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Abstract

Water consumption has become a serious concern in renewable fuel production as demand for biofuels increases to address environmental issues associated with the use of fossil fuels. In recent years, several research groups have suggested seawater (SW) as a promising alternative to fresh water (FW) for the production of biofuels. However, the use of seawater rather than fresh water in the fermentation process is still a relatively unexplored area of research. In this paper, the tolerance of the marine yeast *S. cerevisiae* AZ65 to the presence of salts in seawater was investigated. The results indicated that *S. cerevisiae* AZ65 grew well in media containing up to 10.5% (w/v) sea salts and 20% (w/v) glucose compared with an industrial distiller's strain, *S. cerevisiae* NCYC2592. A multi-stage batch fermentation process was also investigated to increase ethanol productivity. Two different seawater based media were used: SW-YPD medium and SW-molasses medium. *S. cerevisiae* AZ65 achieved an ethanol concentration of 113.52 gL^{-1} with a productivity of 4.15 $g\ L^{-1}h^{-1}$ using SW-YPD medium and an ethanol concentration of 50.32 gL^{-1} with a productivity of 2.46 $g\ L^{-1}h^{-1}$ using SW-molasses medium. These results confirmed the potential of seawater and marine yeasts for implementation in the bioethanol industry using a multi-stage fermentation process.

Keywords:

Bioethanol; marine yeast; marine fermentation; seawater; *Saccharomyces cerevisiae*; water footprint; Bioenergy; biofuels

1. Introduction

The global demand for energy is continuously increasing due to the modernization of people's lifestyles and the rise in the world population. Fossil fuels including petroleum, coal and natural gas are traditionally used to satisfy the demand for energy. However, the world has realised the environmental damage caused by the excessive use of fossil fuels and a shift towards a renewable energy-based economy is globally encouraged. Biofuels are thought to provide the best alternative to fossil fuel because of their capacity for atmospheric CO_2 capture [1, 2]. According to the International Energy Agency (IEA), global biofuel production reached 152 billion litres in 2018 with an anticipated increase of 3% annually until 2024. However, IEA stated that the sustainable annual growth rate of biofuels should be 10% until 2030 to keep pace with the Sustainable Development Scenario (SDS), (www.iea.org/tcep/transport/biofuels/).

Several reports have indicated that energy demands and water consumption are interrelated [3-5]. The expansion in biofuel production has raised, for decades, the food versus fuel debate. Currently, a particular focus is being given to the water consumption of bioenergy, especially for biofuel production [6, 7]. This could soon divert the debate into water versus fuel. The global average water footprint (WF) of 1st generation bioethanol has been estimated at around 2500 L H_2O/L EtOH [8]. The vast majority of this water is consumed during the agricultural process for the biomass production; however, a considerable amount of water is consumed during the industrial process (hydrolysis, fermentation and distillation) for bioethanol production. Although water is recycled during the process, 1st generation bioethanol production requires 2.7 to 5.8 L of water for every L of ethanol produced. Similarly, 2nd generation bioethanol production consumes 4.5 to 5.3 L water for every L of ethanol produced [9].

Seawater is abundant and contains a variety of minerals; hence, it is a promising sustainable alternative to fresh water for bioethanol production. Using seawater in the fermentation process

will reduce the WF of bioethanol [10-12]. There is no need for water recycling when using seawater and therefore, the distilled water produced during ethanol distillation could be considered an additional product. This will convert bioethanol production from a water consuming to a water producing process. The use of seawater in bioethanol production will potentially increase the overall efficiency and profitability of the process by producing distilled water and sea salts as additional products. It will also reduce the cost of adding minerals into the fermentation media. In addition, the dried distillers' grains with solubles (DDGS) produced from the seawater-based bioethanol production will contain sea salts, potentially adding additional value to this DDGS as an animal feed (Figure 1).

Seawater contains a high level of salt (about 35 gL^{-1}) along with various inhibitors [13]. Hence, a halotolerant yeast strain with high fermentation capacity is required for seawater-based bioethanol production. The special features of marine yeasts, particularly high osmotolerance and halotolerance, make them an ideal choice for bioethanol production especially when seawater is used instead of freshwater for preparing the fermentation medium [14]. The use of marine yeast strains for bioethanol production using freshwater has already been demonstrated [15-17]. The current study aims to investigate an industrially sound process for the production of bioethanol using seawater-based media and marine yeasts. The growth rate of marine yeast in the presence of high concentrations of sea salts and a very high concentration of glucose was investigated. Then, a multi-stage fermentation procedure using very high gravity seawater-based fermentation media was carried out to achieve high ethanol productivity. Sugarcane molasses prepared in seawater was used in this study as an example of an industrial substrate for bioethanol production. Sugarcane molasses is a by-product of the sugar industry and contains up to 50% fermentable sugars. It is widely used as a carbohydrate substrate in the production of baker's yeast and bioethanol production [18-20]. During molasses clarification, large quantities of salts are generated [14, 18] which is similar to the case for lignocellulosic

hydrolysis in 2nd generation bioethanol production and seaweed hydrolysis in the 3rd generation bioethanol production, where salts are generated as a result of the neutralisation step. Hence, molasses is an ideal substrate to use as an example for investigating the tolerance and fermentation ability of a new yeast strain.

2. Materials and Methods:

2.1. Microorganisms

Two marine yeast strains, *S. cerevisiae* AZ65 and *S. cerevisiae* AZ118, were used in this study. These strains were previously isolated and identified by Zaky et al. (2016) [21] . Propagation of each strain was as described previously in Zaky et al., (2016) [21]. Briefly, seawater-YPD (SW-YPD) medium was used, which contains (w/v) 1% yeast extract, 2% peptone, and 2% glucose at pH 6 ±0.20. The propagation was performed under aerobic conditions in an orbital shaker (150 rpm) at 30°C for 48 h. A working stock culture of each strain was prepared from cultures propagated on SW-YPD agar slants (as above + 2% agar) and kept at 4°C until required.

The terrestrial yeast strain *S. cerevisiae* NCYC2592 was used in this study as the reference strain. It was maintained using the same method as the marine strains, but the YPD media were prepared using distilled water. *S. cerevisiae* NCYC2592 is an industrial distiller's strain that has been well characterised previously for sugar utilisation and tolerance to various inhibitors [13, 22]

2.2. Natural Seawater

Natural Seawater (SW) used in this study was obtained from Skegness, which is located on the North Sea coast of Lincolnshire, UK. Seawater was filtered using glass microfiber filters (pore

size, 1.20 µm; Whatman®) to remove suspended particles, then autoclaved at 121°C for 15 min, and stored at 4°C until required.

2.3. Synthetic seawater (SSW)

Synthetic seawater (SSW) was prepared according to the formula suggested by Fang et al. (2015) [23] which contains NaCl, $MgCl_2$, $MgSO_4$, $CaCl_2$, KCl, $NaHCO_3$, and NaBr at concentrations of (gL^{-1}) 27.13, 2.50, 3.38, 1.17, 0.74, 0.21, and 0.09 respectively. Double strength (2x SSW) and triple strength (3x SSW) Synthetic seawater was also prepared (Table 1).

2.4. Seawater glucose solution 100% (w/v)

1000 g glucose was transferred into a 2-L Duran bottle containing 425 mL of filtered preheated seawater (at 80°C). A sugar suspension was obtain after closing and shaking the 2-L Duran bottle. The 2-L Duran bottle was then placed in a water bath at 70°C and shaken from time to time until the sugar was totally dissolved and formed a clear colourless glucose syrup. The syrup was left to cool down to room temperature, then transferred into a 1-L volumetric flask. Filtered seawater (at 25°C) was added to the syrup to a final volume of 1 litre resulting in a stable supersaturated solution containing 1 kg glucose per litre of solution, that is, 100% w/v. Note that it is necessary to use a 2 litre bottle in the initial preparation step since the volume of solution at 70°C is considerably greater than after it is cooled to 25°C. Sterilisation was conducted using a disposable vacuum filter with a pore size of 0.45µm, (Fisher Scientific UK Ltd).

2.5. Sugarcane Molasses (SM)

The SM used in this study was a commercial product (animal feed supplement grade) called 'NAF Molasses' that was purchased online from Amazon.co.uk. This product is a crude natural

sugarcane molasses containing 45% (w/w) total sugar as stated on the label. Clarification of crude molasses was carried out using seawater to produce 50% (w/v) SW-Molasses solution as described previously [14].

2.6. Assessing the tolerance of marine *S. cerevisiae* to osmotic and salt stress

YPD media containing 5, 10, 15 and 20% glucose were prepared in Reverse Osmosis Water (ROW) and SSW of different compositions (1x SSW, 2x SSW and 3x SSW) (Table 1). A 96-well cell culture plate was loaded with the media at 200 µL per well. The two marine *S. cerevisiae* strains (AZ65 and AZ118) and the reference strain *S. cerevisiae* NCYC2592 were grown aerobically to exponential phase in YPD broth at 30°C using the working stock cultures. Cells were harvested by centrifugation at 1811× *g* for 3 min (Eppendorf 5810 R, UK) and washed 3 times using ROW. Yeast suspension with, Optical Density measured at 600 nm wavelength, (OD_{600}) of 2.00 was prepared from each strain using ROW. The 96-well cell culture plate was inoculated by pipetting 5 µL of the yeast suspension into each well to reach a starting OD_{600} of approximately 0.05.

Plates were incubated in a TECAN Infinite M200 Pro plate reader (Mannedorf, Switzerland) at 30°C. The plate reader records OD_{600} every 30 minutes using Magellan (7.1, SP1) software. The plates were orbitally shaken for 1 minute before converting pixel density to a signal value reflecting cell growth. After completion of the run, the signal data was compiled and automatically converted into Microsoft® Excel compatible data by Magellan software. The assay was performed in triplicate and the average reading was plotted.

2.7. Fermentation using 15 L bioreactors

Fermentations for bioethanol production were conducted in a modified batch mode using 15 L, *in-situ* sterilisable, stainless steel bioreactors (Techfors-S, Infors-HT, Bottmingen, Switzerland) with 10 L working volumes.

2.7.1. Inoculum preparation

Inocula of the marine *S. cerevisiae* AZ65 were prepared for 10 L fermentation media using the following steps: i) a 50-mL conical flask contains 20 mL SW-YPD medium was inoculated with a loopful of yeast from the yeast slope culture, then incubated in an orbital shaker at 30ºC and 150 rpm for 48 h, ii) the culture was then transferred into a 500-mL conical flask containing 200 mL SW-YPD medium and incubated under the previous conditions, iii) The yeast culture obtained was used to inoculate two 2000-mL conical flasks, each containing 1000 mL of SW-YPD medium, and these were incubated under the previous conditions, iv) yeast cells were harvested using a centrifuge (Beckman, Model-J2-21) at 10,714× *g* for 5 min) harvested yeast cells were washed 3 times using sterile SW and centrifugation, then suspended in sterilised SW to form a concentrated yeast inoculum with OD_{600} of about 500. Aseptic conditions were maintained in every step.

2.7.2. Three-stage batch fermentation using YPD medium prepared using seawater (SW-YPD medium)

The first stage was starting by transferring 8 L of the propagation medium, composed of 1% yeast extract, 2% peptone, and 4% glucose dissolved in seawater, into the bioreactors. The pH of the medium was adjusted to 6.0 using NaOH (50% w/v). The sterilisation for the medium and bioreactor was carried out at 121°C for 15 min. Vessels were cooled down to 30ºC and aseptically inoculated using the yeast inoculum to achieve an initial cell concentration of about OD_{600} 2.0. The propagation stage (1st stage) was carried out aerobically using compressed air at a rate of 10 L/min and stirring rate of 200 rpm. This stage was conducted for 10 h at 30ºC. Samples were collected at regular time points to determine the growth rate by monitoring the OD changes using a spectrophotometer. At the end of the propagation stage, air supply was stopped and the bioreactors were flushed with nitrogen (at a rate of 10 L/min) for 30 min to establish anaerobic condition for the second stage. The temperature of the reactors was also increased to 35ºC.

The second stage was started by adding 2 L of sterilised glucose solution (100% w/v) prepared in seawater to the bioreactors in order to obtain a glucose concentration around 20% (w/v) in the fermentation medium. A sample was taken directly after glucose addition to analyse the concentrations of glucose, glycerol, ethanol and yeast cell density at the beginning of the ethanol fermentation stage. Then, samples were withdrawn at regular time intervals over 20 h to assess the fermentation kinetics by monitoring the changes in the concentrations of glucose, glycerol, ethanol and yeast cell density. The third stage was started by the further addition of 1 L of the sterilised glucose solution (100% w/v) prepared in seawater to the bioreactors. The fermentation conditions and sampling were conducted as in the 2nd stage. The pH was adjusted to 6.0 at the beginning of each stage by adding concentrated NaOH (50% w/v).

2.7.3. Two-Stage Batch fermentation using molasses media prepared using seawater (SW-Molasses medium)

Bioreactors were supplied with 4 L of seawater, then sterilised at 121°C for 15 min. One L of the 50% (w/v) SW-Molasses solution was transferred aseptically into the bioreactors to obtain a propagation medium of 10% (w/v) molasses concentration. The medium was supplemented with 3 mL of antifoam (50% w/v in seawater) and 10 mL of urea solution (20% w/v, in seawater). The medium was aerated for 1 h using compressed air at a rate of 10 L/min after adjusting the pH to 5.5 using NaOH (50% w/v). The propagation stage (1st stage) was started by inoculating the fermentation medium with yeast to achieve a cell density of an OD_{600} of about 2.0. The fermentation was carried out for 14 h at 30ºC with a stirring rate of 200 rpm. Samples were collected at regular time points to determine the growth rate by monitoring OD_{600} using a spectrophotometer. At the end of the propagation stage, the air supply was stopped and the bioreactors were flushed with nitrogen (at a rate of 10 L/min) for 30 min to establish anaerobic conditions for the second stage; also, the temperature of the reactors was increased to 35ºC. The second stage (ethanol production stage) was started by adding 1 L of sterilized seawater

and 4 L of the 50% (w/v) SW-Molasses solution to the bioreactors in order to obtain a fermentation medium with 20% (w/v) molasses. A sample was taken directly after sugar addition to analyse the concentrations of glucose, glycerol, ethanol and yeast cell density at the beginning of the ethanol fermentation stage. Samples were withdrawn at regular time intervals over 30 h to assess the fermentation kinetics by monitoring the changes in the concentrations of sugars (glucose, fructose, and sucrose), glycerol and ethanol as well as the pH and yeast cell density.

2.8 HPLC analysis

Samples were obtained from the bioreactors manually through a sampling port. The OD and pH were measured directly after sampling, then samples were centrifuged at 1811× *g* for 5 min. The supernatant was stored at -20°C until required for analysis. Ethanol, glycerol, acetic acid, glucose, sucrose and fructose were analysed using the HPLC method as developed by Zaky et al (2017) [24].

3. Results

3.1 Growth of marine yeast exposed to increased osmotic stress induced by glucose and seawater salts

Results of growth using increasing osmotic stress (up to 20% glucose) in ROW water revealed that there were no differences in growth between the reference and marine strains. All strains maintained a good growth rate and reached stationary phase in less than 20 h of propagation (Figure 2 Aa, Ba & Ca). When 1x SSW was used for preparing the media, marine strains grew faster than the reference strain especially with the media containing 15 and 20% glucose (Figure 2 Ab, Bb & Cb). Growth using 2x SSW showed that marine yeast grew faster than the reference strain (Figure 2 Ac, Bc & Cc). Using YPD media containing 2x SSW salts and 20%

glucose showed that *S. cerevisiae* AZ65 grew faster than AZ118 while a significant reduction in the growth of *S. cerevisiae* NCYC2592 was recorded (Figure 2 Cc). Growth results using 3x SSW demonstrated that the marine strain AZ65 was more tolerant to osmotic stress induced by glucose and salts compared with the other marine strain, AZ118 (Figure 2 Ad, Bd & Cd). Using 3x SSW and 20% glucose almost completely inhibited the growth of the reference strain (Figure 2 Cd).

3.2 Ethanol production by the marine yeast strain *S. cerevisiae* AZ65 in a 15 L bioreactor using SW-YPD medium in Three-Stage batch fermentation process

The first stage (yeast propagation) was conducted to encourage rapid production of active yeast cells for the ethanol production stage (the 2nd stage). The 2nd stage (ethanol production) was conducted at a high concentration of active yeast obtained from the 1st stage and high glucose concentration (20% w/v). The 3rd stage was conducted to test the tolerance and fermentation ability of the marine yeast strain AZ65 in the presence of high ethanol, glucose, and salt concentrations.

Figure 3 shows the relationship between glucose utilisation, yeast growth, ethanol production and glycerol production throughout the three stages of the process. In the 1st stage, yeast density increased rapidly from an OD of 2.22 ±0.11 to an OD of 15.25 ±0.35 during 8 hours of propagation, at which time the glucose had been almost fully utilised and ethanol reached its maximum concentration in this stage at 16.08 ±0.63 gL^{-1}. The OD continued to increase for the next 2 h reaching an OD of 17.75 ±0.92 while the ethanol concentration slightly decreased. The increase in OD during the last 2 h may be due to the maturation of the cells as the glucose had already been consumed and ethanol production stopped. The highest ethanol yield and productivity were achieved after 8 hours of the fermentation and were 74.10% and 2.01 $g\ L^{-1}h^{-1}$ respectively. By the end of this stage, glycerol and acetic acid concentrations reached 4.08 ±0.05 and 0.21 ±0.01 gL^{-1}, respectively (Table 2 & Figure 3).

In the 2nd stage, the addition of the glucose solution diluted the concentrations of yeast cells, ethanol, glycerol, and acetic acid that were obtained from the first stage. Hence, this stage started at a yeast cell concentration with OD value of 13.95 ±0.21, ethanol 13.41 ±0.26 gL^{-1}, glycerol 3.03 ±0.06 gL^{-1}, acetic acid 0.18 ±0.02 gL^{-1} while glucose concentration was recorded as 196.23 ±1.48 gL^{-1}. During 20 hours of fermentation in this stage, around 73.32 ±1.07 gL^{-1} of ethanol was produced making the total ethanol in the reactor 86.72 ±1.33 gL^{-1}. However, glucose was not fully utilised by the end of this stage, so the maximum ethanol yield was only 73.26%, but the yield based on the utilised glucose ranged between 89.64 - 85.84% throughout the second stage. Excellent ethanol productivity was achieved during this stage, ranging from 3.67 - 4.15 $g\ L^{-1}h^{-1}$. By the end of this stage, glycerol and acetic acid concentrations were recorded as15.16 ±20 and 0.86 ±0.06 gL^{-1} respectively. In addition, yeast cells continued to increase especially during the first 3 h of this stage benefiting from the remaining oxygen of the 1st stage. The yeast cells reached an OD of 24.65 ±0.78 by the end of this stage (Table 2 & Figure 3).

The 3rd stage of this fermentation experiment started at 129.62 ±0.62 gL^{-1} glucose, 13.24 ±0.91 gL^{-1} glycerol, 0.76 ±0.02 gL^{-1} acetic acid, 75.50 ±1.79 gL^{-1} ethanol and OD of 21.60 ±0.85. The reduction of the OD and concentrations of glycerol, acetic acid and ethanol was a result of adding the glucose solution. Yeast continued to produce ethanol but at a slower rate compared with the second stage. The ethanol concentration reached 113.52 ±0.01 gL^{-1} after 24 hours of fermentation. Only 65.25% of the glucose had been utilised during the 3rd stage, hence the maximum ethanol yield in this stage was 57.51%; however, the yield based on the utilised glucose ranged from 74.31 to 90.73% throughout the stage. The highest ethanol productivity was 1.86 $g\ L^{-1}h^{-1}$, which was recorded after 4 h of fermentation, then slightly decreased to around 1.60 $g\ L^{-1}h^{-1}$ throughout this stage. Yeast concentration stayed almost constant at an

OD of around 21.5. By the end of this stage, glycerol and acetic acid concentrations were 18.31 ±0.77 and 0.98 ±0.13 gL^{-1} respectively (Table 2 & Figure 3).

3.3 Ethanol production by the marine yeast strain *S. cerevisiae* AZ65 in a 15 L bioreactor using SW-Molasses medium in a Two-Stage batch fermentation process

In this experiment, a 2-stage batch fermentation process was investigated to improve the bioethanol productivity using molasses media. The first stage was conducted to propagate the yeast to obtain high numbers of active yeast cells for the ethanol production stage (the 2nd stage).

In the first stage, yeast density increased from an OD of 1.65 ±0.04 to an OD of 9.79 ±0.02 over 14 hours at which time 98.68% of sugars had been utilised. By the end of this stage, 11.32 ±1.67 gL^{-1} of ethanol was produced which accounted for 49.13% of the theoretical yield, and the productivity was 0.81 $g\ L^{-1}h^{-1}$, and the concentration of glycerol reached 3.40 ±0.10 gL^{-1} (Table 3 & Figure 4). Although this stage was conducted aerobically to produce yeast biomass, the results indicated that almost 50% of the sugars were consumed to produce ethanol. This highlights the importance of the fed batch approach with low sugar concentration throughout the fermentation time when using SW-molasses media for baker's yeast production to keep ethanol production at the lowest level.

The second stage started with the removal of 4 L of the yeast culture obtained from the 1st stage. The removed culture was centrifuged to extract the yeast biomass which was then re-suspended in 4 L of 50% (w/v) molasses and returned to the reactor. As a consequence; the total sugar concentration at the start of this stage was 91.27 ±5.90 gL^{-1} while the concentrations of yeast cells, glycerol and ethanol were diluted to 5.89 ±0.83 OD, 2.73 ±0.19 gL^{-1} and 7.97 ±0.60 gL^{-1} respectively. During 30 hours of fermentation, yeast cells continued to increase - especially in the first 9 hours - and reached an OD of 10.39 ±1.44 by the end of this stage.

Around 42.35 gL^{-1} of ethanol was produced during this stage making the total ethanol in the fermenter 50.32 ±1.95 gL^{-1} when 98.12% of the sugars were utilised. Ethanol yield reached 95.35% after 30 hours of fermentation and ethanol productivity was recorded as 2.46 $g\ L^{-1}h^{-1}$ after 3 hours of fermentation, then decreased gradually to 1.41 $g\ L^{-1}h^{-1}$ at the end of the fermentation. The production of glycerol was recorded as 10.55 ±0.14 gL^{-1} by the end of this stage (Table 3 & Figure 4).

4. Discussion

The results revealed that the growth rate of *S. cerevisiae* decreased as sea salts concentration increased in the propagation media. However, the marine *S. cerevisiae* strains (AZ65 and AZ118) had higher tolerance to the presence of salts when compared with the terrestrial yeast strain *S. cerevisiae* NCYC2592. *S. cerevisiae* is only moderately salt tolerant and other yeast species such as *Zygosaccharomyces rouxii* have been shown to be more tolerant to the presence of salt than *S. cerevisiae* [25]. Capusoni et al. (2019) also reported that the growth of two marine *Debaryomyces hansenii* strains was reduced by 42 - 46% when cultivated in media containing sea salts with 2 M NaCl [12]. Their investigation suggested that this inhibition occurred mainly due to membrane depolarization and reduced membrane permeability induced by the presence of hyper-osmotic stress on the cell [12]. This reduction in biomass yield may be a reflection of the cell's greater energy expenditure to maintain the balance of the cell's internal osmotic pressure. We previously reported that the presence of high amounts of salts, especially NaCl, slowed down the fermentation rates of marine and terrestrial *S. cerevisiae* at different levels [14]. On the other hand, Petrovic et al., (2002) reported that the black yeast *Hortaea werneckii* grew well in a medium containing high concentrations of NaCl; however, the growth rate decreased by up to 60% in minimal medium at a very high salinities between 10 and 17% NaCl [26].

Bioethanol is usually produced in a batch fermentation processes. This is simple, has less chance of contamination, is easy to manage the feedstock and does not require skilled labour. However, the productivity is low due to low yeast concentration at the beginning of the process and the high concentration of the fermentation substrate. Fed-batch and continuous fermentation are two processes applied in bioethanol fermentation in order to overcome the limitations associated with the convenient batch fermentation process. Both processes provide higher productivity, shorter fermentation time and lower toxic effect of the medium components compared with batch fermentation. However, they are more sophisticated processes and are associated with many disadvantages including the requirement for skilled labour, increased possibility of contamination and the loss of substrate [27].

The Two-Stage and Three-Stage batch fermentation processes investigated in this paper provide an easy solution to overcome the low ethanol productivity in batch fermentation by increasing the yeast density in the fermenter before starting the ethanol production stage. Maximum ethanol productivity in the 2nd stage (the ethanol production stage) in this study reached 4.15 and 2.46 g $L^{-1}h^{-1}$ from SW-YPD media and SW-Molasses media respectively. By contrast, it was reported that the maximum ethanol productivity achieved in a single batch fermentation for bioethanol production using SW-YPD media and SW-Molasses media was 2.49 and 1.43 g $L^{-1}h^{-1}$ respectively [14]. Thus, almost a 2-fold increase in ethanol productivity was achieved when using the Two-Stage and Three-Stage batch fermentation process. This is mainly due to the high yeast cell density at the start of the ethanol production stage. The concentration of yeast cells reached more than OD 17 in 10 h propagation allowing the ethanol stage to start at almost OD 14 yeast cell concentration, while in a single stage fermentation, the yeast cell concentration reached OD 17 after 42 [14]. Providing suitable conditions for yeast propagation including aeration and low sugar concentration during the 1st stage allowed the rapid production of yeast cells. Also, dividing the total sugar concentration into 2 or 3 doses

reduced the osmotic effect of the total concentration on the yeast cells. The results could be further improved by optimising other fermentation conditions such as supplying a low aeration rate during the fermentation stage. Liu et al., (2016) found that supplying low amounts of O_2 during fermentation enhanced the yield and productivity of ethanol in a very high gravity medium [28]. This is because O_2 enhances cell recovery through respiration and the TCA cycle by retaining pivotal cellular components during carbon utilisation and synthesis. Oxygen also helps yeast to synthesise sterols and unsaturated lipids which are required for maintaining a healthy cell membrane [29].

Ethanol productivity decreased dramatically during the 3rd stage. This is presumably due to the increasing stress resulting from adding high amount of glucose to the fermentation medium, which already contained very high concentrations of ethanol and salts. It was reported that osmotic stress and high ethanol concentration inhibits yeast cell growth and represses glucose transporters [27, 30-32]. It was also reported that initial ethanol at high concentration would reduce the sugar assimilation of *S. cerevisiae* by limiting the transport of glucose into the cells [33, 34]. Nguyen et al (2015) show that the glucose uptake rate by the *S. cerevisiae* decreased when the initial ethanol concentration in the medium increased from 23.7 to 94.7 gL^{-1}. In line with that, yeast formation and ethanol production decreased gradually as the initial ethanol concentration increased in the medium from 0 to 94.7 gL^{-1} [35]. Hence, we anticipate that a higher ethanol productivity could have been archived during the 3rd stage if less glucose was added as this means less osmotic stress on the yeast cells. The 3rd stage was conducted mainly to test whether the marine yeast is able to continue the fermentation at such high stresses, and this was found to be the case, as the ethanol concentration reached 113.52 gL^{-1} compared to 93.50 gL^{-1} in the single stage fermentation [14].

Traditional freshwater-based batch bioethanol fermentation using terrestrial *S. cerevisiae* usually yields a maximum concentration of 7 - 8% (v/v) ethanol from glucose concentration of

up to 180 gL^{-1}. Ethanol concentration of 10 - 12% (v/v) could be achieved using a high gravity (HG) ethanol fermentation which has initial glucose concentration ranging from 180 to 220 gL^{-1}. Both, the traditional and HG fermentation methods usually require 50 - 70 hours to reach the maximum ethanol concentration. Only specific strains of *S. cerevisiae* or other microorganisms with higher thresholds for ethanol stress can performing the HG ethanol fermentation as ordinary strains cannot tolerate the targeted ethanol concentration [41, 42]. In comparison, ethanol concentration reached 14.46% (v/v) in 54 hours using the 3-stage fermentation investigated in this study. This indicates the potential of the multi-stage fermentation technique investigated in this study as well as the potential of marine yeast in bioethanol industry especially when seawater-based fermentation media is used.

Acetic acid is one of the most potent inhibitors of yeast growth and fermentation process as it inhibits yeast cell growth by impeding the metabolic functions through intracellular acidification [36, 37]. However, the maximum production here was around 1 gL^{-1}, which is not very toxic, especially considering that marine yeast was reported to be tolerant to many inhibitors including acetic acid compared with terrestrial yeast [13]. On the other hand, glycerol is an osmolyte and is formed in yeast in response to stress factors (especially osmotic stress) [30]. Although glycerol is the main by-product of ethanol fermentation accounting for 4 to 5% of the carbon substrate consumption [32, 38], its formation is inversely linked to ethanol yield. Hence, a low-glycerol-producing strain would have a great positive impact on ethanol yields on an industrial scale [30]. Sucrose is the major sugar in raw molasses (about 50% of the total sugars), however, the chemical analysis of the clarified molasses showed lower sucrose concentration compared with the concentrations of glucose and fructose. This was due to the addition of concentrated sulphuric acid and heating for 1 hour during the clarification of molasses. We noticed that yeast favours the utilisation of glucose, then sucrose and finally fructose during fermentation. This was in line with results obtained by D'Amore et al. (1989)

[39]. The fermentations in this study used seawater and molasses without any added minerals, indicating that seawater can provide the essential minerals required for cell growth and ethanol production [40].

The results obtained in this paper support our previous findings which indicated that seawater can successfully substitute freshwater for ethanol production without compromising the production rate [14]. They also elucidate the importance of marine yeast as a potential candidate for the bioethanol industry. In addition, this paper demonstrated that applying a multi-stage batch fermentation process significantly improved ethanol productivity. This paper also indicates the potential of marine yeast for bioethanol production using 2^{nd} and 3^{rd} generation biomass substrates which generate salts during hydrolysis. Practically, in these experiments, the fermentations using seawater-based media were carried out in the same manner as fermentation using fresh water on lab scale. However, at the industrial scale, higher corrosion rate is possible because of the high salt content of seawater. This could be avoided by applying a suitable coating layer inside the existing bioreactor and considering the use of corrosion resistant steel when building a new system. Also pipes made from Chlorinated Polyvinyl Chloride (CPVC) or Polyvinyl Chloride (PVC) should be used instead of conventional metal pipes [14]. We anticipate that use of seawater will encourage rapid advances in material science to provide alternative materials to solve the corrosion issue in bioreactors.

Seawater fermentation is a sustainable approach that aims to reduce the water footprint of industrial biotechnology products. In addition to bioethanol production, a seawater fermentation approach has been recently proposed for other industrial biotechnology applications including production of succinic acid [43], baker's yeast [40] carotenoids [44], lipids [45], polyhydroxyalkanoates [46] and others [47]. Successful seawater-based biorefineries may be established as a coastal industry with direct access to clear and clean

seawater. There are several other potential benefits that could be obtained from the proposed coastal-seawater-based biorefineries including; a) easy and direct access to an abundant source of water and minerals; b) direct access to a safe site for biological waste disposal after appropriate waste treatment procedures; c) easy access to low cost and low carbon footprint transportation by sea freight; d) easy access to marine biomass which is a potential negative WF substrate for biofuel production; e) potential for safe storage for the excess CO_2 produced (Figure 1). Seas and oceans have the potential to store huge amounts of CO_2 as an indefinite carbon sink [48, 49]. Storing the CO_2 emitted from bioethanol production under seawater could immensely reduce the carbon footprint of bioethanol and increase the positive environmental impact of bioethanol as a green fuel.

Conclusion

The marine environment has a huge potential as a source of abundant water and halotolerant yeast isolates for biorefineries. The marine *S. cerevisiae* AZ65 strain showed high growth rate under osmotic stress induced by the presence of high concentration of sea salts and/or glucose compared with the reference terrestrial yeast *S. cerevisiae* NCYC2592. Results showed that *S. cerevisiae* AZ65 tolerated up to 10.5% sea salts and YPD medium containing 20% glucose. Applying 3-stage batch fermentation in 15-L bioreactors using seawater media, AZ65 produced 73 gL^{-1} of ethanol from 165 gL^{-1} of glucose within 20 h of fermentation with an ethanol productivity of around 4 $g\ L^{-1}h^{-1}$ in the main ethanol production stage. The concentration of ethanol in the bioreactor reached 113.52 gL^{-1} by the end of the 3rd fermentation stage. Using sugarcane molasses prepared in SW, *S. cerevisiae* AZ65 produced 50.32 gL^{-1} of ethanol with an ethanol productivity ranging from 2.46 to 1.41 $g\ L^{-1}h^{-1}$. These results indicated the potential use of seawater-based media for ethanol production using the marine yeast strain *S. cerevisiae*

AZ65. Full sustainability can be achieved by utilising marine biomass as a carbon substrate in the seawater fermentation process, accommodating the concept of marine fermentation where the whole system runs on marine elements (seawater, marine substrates, and marine microorganisms). Hence, our future research will investigate the use of seaweed in seawater-fermentation systems for the production of bioethanol and bio-based high-value chemicals.

Declarations

Authors' contributions

ASZ proposed, designed, performed the research, and drafted the first manuscript; CEF provided technical advice and reviewed the manuscript; GAT co-supervised the research and reviewed the manuscript; CD supervised the research and reviewed the manuscript. All the authors discussed data, revised the manuscript and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets and yeast strains used in the current study are available from the corresponding author (ASZ) on reasonable requests.

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Tables:

Table 1: Components (gL^{-1}) of YPD media with different glucose and salts concentrations in ROW and SSW of different salt concentrations

	Glucose	Peptone	Yeast Extract	NaCl	$MgCl_2$	$MgSO_4$	$CaCl_2$	KCl	$NaHCO_3$	NaBr
YPD in ROW	100.00	20.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	150.00	20.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	200.00	20.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
YPD in 1x SSW	100.00	20.00	10.00	27.13	2.50	3.38	1.17	0.74	0.21	0.09
	150.00	20.00	10.00	27.13	2.50	3.38	1.17	0.74	0.21	0.09
	200.00	20.00	10.00	27.13	2.50	3.38	1.17	0.74	0.21	0.09
YPD in 2x SSW	100.00	20.00	10.00	54.27	5.01	6.76	2.33	1.48	0.41	0.17
	150.00	20.00	10.00	54.27	5.01	6.76	2.33	1.48	0.41	0.17
	200.00	20.00	10.00	54.27	5.01	6.76	2.33	1.48	0.41	0.17
YPD in 3x SSW	100.00	20.00	10.00	81.40	7.51	10.15	3.50	2.23	0.62	0.26
	150.00	20.00	10.00	81.40	7.51	10.15	3.50	2.23	0.62	0.26
	200.00	20.00	10.00	81.40	7.51	10.15	3.50	2.23	0.62	0.26

Table 2: HPLC analysis for 3-stage batch fermentation using SW-YPD medium

Stage	Time (h)	Growth (OD)	Glucose (gL^{-1})	Utilized Glucose (%)	Glycerol (gL^{-1})	Acetic (gL^{-1})	EtOH (gL^{-1})	EtOH/ Cycle (gL^{-1})	EtOH Yield[a] (%)	EtOH Yield[b] (%)	EtOH Produc. ($g\ L^{-1}h^{-1}$)
S1	0	2.22 ±0.11	42.55 ±0.14	0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00	0.00	0.00
	2	2.82 ±0.03	9.45 ±1.00	0.58	0.58 ±0.05	0.06 ±0.00	2.89 ±0.36	2.89 ±0.36	13.34	59.96	1.45
	4	4.65 ±0.08	27.12 ±0.47	36.27	1.13 ±0.10	0.09 ±0.00	5.60 ±0.13	5.60 ±0.13	25.81	71.14	1.40
	8	15.25 ±0.35	1.21 ±0.16	97.15	3.70 ±0.19	0.18 ±0.01	16.08 ±0.63	16.08 ±0.63	74.10	76.28	2.01
	10	17.75 ±0.92	0.00 ±0.00	100.00	4.08 ±0.05	0.21 ±0.01	15.92 ±0.11	15.92 ±0.11	73.36	73.36	1.59
S2	0	13.95 ±0.21	196.23 ±1.48	0.00	3.03 ±0.06	0.18 ±0.02	13.41 ±0.26	0.00 ±0.00	0.00	0.00	0.00
	3	18.20 ±0.00	169.22 ±0.89	13.76	6.22 ±0.89	0.34 ±0.02	25.78 ±1.97	12.37 ±1.71	12.36	89.64	4.12
	9	22.05 ±0.07	114.32 ±3.03	41.73	11.41 ±0.88	0.66 ±0.07	50.36 ±3.84	36.95 ±3.58	36.91	88.36	4.11
	12	23.85 ±0.07	84.26 ±0.65	57.06	12.73 ±0.65	0.77 ±0.07	63.15 ±3.33	49.75 ±3.07	49.70	87.10	4.15
	15	24.45 ±0.07	62.71 ±0.46	68.04	14.15 ±0.07	0.80 ±0.04	71.86 ±1.89	58.45 ±0.63	58.41	85.84	3.90
	20	24.65 ±0.78	29.99 ±0.30	84.72	15.16 ±0.20	0.86 ±0.06	86.72 ±1.33	73.32 ±1.07	73.26	86.48	3.67
S3	0	21.60 ±0.85	129.62 ±0.62	0.00	13.24 ±0.91	0.76 ±0.02	75.50 ±1.79	0.00 ±0.00	0.00	0.00	0.00
	4	21.75 ±0.78	110.00 ±0.21	15.14	15.13 ±0.24	0.86 ±0.01	82.95 ±0.78	7.45 ±1.01	11.27	74.31	1.86
	8	21.70 ±0.71	101.60 ±1.20	21.61	17.39 ±0.11	1.00 ±0.06	88.47 ±0.79	12.97 ±1.01	19.62	90.73	1.62
	15	22.15 ±0.07	75.05 ±0.28	42.10	17.87 ±0.92	1.05 ±0.06	99.83 ±2.32	24.33 ±0.53	36.80	87.43	1.62
	20	21.60 ±0.28	58.00 ±0.21	55.25	18.07 ±0.77	1.08 ±0.04	107.46 ±3.12	31.95 ±1.32	48.34	87.51	1.60
	24	21.45 ±0.35	45.05 ±0.28	65.25	18.31 ±0.77	0.98 ±0.13	113.52 ±0.01	38.02 ±1.79	57.51	88.13	1.58

[a] Calculated as a percentage of the theoretical yield (0.51) based on the total glucose of each stage.

[b] Calculated as a percentage of the theoretical yield (0.51) based on the utilised glucose.

Table 3: HPLC analysis for 2-stage batch fermentation using SW-SM Medium

Stage	Time (h)	(OD)	Sugars (gL^{-1})				Utilised Sugars (%)	Glycerol (gL^{-1})	EtOH (gL^{-1})	EtOH/ Cycle (gL^{-1})	EtOH Yield[a] (%)	EtOH Yield[b] (%)	EtOH Prod. ($g\ L^{-1}h^{-1}$)
			Sucrose	Glucose	Fructose	Total							
S1	0	1.65 ±0.04	12.90 ±0.97	14.96 ±1.51	17.22 ±1.57	45.07 ±4.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	14	9.79 ±0.02	0.17 ±0.06	0.00	0.21 ±0.20	0.38 ±0.26	98.68	3.40 ±0.10	11.32 ±1.67	11.32	49.13	49.56	0.81
S2	0	5.89 ±0.83	24.91 ±4.93	33.45 ±2.28	32.91 ±1.32	91.27 ±5.90	0.00	2.73 ±0.19	7.97 ±0.60	0.00	0.00	0.00	0.00
	3	6.68 ±1.05	22.65 ±2.94	15.24 ±0.23	31.68 ±3.76	69.57 ±1.05	27.88	4.69 ±0.00	15.36 ±1.05	7.39	16.64	71.09	2.46
	6	7.88 ±0.93	18.02 ±3.70	11.10 ±1.14	30.06 ±2.83	59.19 ±0.26	38.43	6.50 ±0.10	21.90 ±1.88	13.93	31.35	85.24	2.32
	9	9.17 ±0.62	9.25 ±0.65	7.76 ±1.55	27.78 ±2.42	44.79 ±3.32	55.52	7.33 ±0.12	27.30 ±1.50	19.33	43.51	83.57	2.15
	12	9.44 ±1.13	5.49 ±0.72	4.00 ±2.36	23.06 ±1.76	32.55 ±4.84	69.50	8.30 ±0.60	32.93 ±1.94	24.96	56.18	83.97	2.08
	24	10.35 ±1.43	3.16 ±0.96	0.00	2.39 ±2.90	5.55 ±3.86	97.05	9.83 ±0.62	48.56 ±0.73	40.59	91.38	93.43	1.69
	30	10.39 ±1.44	2.17 ±0.53	0.00	0.60 ±0.85	2.77 ±1.38	98.12	10.55 ±0.14	50.32 ±1.95	42.35	95.35	94.28	1.41

[a] Calculated as a percentage of the theoretical yield (0.51) based on the total sugar in each stage.

[b] Calculated as a percentage of the theoretical yield (0.51) based on the utilised sugars.

Figures:

Figure 1: Schematic Diagram for Industrial Seawater-Based Bioethanol Production (100,000 L Bioreactor)

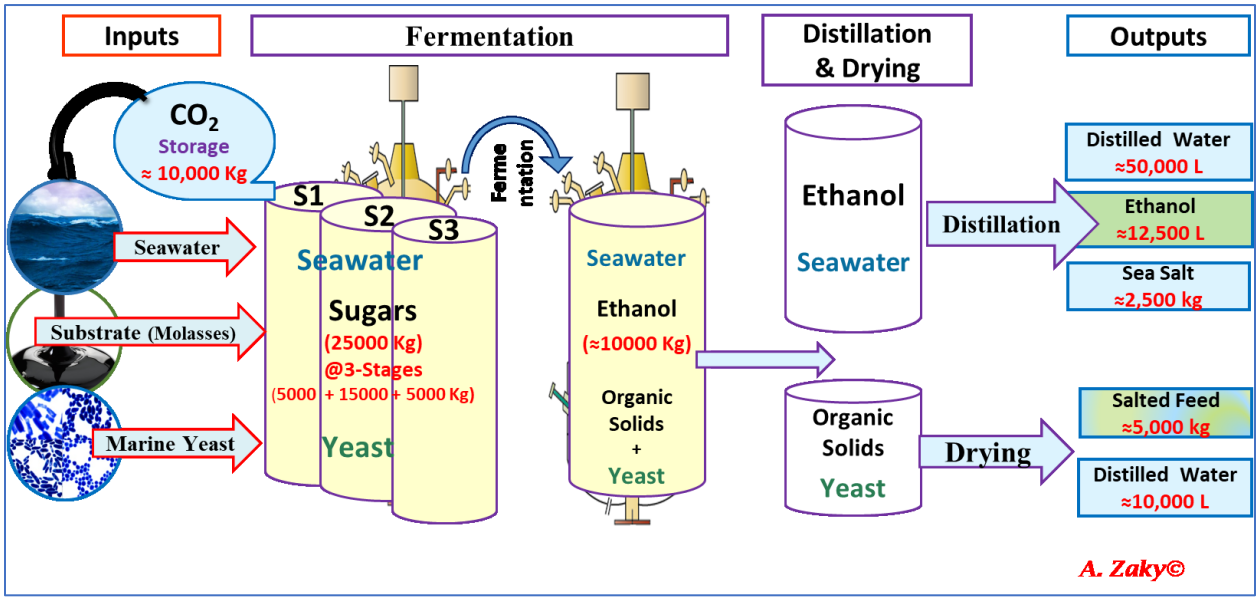
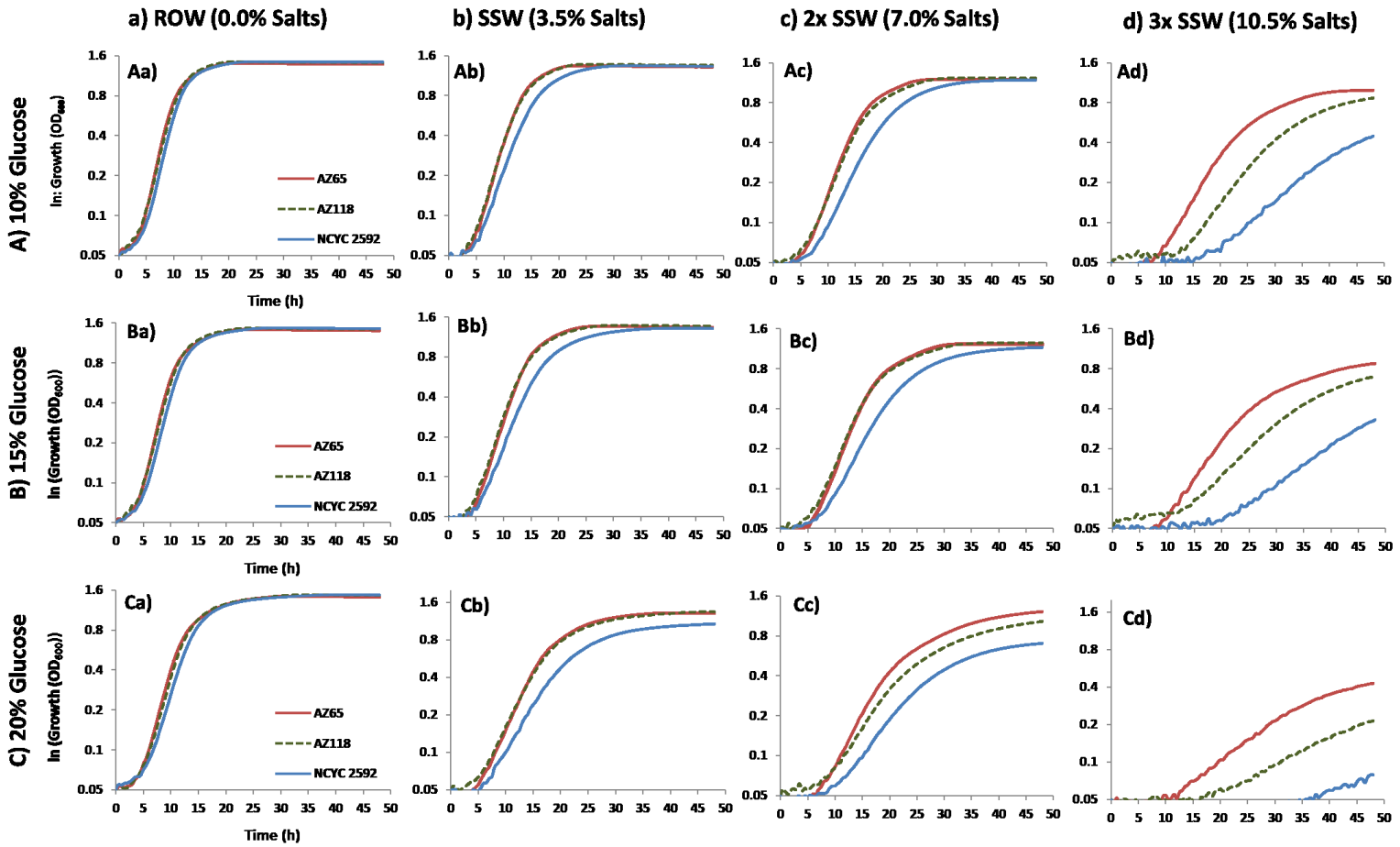


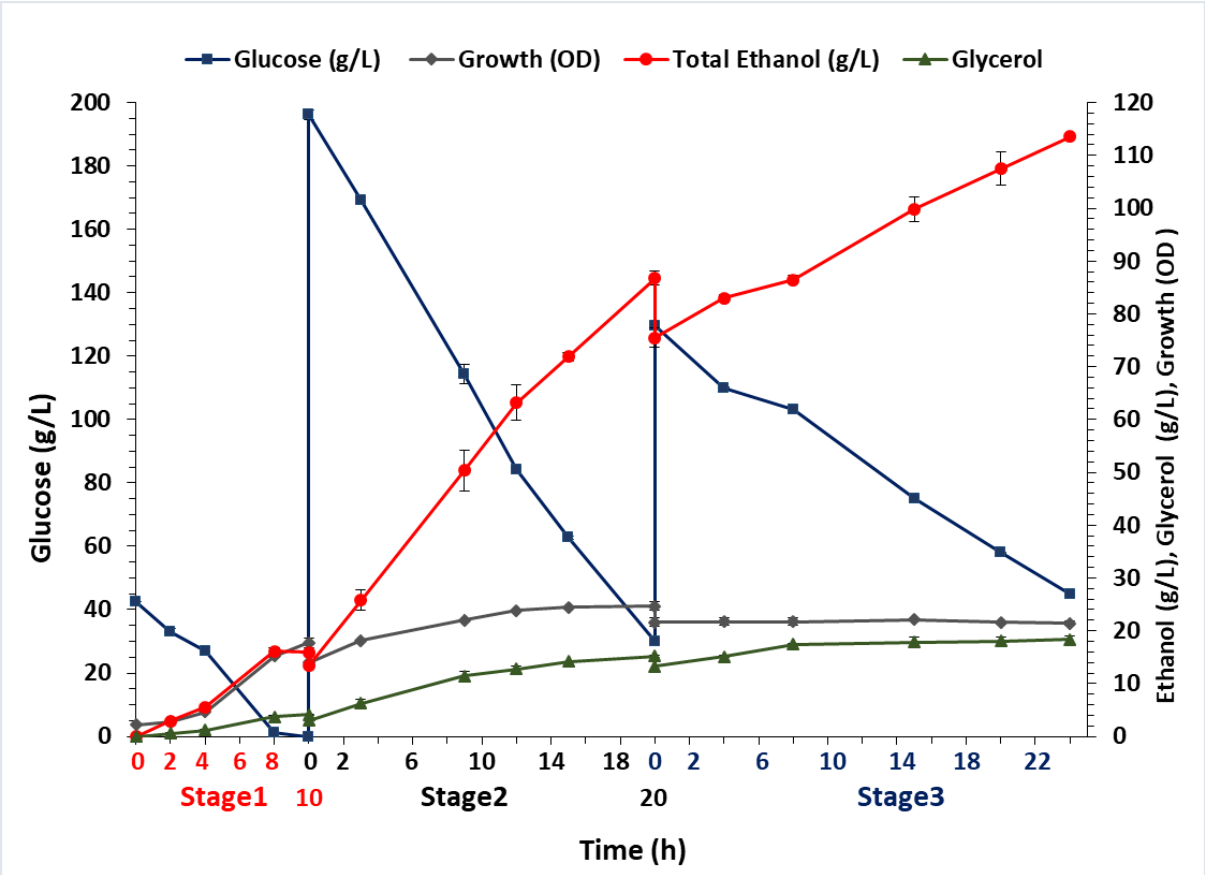
Figure 2: Growth of marine yeast strains when exposed to increased osmotic stress (0 - 20% glucose and 0 - 10.5% sea salts) using YPD media in ROW, SSW, 2x SSW and 3x SSW.



ROW: Reverse Osmosis Water - **SSW:** Synthetic Seawatwer - **AZ65 & AZ118:** two marine *S. cerevisiae* strains - **NCYC2592:** a terrestrial *S. cerevisiae* strain used as a reference for comparison.

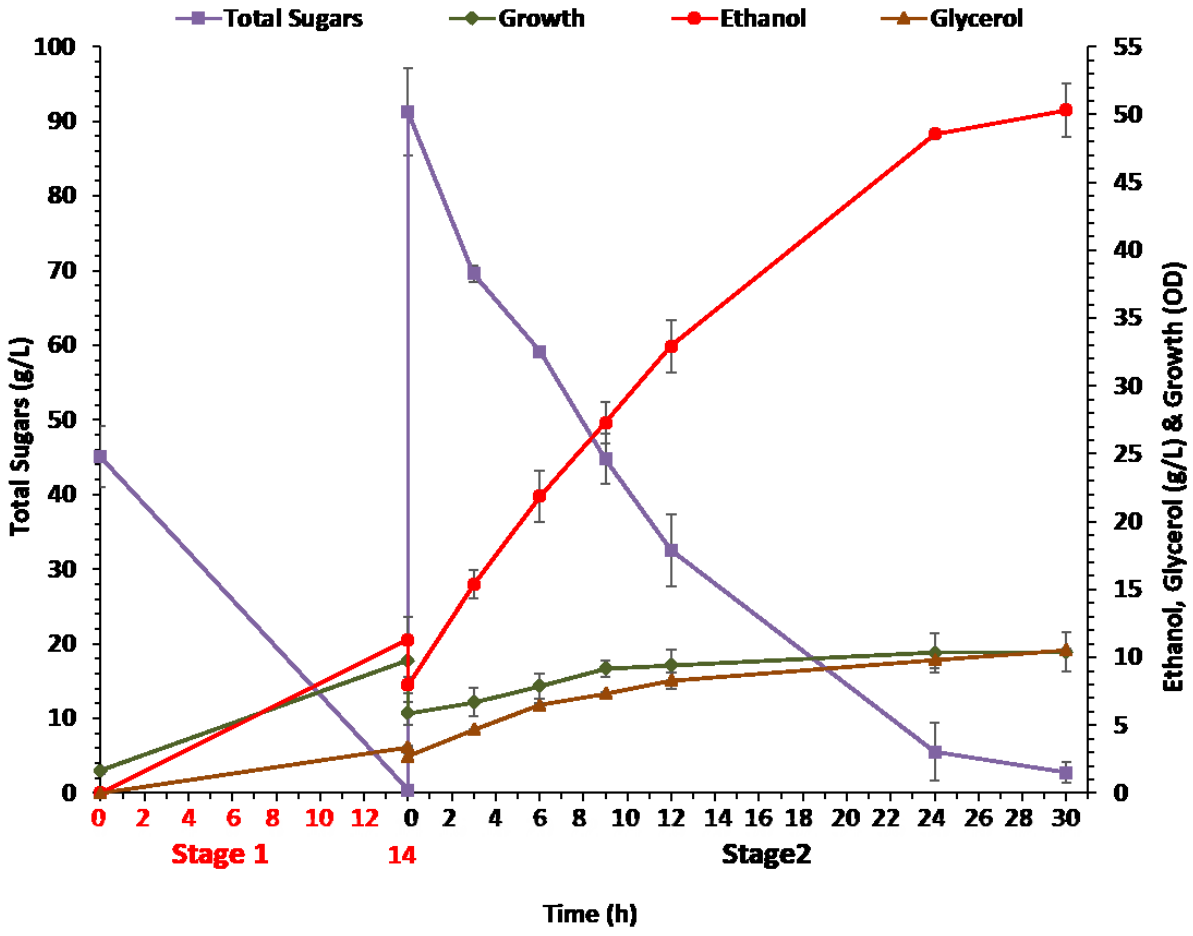
The experimental error bars represent the standard deviation of three replicates.

Figure 3: Changes in the concentration of glucose, ethanol, glycerol and biomass in 3-stage batch fermentation conducted using *S. cerevisiae* AZ65 in SW-YPD medium.



The experimental error bars represent the standard deviation of the three replicates.

Figure 4: Changes in the concentration of sugars, ethanol, glycerol and biomass in 2-stage batch fermentation conducted using *S. cerevisiae* AZ65 in SW-Molasses medium.



The experimental error bars represent the standard deviation of the three replicates.

Graphical Abstract