3):878–893

Kuhn J, Müller H, Salzig D, Czermak P (2015) A rapid method for an offline glycerol determination during microbial fermentation. Electron J Biotechnol 18(3):252–255

Kumar A, Poonam B, Lal CR (2010) Isolation and molecular characterization of phosphate solubilizing *Enterobacter* and *Exiguobacterium* species from paddy fields of Eastern Uttar Pradesh, India. Afr J Microbiol Res 4:820–829

Kumari R, Pramanik K (2012) Improvement of multiple stress tolerance in yeast strain by sequential mutagenesis for enhanced bioethanol production. J Biosci Bioeng 114(6):622–629

Kurtzman CP, Fell JW, Boekhout T (2011) The yeasts, a taxonomy study, 5th edn. Elsevier Science Publisher, Amsterdam, p 2354

Kurtzman CP, Fell JW (1998) The yeasts-a taxonomic study, vol 149, 4th edn. Elsevier Science Publ BV, Amsterdam, p 1055

Leandro MJ, Sychrova H, Prista C, Loureiro-Dias MC (2011) The osmotolerant fructophilic yeast *Zygosaccharomyces rouxii* employs two plasma-membrane fructose uptake systems belonging to a new family of yeast sugar transporters. Microbiology 157(2):601–608

Lin Y, Tanka S (2006) Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol 69:627–642

Lodder J, Kreger-Van Rij NJW (1952) The yeasts: a taxonomic study. North-Holland Pub, Amsterdam, p 713

Martinez F, Corio-Costet MF, Levis C, Coarer M, Fermaud M (2008) New PCR primers applied to characterize distribution of *Botrytis cinerea* populations in French vineyards. Vitis 47:217–226

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Biochem 31:426–428

Mirończuk AM, Rzechonek DA, Biegalska A, Rakicka M, Dobrowolski A (2016) A novel strain of *Yarrowia lipolytica* as a platform for value-added product synthesis from glycerol. Biotechnol Biofuels 9:180

Nancib N, Nancib A, Boudjelal A, Benslimane C, Blanchard F, Boudrant J (2001) The effect of supplementation by different nitrogen sources on the production of lactic acid from date juice by *Lactobacillus casei* subsp, *rhamnosus*. Bioresour Technol 78:149–153

Nissen T, Hamann CW, Kielland-Brandt MC, Nielsen J, Villadsen J (2000) Anaerobic and aerobic batch cultivations of *Saccharomyces cerevisiae* mutants impaired in glycerol synthesis. Yeast 16:463–474

Orlic S, Arroyo-Lopez FN, Huic-Babic K, Lucilla I, Querol A, Barrio E (2010) A comparative study of the wine fermentation performance of *Saccharomyces paradoxus* under different nitrogen concentrations and glucose/fructose ratios. J Appl Microbiol 108:73–80

Overkamp KM, Bakker BM, Kötter P, Luttik MAH, van Dijken JP, PronK JT (2002) Metabolic engineering of glycerol production in *Saccharomyces cerevisiae*. Appl Environ Microbiol 68(6):2814–2821

Padilla B, Gil J, Manzanares P (2018) Challenges of the non-conventional yeast *Wickerhamomyces anomalus* in winemaking. Ferment 4(3):68

Passoth V, Fredlund E, Druvefors UÄ, Schnürer J (2006) Biotechnology, physiology and genetics of the yeast *Pichia anomala*. FEMS Yeast Res 6(1):3–13

Patil SV (2013) Kinetics, simulation and scale up of process for fermentative production of glycerol and arabitol using *Hansenula anomala*. PhD thesis in biotechnology, University of Pune, India, p 258

Petrovska B, Winkelhausen E, Kuzmanova S (1999) Glycerol production by yeasts under osmotic and sulfite stress. Can J Microbiol 45:695–699

Piddocke M, Kreisz S, Heldt-Hansen H, Nielsen K, Olsson L (2009) Physiological characterization of brewer's yeast in high-gravity beer fermentations with glucose or maltose syrups as adjuncts. Appl Microbiol Biotechnol 84:453–464

Remize F, Barnavon L, Dequin S (2001) Glycerol export and glycerol-3-phosphate dehydrogenase, but not glycerol phosphatase, are rate limiting for glycerol production in *Saccharomyces cerevisiae*. Metab Eng 3:301–312

Revin V, Atykyan N, Lyovina E, Dragunova Y, Ushkina V (2018) Effect of ultraviolet radiation on physiological and biochemical propertiesof yeast *Saccharomyces cerevisiae* during fermentation of ultradispersed starch raw material. Electron J Biotechnol 31:61–66

Sahoo DK, Agarwal GP (2001) An investigation on glycerol biosynthesis by an osmophilic yeast in a bioreactor. Process Biochem 36:839–846

Sancar GB, Smith FW (1989) Interactions between yeast photolyase and nucleotide excision repair proteins in *Saccharomyces cerevisiae* and *Escherichia coli*. Mol Cell Biol 9(11):4767–4776

Savergave LS, Gadre RV, Vaidya BK, Jogdand VV (2013) Two-stage fermentation process for enhanced mannitol production using *Candida magnoliae* mutant R9. Bioprocess Biosyst Eng 36:193–203

Shahsavarani H, Sugiyama M, Kaneko Y, Chuenchit B, Harashima S (2012) Superior thermotolerance of *Saccharomyces cerevisiae* for efficient bioethanol fermentation can be achieved by overexpression of RSP5 ubiquitin ligase. Biotechnol Adv 30(6):1289–1300

Shen B, Hohmann S, Jensen RG, Bohnert HJ (1999) Roles of sugar alcohols in osmotic stress adaptation. replacement of glycerol by mannitol and sorbitol in yeast. Plant Physiol 121:45–52

Silva-Graça M, Lucas C (2003) Physiological studies on long-term adaptation to salt stress in the extremely halotolerant yeast *Candida versatilis* CBS 4019 (syn. *C. halophila*). FEMS Yeast Res 3(3):247–260

Sivasankaran C, Ramanujam PK, Shanmugam S, Sathendra R, Balasubramanian B, Mani J (2014) Comparative study on *Candida* sp. for the production of glycerol. Chem Tech 6(12):5058–5063

Snoep L, Mrwebi M, Schuurmans M, Rohwer JM, Teixeira de Mattos MJ (2009) Control of specific growth rate in *Saccharomyces cerevisiae*. Microbiology 155:1699–1707

Sulieman A, Esra AM, Abdelgadir WS (2015) Isolation and identification of yeasts from the different stages of *Hulu-mur* fermentation. J Microbiol Res 5(2):71–76

Taherzadeh MJ, Adler L, Lidén G (2002) Strategies for enhancing fermentative production of glycerol—a review. Enz Microb Technol 31:53–66

Tapia VE, Anschau A, Coradini AL, Franco T, Deckmann AC (2012) Optimization of lipid production by the oleaginous yeast *Lipomyces starkeyi* by random mutagenesis coupled to cerulenin screening. AMB Express 2:1–8

Thome PE (2007) Cell wall involvement in the glycerol response to high osmolarity in the halotolerant yeast *Debaryomyces hansenii*. Antonie Leeuwenhoek 91:229–235

Varela C, Pizarro F, Agosin E (2004) Biomass content governs fermentation rate in nitrogen-deficient wine musts. Appl Environ Microbiol 70(6):3392–3400

Vijaikishore P, Karanth NG (1986) Glycerol production by immobilized cells of *Pichia farinosa*. Biotechnol Lett 8:257–260

Walker GM (1998) Yeast physiology and biotechnology. Wiley, London, p 362 **(ISBN: 978-0-471-96446-9)**

Walker GM (2011) *Pichia anomala:* cell physiology and biotechnology relative to other yeasts. Antonie Leeuwenhoek 99(1):25–34

Wang ZX, Zhuge J, Fang H, Prior BA (2001) Glycerol production by microbial fermentation: a review. Biotechnol Adv 19:201–223

Watanabe T, Watanabe I, Yamamoto M, Ando A, Nakamura T (2011) A UV-induced mutant of *Pichia stipitis* with increased ethanol production from xylose and selection of a spontaneous mutant with increased ethanol tolerance. Bioresour Technol 102(2):1844–1848

White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press Inc, New York, pp 315–322

Yaçlin SK, Özbas ZY (2005) Determination of growth and glycerol production kinetics of a wine yeast strain *Saccharomyces cerevisiae* Kalecik1 in different substrate media. World J Microb Biot 21:1303–1310

Yaçlin SK, Özbas ZY (2008) Effects of pH and temperature on growth and glycerol production kinetics of two indigenous wine strains of *Saccharomyces cerevisiae* from Turkey. Braz J Microbiol 39:325–332

Yin Y, Petes TD (2013) Genome-wide high-resolution mapping of UV-induced mitotic recombination events in *Saccharomyces cerevisiae*. PLoS Genet 9(10):e1003894

Zhang J, Liu D, Xie D, Wang Y, Sun Y (2002) Production of glycerol by fermentation using osmophilic yeast *Cadida krusei* with different starchy substrates. Enz Microbiol Technol 30(6):758–762

Zhang G, Lin Y, Qi X, Wang L, He P, Wang Q, Ma Y (2015a) Genome shuffling of the nonconventional yeast *Pichia anomala* for improved sugar alcohol production. Microb Cell Fact 14(1):112

Zhang M, Shi J, Jianga L (2015b) Modulation of mitochondrial membrane integrity and ROS formation by high temperature in *Saccharomyces cerevisiae*. Electron J Biotechnol 18(3):202–209

Zhang M, Wu W, Gu, X, Weichen Y, Qi F, Jiang X, Huang J (2018) Mathematical modeling of fed-batch fermentation of *Schizochytrium* sp. FJU-512 growth and DHA production using a shift control strategy. Biotech 3:162. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5840084/

Zhao XQ, Bai FW (2009) Mechanisms of yeast stress tolerance and its manipulation for efficient fuel ethanol production. J Biotechnol 144(1):23–30

Zhao X, Procopio S, Becker T (2015) Flavor impacts of glycerol in the processing of yeast fermented beverages: a review. J Food Sci Technol 52(12):7588–7598

Zhuge J, Fang HY, Wang ZX, Chen DZ, Jin HR, Gu HL (2001) Glycerol production by a novel osmotolerant yeast *Candida glycerinogenes*. Appl Microbiol Biotechnol 55:686–692