Comparative assessment of yeast fermentation performance, ethanol tolerance and membrane fluidity

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Abstract

Yeast nitrogen base (YNB) is a chemically defined fermentation medium that does not interfere with fluorescence spectroscopy measurements in the UV to blue range. It is a useful medium for in situ monitoring during fermentation of cell physiology by fluorescence methods. However, compared to rich media, it is considered to have poor nutritional availability. Overall nutrition affects yeast ability to convert sugar to ethanol. This study investigated growth and fermentation performance of three Saccharomyces cerevisiae strains in YNB. Three different glucose concentrations ranging from 5 to 15% (w/v) were applied to investigate the highest concentration of glucose able to be efficiently converted to ethanol by each yeast strain.

Growth and fermentation performance of the yeast strains were different. The fermentation performance could be ranked (highest to lowest) as strains A15, A12 and K7, while the growth performance could be ranked K7, A12, and A15. In general, fermentation with 15% initial sugar in the minimal medium led to lower sugar conversion to ethanol. The medium containing 10% glucose was considered the best to optimally differentiate fermentation performance of yeast strains.

Keywords: Ethanol fermentation, poor nutrition, fermentation performance, fluorescence spectroscopy.

Introduction

Fermentation performance of the yeast *Saccharomyces cerevisiae* during ethanol production is influenced by many factors. In addition to fermentable sugar, availability of key nutrients is one of the most important factors in fermentation.[1] For an effective and efficient fermentation, a nutritionally rich medium is required.[2,3] Nitrogen is one of main elements that can be found in many macromolecules of living organisms and plays crucial roles in structure and function of these molecules. Most organisms have mechanisms to provide a continuous supply of nitrogen. Therefore, many cellular activities are dependent upon nitrogen uptake and assimilation.[3]

Media with structurally complex nitrogen sources were found to promote fermentation efficiency.[3,4] It was found that media with ammonium salts as the only nitrogen source (termed poor media) led to poor yeast fermentation performance. Fermentation performance was improved in media containing peptides (peptone) and further improved in media containing casamino acids.[3] In complex media such as those using yeast extract as the nitrogen source, yeast cells can utilize high initial sugar concentrations (e.g. very high gravity fermentations with ≥ 27% w/v sugar) and efficiently convert sugar to ethanol.[2,5-7]

However, certain experimental investigations of yeast physiology during fermentation e.g. study of proteins or membranes using fluorescence spectroscopy, yeast extract or other complex sources of nitrogen cannot be used as their high fluorescence background interferes with the labeled fluorescence.[8] Such spectrofluorimetry studies must utilize "poor" media (i.e. with simple ammonium salts) such as yeast nitrogen base which has relatively low interfering fluorescence background.[8-11]

When such poor media are used, very low glucose concentrations are usually used in the initial stage of fermentation.[9-13] While enabling yeasts to be studied under standardized conditions without the batch-to-batch variations of complex medium ingredients; poor media can lead to lower tolerance to stresses including high osmotic pressure which will limit the initial glucose concentration in fermentation studies.[10]

Material and Methods

Yeast Strains and Maintenance: Three *S. cerevisiae* strains, A12[14], A15 (ATCC 38554) and K7 (ATCC26422)[15] were used in the present study. Yeast strains were maintained on slopes of a complete medium, yeast extract peptone (YEP) containing (w/v) 0.5% yeast extract, 0.5% bacteriological peptone, 0.3% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 1% glucose and 1.5% agar. Slopes were stored at 4°C and sub-cultured every 6 months. Master cultures were stored in a Sanyo -80°C freezer.

Growth media and culture conditions: Cells were grown in the defined medium YNB (Yeast Nitrogen Base, Difco) broth containing 0.67% (w/v) YNB with 5% (w/v) glucose. Starter cultures were inoculated from slopes and grown overnight (~16 h) at 30°C and 180 opm in an orbital shaker (Paton).

Experimental batch culture conditions and sampling: Experimental cultures were prepared in YNB with either 5,

10 or 15% (w/v) glucose. Aerobic cultures were prepared by aseptically adding media to sterile Erlenmeyer flasks, each sealed with an oxygen-permeable cotton wool bung, and then inoculating with one of three yeast strains to give an initial viable cell number of ~10^6 cells/mL. The ratio of flask size to culture volume was 4:1 to ensure adequate oxygen mixing.

Culture samples were aseptically removed by drawing off with a sterile micropipette every 6 h from 0 to 30 h, followed by 12 h interval from 48 – 168 h. Examination of the samples included optical density, viable cell numbers, and glucose and ethanol concentrations. Detailed analysis including ethanol tolerance and membrane fluidity was performed at 6 and 24 h.

Growth Rate: Yeast growth was monitored by measuring optical density of the culture at 600 nm (OD_{600nm}) using a Beckman DU 650 spectrophotometer making dilutions where necessary. Measurements were made using 1 mL (10 mm path length) PMMA cuvettes (Sarstedt).

Viable Cell Numbers: Viable cell numbers were assessed using the methylene violet staining method and light microscopy (400× magnification) using a Neubauer-type haemocytometer. Methylene violet staining has been proposed as a better method for monitoring yeast cell viability compared to the traditional methylene blue staining method.[16]

Determination of membrane fluidity by spectrofluorometric analysis: Membrane fluidity was assessed using steady-state fluorescence spectroscopy, measuring generalized polarization of 6-dodecanoyl-2-dimethylamino-naphthalene (laurdan) following incorporation of the probe into yeast plasma membranes as outlined by Learmonth.[8] Cell suspensions were standardized by diluting with centrifuged (10000 *g*) supernatant to OD_{600nm} of 0.4 and a volume of 3 mL in a cuvette.

Incorporation of the fluorescent probe into yeast cell membranes was accomplished by incubating the standardized washed cell sample with a final concentration of 5 µM laurdan for 60 min at 30°C in the dark with stirring. Unlabeled cell suspension at the same cell density was used to measure background fluorescence which was subtracted from the experimental readings. The results were expressed as Generalized Polarization (GP) determined using equation 1:

$$GP = \frac{I_{440nm} - I_{490nm}}{I_{440nm} + I_{490nm}} \quad (1)$$

where I_{440nm} : Emission intensity at 440 nm
I_{490nm} : Emission intensity at 490 nm

Membrane fluidity after exposure to ethanol: The GP value was monitored as described earlier over a period of 10 minutes, with measurements taken every minute. After 10 minutes absolute ethanol was added to the sample cuvette to bring the final concentration of ethanol to 18% (v/v). The GP was then monitored for another 10 minutes. The GP initial drop was calculated as the percentage difference of GP values immediately after ethanol exposure compared to GP value before ethanol exposure. The GP recovery was determined using equation 2:

$$\text{GP Recovery (\%)} = \frac{GP_{final} - GP_{initial}}{GP_{start} - GP_{initial}} \text{ X } 100\% \quad (2)$$

where GP_{final} = Final GP value at 20 minutes, $GP_{initial}$ = GP value immediately after ethanol addition (11^{th} minute) and GP_{start} = The GP values immediately before ethanol addition (10 minutes).

Determination of glucose: Glucose concentration in fermentation media was determined using the alkaline ferricyanide method of Walker and Harmon.[17] Absorbance of the standards and samples was read at 420 nm with a Beckman DU650 spectrophotometer.

Determination of ethanol: Ethanol concentration was determined using an enzymatic assay by alcohol dehydrogenase as proposed by Ough and Amerine[18] and modified by Ishmayana et al.[19] Sample absorbance was read at 340 nm using Beckman DU650.

Determination of glycerol: Glycerol was determined using a HPLC system (Shimadzu) with monitoring of absorbance at 195, 200, 210 and 220 nm by a photodiode array detector and also using a refractive index detector. Phenomenex Rezek ROA Organic acid H^+ (8%) column (300 × 7.8 mm 8 µm particle size) was used for the separation of analytes with matching guard column. Separations were conducted at 65°C with the mobile phase sulfuric acid (0.005 M) in deionized MilliQ water (resistivity ~ 18 Mohm) filtered through a 0.45 µm filter. The mobile phase was passed through an in-line degasser to ensure that the phase was gas free. The flow rate was maintained at 0.6 mL/min. Crude sample from fermentation was filtered using a 0.22 µm filter and aliquots of 10 µL were injected and analyzed for 24 min.

Results and Discussion

Cell Growth: Nitrogen is one of the macronutrients required for yeast cell growth. Complex nitrogen sources such as yeast extract, casamino acids or peptone promote cell growth and ethanol productivity.[2-4] However, these media have very high fluorescence backgrounds, and therefore fluorescence-based studies of yeast physiology in situ during fermentation are not possible. Despite its poor nutrition in terms of complexity of nitrogen source, yeast nitrogen base (YNB) has the advantage that it does not have high fluorescence background. Therefore, YNB is more preferred for experiments involving fluorescence measurements. In the present study, we used YNB and different concentrations of initial glucose to investigate the difference in fermentation performance of three *S. cerevisiae* strains.

Cell growth of the three strains in different initial glucose concentration is presented in figure 1. The figure shows that the three yeast strains used in the present study have different growth patterns. In term of maximum growth rate, K7 seems to have the fastest while A15 has the slowest maximum growth rate. No significant differences were observed for A12 to either K7 or A15 (Table 1). Interestingly, A15 reached higher OD compared to A12 as presented in figure 1 and table 1. The maximum growth rate value was obtained from the specific growth rate in respirofermentative phase and in this study, it was calculated as specific growth rate from 0-12 hours.

Plotting Ln value of the OD against time indicates that the highest growth rate occurred during the first twelve hours of the fermentation experiment (data not shown) and therefore the maximum growth rate was calculated based on the OD value change from 0-12 hours. A12 showed the highest growth rate decrease after 12 hours of fermentation, which therefore led to lower OD achieved.

Cell viability: Viability of the three yeast strains used in this study is presented in figure 2. The viability of the strains was different between one strain and another. A12 started at moderately high viability (~80%), increased to ~ 97% at 6 hours and gradually decreased to almost zero percent after 168 h. It was observed that when this strain grown in 15% w/v initial glucose, the viability was substantially lower at around 72-96 hours. For K7, the viability started from the highest point (~95%) and remained high compared to the other strains for up to 48 hours.

As for this strain, the cells grown in the lowest glucose concentration first reached the lowest viability at about 96 h while in the higher glucose concentration, it maintained viability up to about 132 h. The viability of A15 started lowest and rapidly increased to ~90% at 6 hours, followed by a rapid decrease to ~60% for cell grown in 5 and 10% w/v glucose while for cell grown in 15% w/v glucose, the viability dropped to ~50%. Interestingly, the viability of this strain did not decrease gradually as observed for other strains but remained at about 40-50% up to 84 hours.

Cell viability is considered as an important parameter for determining the fermentation performance. Yeast cultures with higher viability were expected to have better fermentation performance because of higher number of active cells. However, the results of the present study indicate that yeast cultures with high viability do not always have higher ethanol production. This result is in agreement with previous study by Manazu et al,[20] who found that a laboratory strain with higher cell viability produced less ethanol compared to a wine strain. This result led us to consider the presence of viable but non-fermenting cells.

Glucose Consumption and Ethanol Production: Fermentation performance is greatly influenced by nutritional availability. The concentration of glucose as main carbon source in the present study was varied to investigate the fermentation performance of the yeast strains in YNB media. Since every strain may have different tolerance against osmotic pressure, the difference of glucose concentration at the initial stage of fermentation was predicted to affect the ability of the yeast cell to consume glucose and convert it to ethanol.

Most studies using YNB as their basal media use 2% (w/v) initial glucose as carbon source.[21,22] In the present study three initial glucose concentrations were used i.e. 5, 10 and 15% w/v, and the glucose consumption is presented in figure 3. It was clear that when higher glucose concentration was applied, more time was required to consume the sugar. When high sugar concentration was used, it is common to observe stuck fermentation.[10,23,24] In industrial processes where a complex nitrogen source was used as basal media, stuck fermentation usually occurs when sugar exceeds 27% w/v.[25,26]

In the present study, stuck fermentation began to be observed at 10% w/v initial glucose for K7, leaving ~3% w/v glucose at the end of fermentation. Furthermore in 15% w/v initial glucose, all strains showed stuck fermentation from about 96 hours. While no substantially different fermentation performance was detected in 5% initial glucose, different performance of the yeast strains was observed when 10% initial glucose was used. At this level, A15 was the fastest strain consuming almost all glucose at 84 h, followed by A12 and finally K7 that showed stuck fermentation. Based on this result, it is most likely that A15 is the most osmotolerant followed by A12 and K7.

In agreement with glucose consumption, ethanol production showed similar patterns. A15 with 10% glucose produced the highest ethanol concentration at around 96 hours (~2.2% w/v), while A12 and K7 produced similar level of ethanol. When initial glucose concentration increased to 15% (w/v), the ethanol produced decreased. The lower ethanol production in higher initial glucose can be caused by the stuck fermentation as previously described. The lowest initial glucose concentration led to the fastest ethanol production, which peaked at 36 hours for A15 and 48 hours for K7 and A12.

Decline of ethanol concentration was observed after the ethanol peaked for all strains and conditions. For the lowest initial glucose concentration, where glucose was exhausted at around 36 hours, the ethanol started to decline at 48 hours indicating diauxic shift where the cell starts to use ethanol as carbon source. For 10% (w/v) initial glucose, the diauxic shift seemed to start at a later stage at around 96 hours. For 15% (w/v) initial glucose, the decline of ethanol was observed after 36, 72 and 96 hours for A12, K7 and A15 strains respectively.

Generally, diauxic shift occurs when sugar is not available as the carbon source and cell starts to use ethanol. For 15%

initial sugar, even though glucose was not exhausted, ethanol started to decline. It seems that the cells might have started to use ethanol as carbon source[27], oxidation of ethanol to acetic acid[28] (in cultures where the glucose concentration decreased to the repression threshold of about 2 mg/mL) or some ethanol evaporated, resulting in the decrease of ethanol produced.

The amount of ethanol detected in the fermentation media was actually much lower than the theoretical amount. When yeast cells are exposed to osmotic stress, they synthesize glycerol as stress protector.[28,29] Glucose in the media is not only used for ethanol production, but also to synthesize glycerol. Therefore, the amount of ethanol cannot reach the theoretical amount due to diversion of ethanol production to glycerol synthesis for protecting the cell. To check this hypothesis, the concentration of glycerol was determined at 96 hours representing stationary phase and presented at figure 5.

It was clear that the glycerol concentration increased with increasing initial glucose concentration. It was also observed that A12 synthesized the most of glycerol followed by A15 and K7. This observation supports the ethanol production result in which A12 produced the least ethanol compared to other strain. This means that for A12, more sugar was converted to glycerol, lowering the amount of ethanol produced. However, for K7, even though it synthesized the least glycerol, the ethanol produced was lower compared to the other strains. This led to higher glucose remaining in the medium at the end of fermentation as described previously. A15 seemed to have the best fermentation performance as indicated by higher ethanol and lower glycerol produced and also less residual sugar in the media (figure 3 and table 2).

Fermentation kinetics parameters were evaluated to study whether there were any effects of different sugar concentration on the fermentation performance.

The fermentation kinetics parameters obtained in this study are presented in table 2. The highest glucose consumption rate (Qs) was observed for A12 at 5% and the lowest was for K7 at 15% initial glucose while no significant differences were observed in term of Qs for the other strains and conditions. While there were no significant differences for ethanol production rate (Qp) and ethanol productivity (Yp/s), significant differences were noticed for glucose consumption where fermentation of 5% initial glucose showed the highest glucose consumption for all strains. For 10% initial glucose, A15 and A12 did not differ significantly but K7 had significantly lower glucose consumption compared to the other strains. For 15% initial glucose, A12 and K7 did not differ significantly while A15 had significantly higher glucose consumption.

The kinetics parameters data indicate that even though the Qs, Qp and Yp/s did not differ significantly, the total amount of ethanol produced or glucose consumed could differ significantly. This may be caused by the diversion of ethanol production to glycerol production to overcome osmotic stress. As previously described, glycerol was detected for all conditions and strains in this study. Furthermore, in media with higher initial glucose concentration, more glycerol was produced leading to similar rates of ethanol production. However, in the end it is most likely that glycerol reached saturation point where the cell ceased producing it, or the glucose concentration decreased below stressful levels, so that the rest of the sugar could be converted to ethanol.

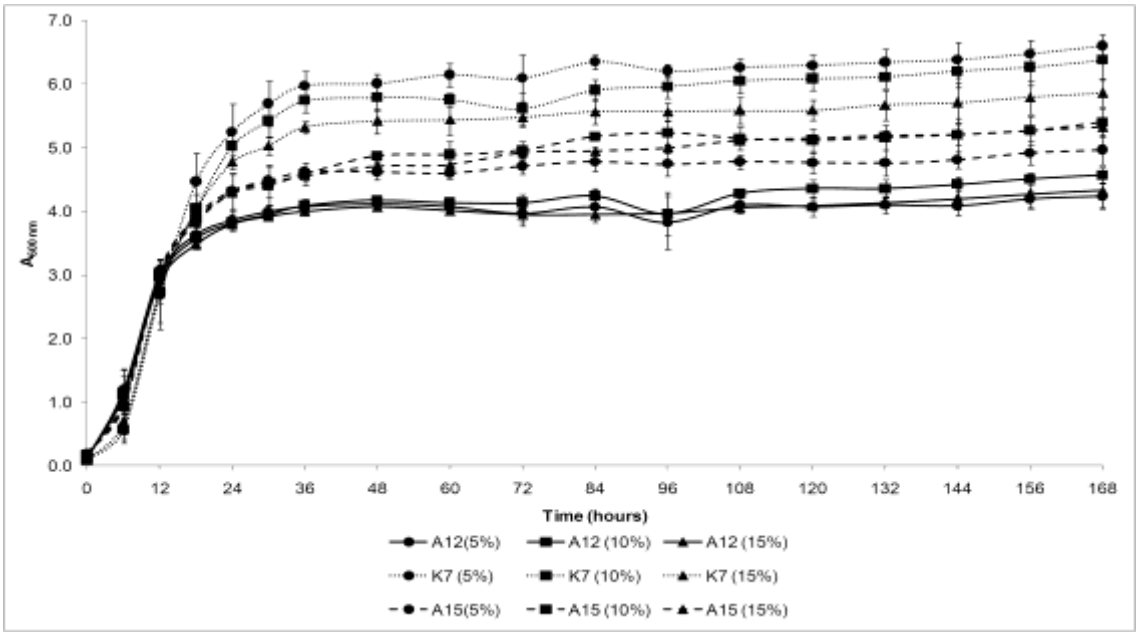

Figure 1: Growth curve of yeast strains A12, K7 and A15 in YNB media with different initial glucose concentration. Numbers in parentheses are initial glucose concentration. Error bars indicate standard deviation of three independent experiments.

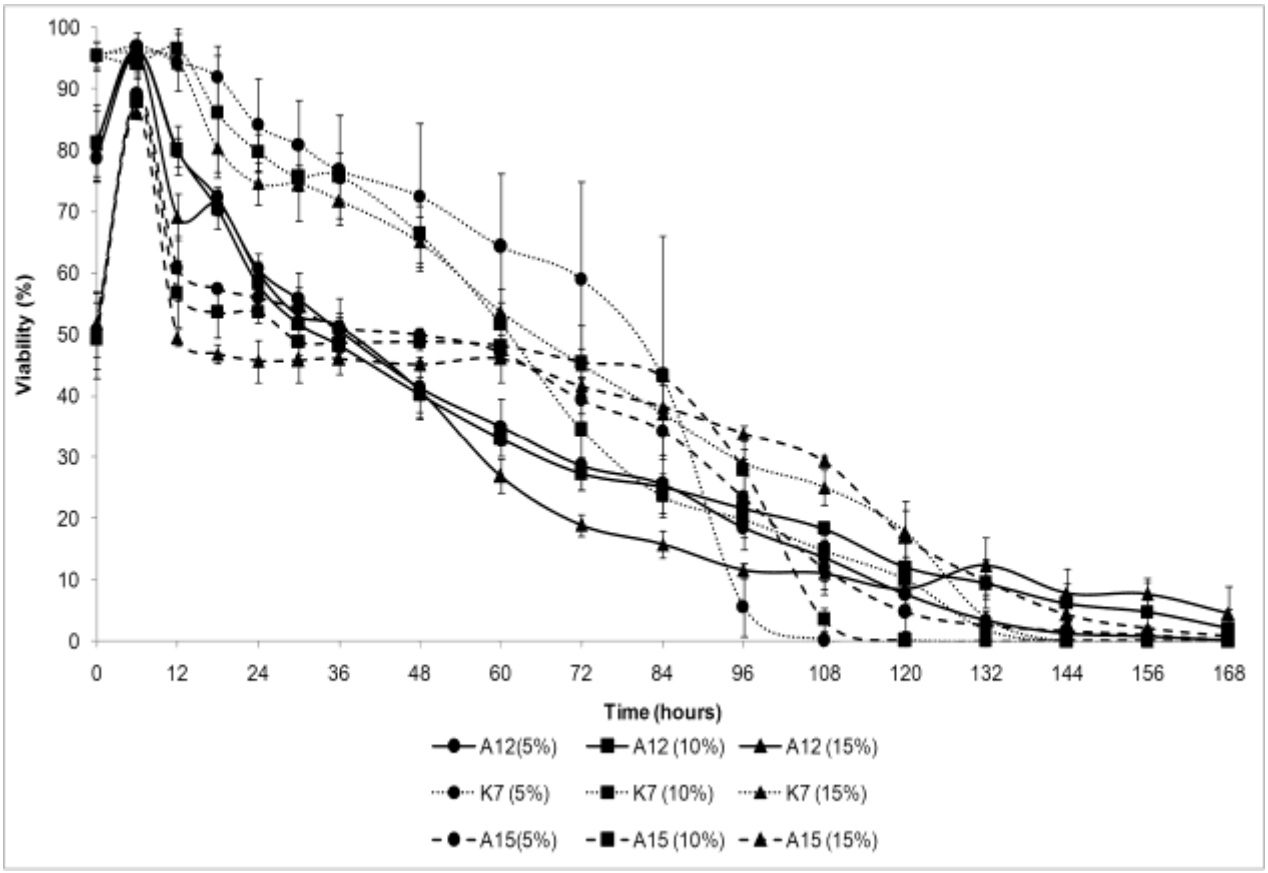

Figure 2: Viability of yeast strains A12, K7 and A15 in YNB media with different initial glucose concentration. Numbers in parentheses are initial glucose concentration. Error bars indicate standard deviation of three independent experiments.

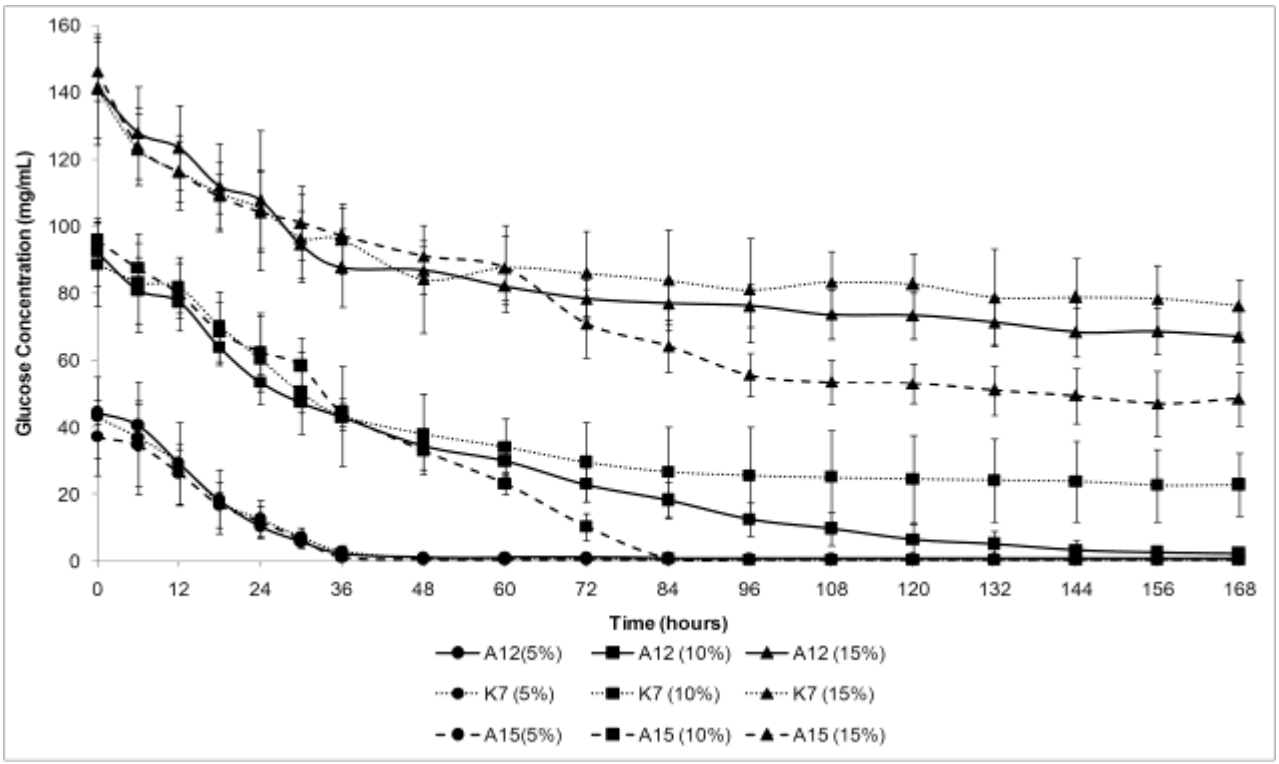

Figure 3: Glucose consumption of yeast strains A12, K7 and A15 in YNB media with different initial glucose concentration. Numbers in parentheses are initial glucose concentration. Error bars indicate standard deviation of three independent experiments.

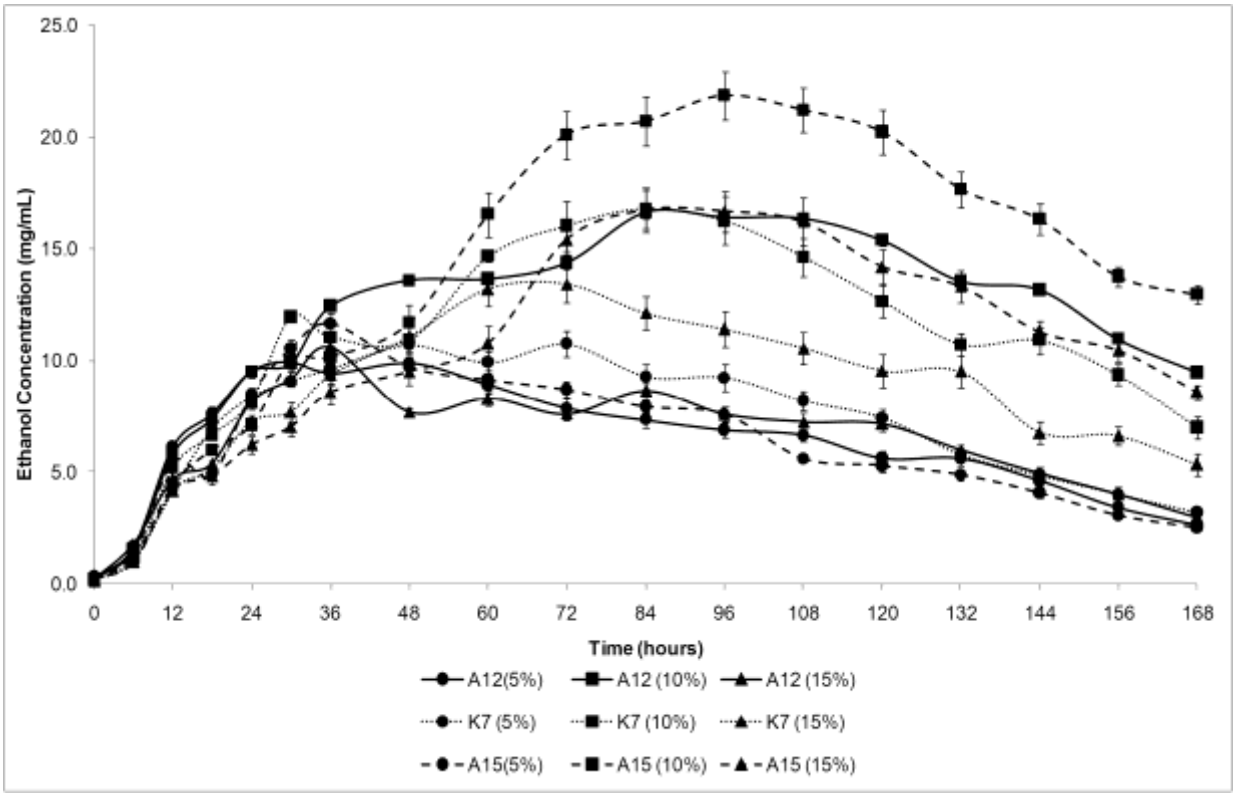

Figure 4: Ethanol production of yeast strains A12, K7 and A15 in YNB media with different initial glucose concentration. Numbers in parentheses are initial glucose concentration. Error bars indicate standard deviation of three independent experiments.

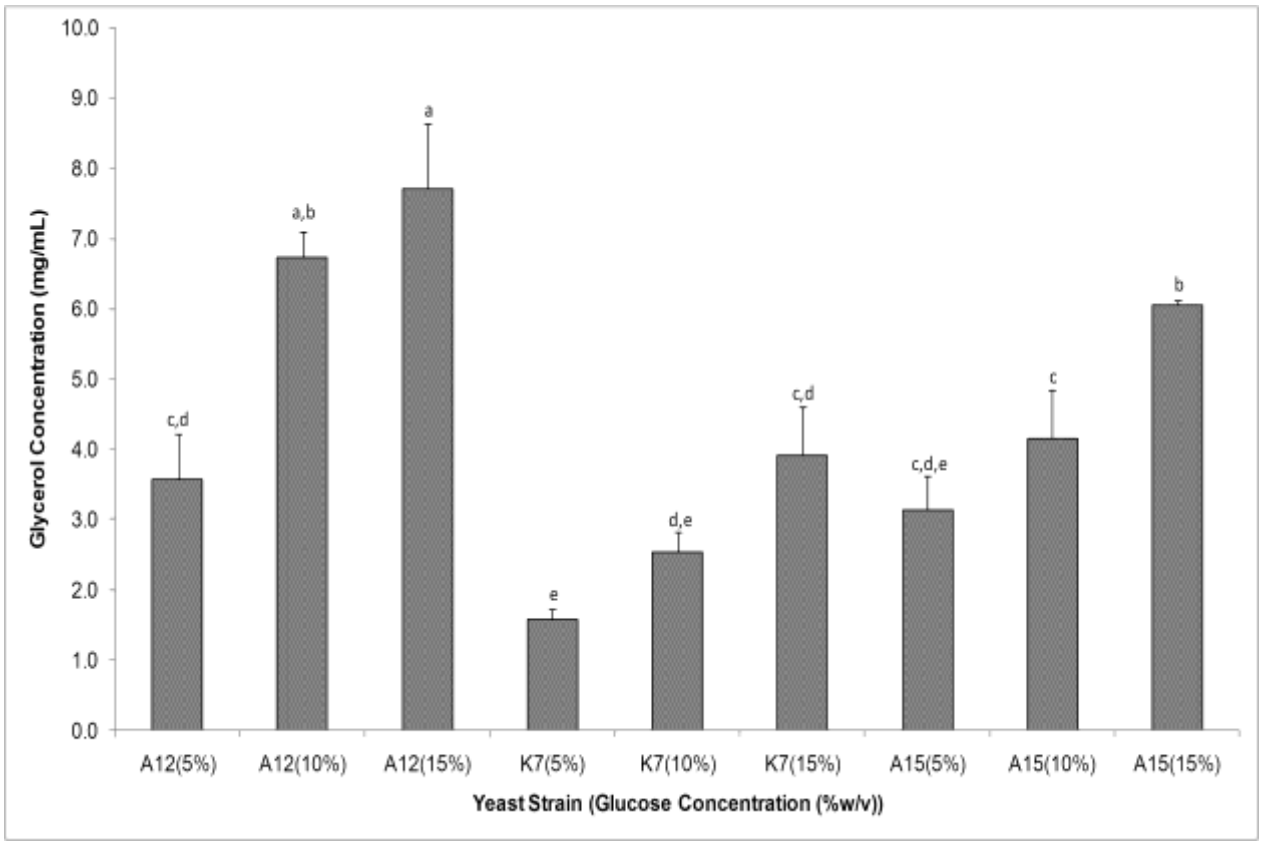

Figure 5. Glycerol concentration at 96 hours of yeast strains A12, K7 and A15 grown in YNB media with different initial glucose concentration at 96 hours. Numbers in parentheses are initial glucose concentration. Bars with same letters are not significantly different. Error bars indicate standard deviation of three independent experiments.

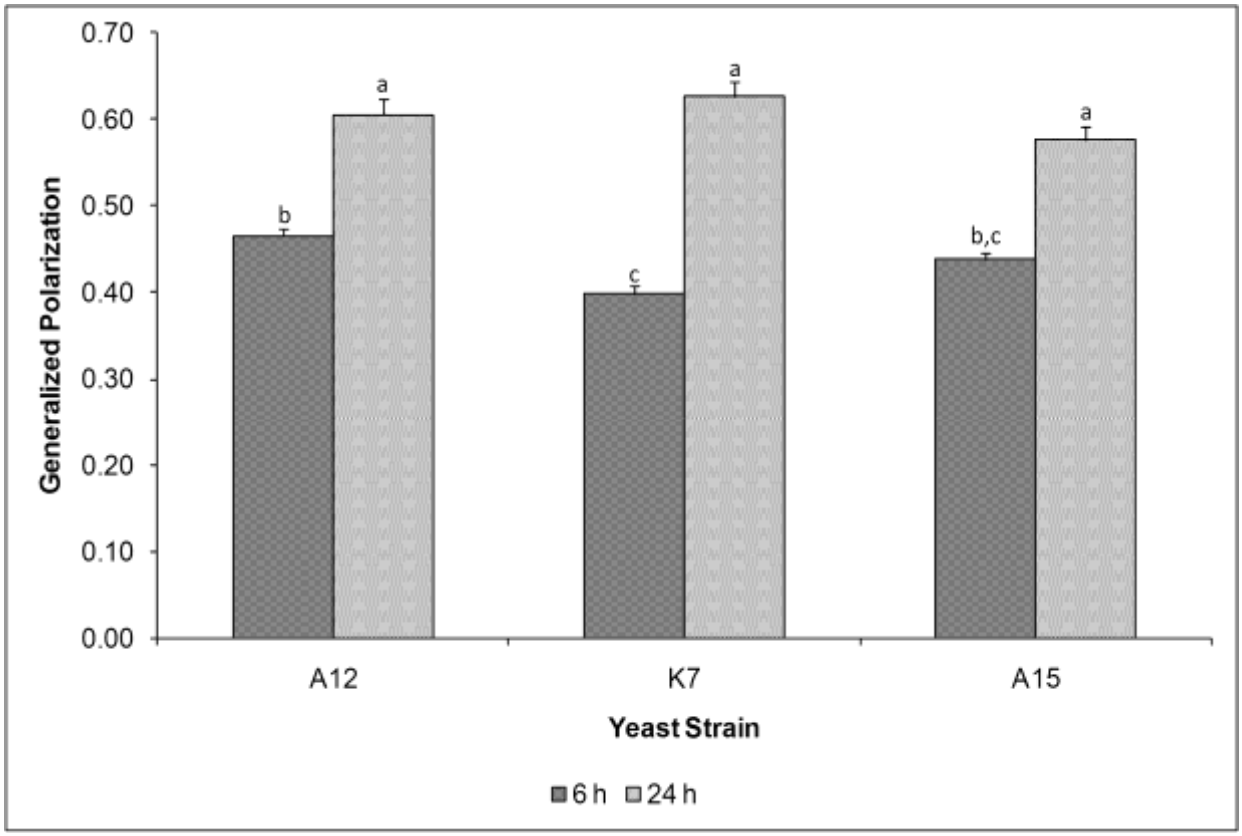

Figure 6: Generalized polarization at 6 and 24 hours of yeast strains A12, K7 and A15 yeast strains grown in YNB media with different initial glucose concentration at 6 and 24 hours. Bars with same letters are not significantly different. Error bars indicate standard deviation of three independent experiments.

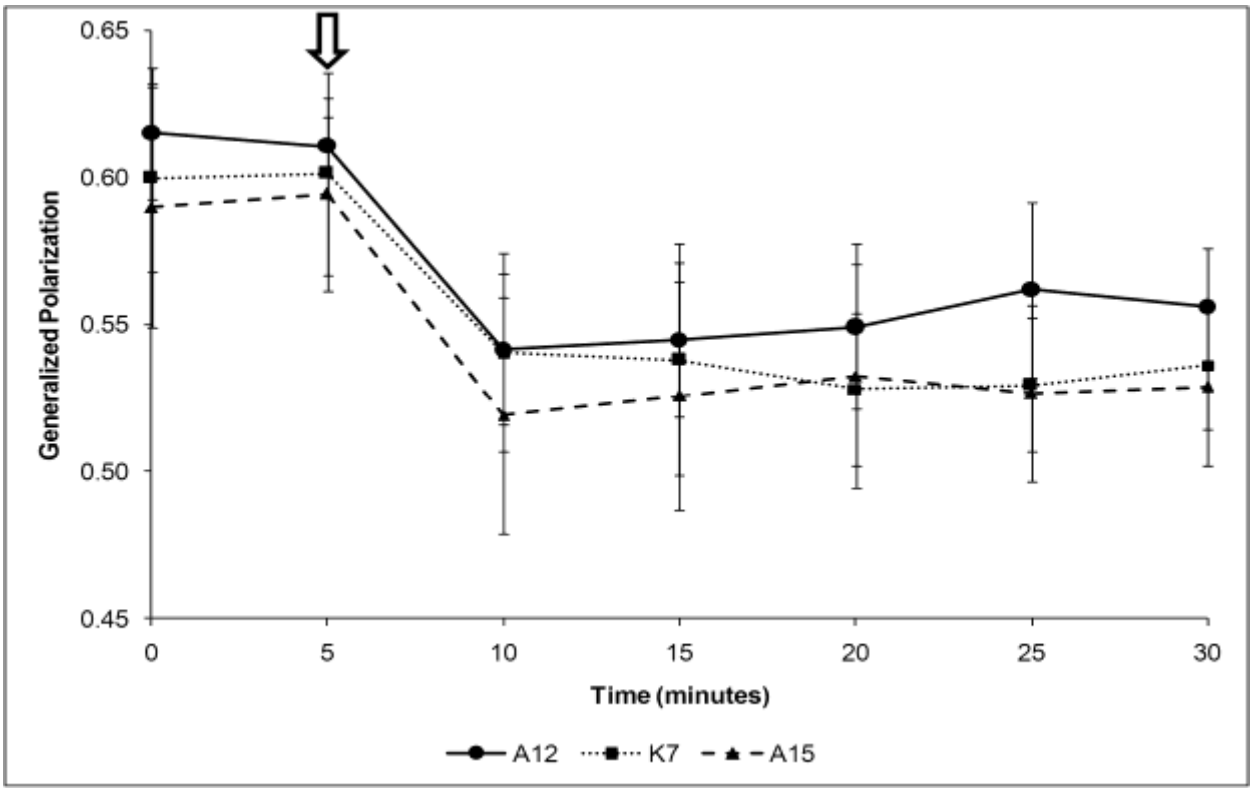

Figure 7: Generalized polarization of yeast strains A12, K7 and A15 yeast strains grown in YNB media before and after ethanol exposure. Arrow sign indicates addition of absolute ethanol to give 18% v/v final concentration. Error bars indicate standard deviation of three independent experiments.

Table 1
Growth parameters of yeast strains A12, K7 and A15 yeast strains in YNB media with different initial glucose concentration. Value are means of three independent experiment followed by standard deviation. Values with the same letter within the column are not significantly different.

Strain	Initial Sugar Concentration (%w/v)	$\mu_{max\ 0\text{-}12}$ (h^{-1})	$OD_{(36\ h)}$
A12	5	0.271 ± 0.005^{abc}	4.081 ± 0.094^{d}
	10	0.268 ± 0.008^{abc}	4.093 ± 0.100^{d}
	15	0.263 ± 0.006^{abc}	4.002 ± 0.056^{d}
K7	5	0.275 ± 0.010^{ab}	5.977 ± 0.235^{a}
	10	0.281 ± 0.010^{a}	5.744 ± 0.187^{a}
	15	0.287 ± 0.024^{a}	5.327 ± 0.101^{b}
A15	5	0.238 ± 0.028^{c}	4.623 ± 0.152^{c}
	10	0.244 ± 0.019^{bc}	4.591 ± 0.187^{c}
	15	0.248 ± 0.008^{abc}	4.566 ± 0.054^{c}

Table 2
Fermentation kinetics parameters of yeast strains A12, K7 and A15 yeast strains in YNB media with different initial glucose concentration. Value are means of three independent experiment followed by standard deviation. Values with the same letter within the column are not significantly different.

Strain	Initial Sugar Concentration (%w/v)	Qs ($g.L^{-1}.h^{-1}$)	Qp ($g.L^{-1}.h^{-1}$)	Yp/s ($g.g^{-1}$)	Glucose Consumption (%)
A12	5	1.285 ± 0.128^{a}	0.322 ± 0.145^{a}	0.246 ± 0.085^{a}	98.34 ± 0.41^{a}
	10	0.883 ± 0.181^{ab}	0.196 ± 0.036^{a}	0.224 ± 0.031^{a}	97.21 ± 1.88^{a}
	15	0.767 ± 0.158^{ab}	0.101 ± 0.044^{a}	0.131 ± 0.053^{a}	52.52 ± 3.46^{c}
K7	5	1.199 ± 0.340^{ab}	0.297 ± 0.066^{a}	0.250 ± 0.022^{a}	98.74 ± 0.22^{a}
	10	0.740 ± 0.232^{ab}	0.198 ± 0.105^{a}	0.256 ± 0.078^{a}	73.71 ± 11.29^{b}
	15	0.678 ± 0.059^{b}	0.143 ± 0.088^{a}	0.205 ± 0.108^{a}	45.61 ± 5.35^{c}
A15	5	1.020 ± 0.296^{ab}	0.342 ± 0.135^{a}	0.329 ± 0.035^{a}	98.53 ± 0.29^{a}
	10	1.134 ± 0.064^{ab}	0.244 ± 0.134^{a}	0.214 ± 0.112^{a}	99.55 ± 0.02^{a}
	15	0.975 ± 0.025^{ab}	0.197 ± 0.115^{a}	0.200 ± 0.112^{a}	66.82 ± 5.17^{b}

Membrane fluidity: One of the applications of fluorescence spectroscopy is to determine plasma membrane fluidity by measuring generalized polarization (GP). Higher GP values indicate lower membrane fluidity.[8,9] In the present study, GP values of the three yeast strains grown in 5% initial glucose were determined at 6 and 24 hours. The results are presented in figure 6. GP values at 6 hours were significantly lower than the GP values at 24 hours. This indicates that the plasma membrane becomes less fluid compared to the cell at 6 hours. At 6 hours, K7 had the lowest and A12 had the highest GP value while A15 was not significantly different from either K7 or A12. This result indicates that during the initial stage of fermentation, fluidity of the plasma membrane was higher than in the later stage. Membrane fluidity becomes lower in line with increasing cell age as indicated by previous study.[30]

Ethanol is known to fluidize the plasma membrane.[31] The extent to which fluidity changes may differ between one strain and another. To investigate the different plasma membrane fluidity of the strains used in this study when exposed to ethanol, the GP value was monitored before and after exposure to 18% v/v ethanol and the result is presented in figure 7. It was observed that the GP value decreased markedly directly after ethanol exposure. This indicated that the fluidity of the yeast plasma membrane increases following ethanol exposure. All strains showed the same trend although no significant differences between strains were detected.

Conclusion

All yeast strains used in this study evidenced different performance when grown in poor media (YNB) with varying initial glucose concentrations ranging from 5-15% (w/v). No significant differences were detected when 5% (w/v) initial glucose was used for fermentation. A15 showed the best glucose consumption followed by A12 and K7 when 10 and 15% (w/v) initial glucose was applied.

Furthermore, stuck fermentation was observed when 15% (w/v) initial glucose was used. Significant differences were

observed in term of GP value of the cell at 6 and 24 hours, indicating reduction in plasma membrane fluidity. More fluid plasma membranes were also detected when yeast cells were exposed to 18% (v/v) ethanol.

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References

1. Batistote M., da Cruz S.H. and Ernandes J.R., Altered patterns of maltose and glucose fermentation by brewing and wine yeasts influenced by the complexity of nitrogen source, *Journal of the Institute of Brewing*, **112(2)**, 84–91 **(2006)**

2. Bafrncová P., Šmogrovičová D., Sláviková I., Pátková J. and Dömény Z., Improvement of very high gravity ethanol fermentation by media supplementation using *Saccharomyces cerevisiae*, *Biotechnology Letters*, **21(4)**, 337-341 **(1999)**

3. da Cruz S.H., Cilli E.M. and Ernandes J.R., Structural complexity of the nitrogen source and influence on yeast growth and fermentation, *Journal of the Institute of Brewing*, **108(1)**, 54-61 **(2002)**

4. Júnior M.M., Batistote M., Cilli E.M. and Ernandes J.R., Production yeasts in media containing structurally complex nitrogen sources, *Journal of the Institute of Brewing*, **115(3)**, 191-197 **(2009)**

5. Chan-u-tit P., Laopaiboon L., Jaisil P. and Laopaiboon P., High level ethanol production by nitrogen and osmoprotectant supplementation under very high gravity fermentation conditions, *Energies*, **6(2)**, 884-899 **(2013)**

6. Deesuth O., Laopaiboon P., Jaisil P. and Laopaiboon L., Optimization of nitrogen and metal ions supplementation for very high gravity bioethanol fermentation from sweet sorghum juice using an orthogonal array design, *Energies*, **5(9)**, 3178-3197 **(2012)**

7. Deesuth O., Laopaiboon P., Klanrit P. and Laopaiboon L., Improvement of ethanol production from sweet sorghum juice underhigh gravity and very high gravity conditions: Effects of nutrient supplementation and aeration, *Industrial Crops and Products*, **74**, 95–102 **(2015)**

8. Learmonth R.P., Membrane fluidity in yeast adaptation – insights from fluorescence spectroscopy and microscopy, In Geddes C.D., eds., Reviews in Fluorescence 2010, Springer, New York, 67-93 **(2012)**

9. Butcher B.A., Yeast adaptation mechanisms, Ph.D. Thesis, University of Southern Queensland **(2008)**

10. Ishmayana S., Learmonth R.P. and Kennedy U., Fermentation performance of the yeast Saccharomyces cerevisiae in media with high sugar concentration, Proceeding of the 2nd International Seminar on Chemistry, Jatinangor, 379-385 **(2011)**

11. Learmonth R.P. and Gratton E., Assessment of membrane fluidity in individual yeast cells by Laurdan Generalised Polarisation and multi-photon scanning fluorescence microscopy, In Kraayenhof R., Visser A.J.W.G. and Gerritsen H.C., eds., Fluorescence spectroscopy, imaging and probes: new tools in chemical, physical and life sciences, Springer-Verlag, Heidelberg, Germany, 241-252 **(2002)**

12. Matsuura K. and Takagi H., Vacuolar functions are involved in stress-protective effect of intracellular proline in *Saccharomyces cerevisiae*, *Journal of Bioscience and Bioengineering*, **100(5)**, 538-544 **(2005)**

13. Poole K., Walker M.E., Warren T., Gardner J., McBryde C., de Baros Lopes M. and Jiranek V., Proline transport and stress tolerance of ammonia-insensitive mutants of the *PUT4*-encoded proline-specific permease in yeast, *Journal of General and Applied Microbiology*, **55**, 427-439 **(2009)**

14. Lewis J.G., Learmonth R., Attfield P. and Watson K., Stress co-tolerance and trehalose content in baking strains of *Saccharomyces cerevisiae*, *Journal of Industrial Microbiology & Biotechnology*, **18**, 30–36 **(1997)**

15. Lewis J.G., Learmonth R.P. and Watson K., Role of growth phase and ethanol in freeze-thaw stress resistance of Saccharomyces cerevisiae, *Applied and Environmental Microbiology*, **59(4)**, 1065-1071 **(1993)**

16. Smart K.A., Chambers K.M., Lambert I., Jenkins C. and Smart C.A., Use of methylene violet staining procedures to determine yeast viability and vitality, *Journal of the American Society of Brewing Chemists*, **57(1)**, 18-23 **(1999)**

17. Walker J.A. and Harmon D.L., Technical note: A simple, rapid assay for a-amylase in bovine pancreatic juice, *Journal of Animal Science*, **74**, 658-662 **(1996)**

18. Ough C.S. and Amerine M.A., Methods for analysis of must and wines, John Wiley & Sons, Davis **(1988)**

19. Ishmayana S., Fadhlillah M., Kristia Y.Y. and Budiman H., Validation of a modified alcohol dehydrogenase assay for ethanol determination, *Current Chemistry Letters*, **4(2)**, 77-84 **(2015)**

20. Mannazzu I., Angelozzi D., Belviso S., Budroni M., Farris G.A., Goffrini P., Lodi T., Marzona M. and Bardi L., Behaviour of *Saccharomyces cerevisiae* wine strains during adaptation to unfavourable conditions of fermentation on synthetic medium: Cell lipid composition, membrane integrity, viability and fermentative activity, *International Journal of Food Microbiology*, **121**, 84-91 **(2008)**

21. Özcan S., Freidel K., Leuker A. and Ciriacy M., Glucose uptake and catabolite repression in dominant *HTR1* mutants of *Saccharomyces cerevisiae*, *Journal of Bacteriology*, **175(17)**, 5520-5528 **(1993)**

22. Sampermans S., Mortier J. and Soares E.V., Flocculation onset in *Saccharomyces cerevisiae*: the role of nutrients, *Journal of Applied Microbiology*, **98(2)**, 525-531 **(2005)**

23. Panchal C.J. and Stewart G.G., The effect of osmotic pressure on the production and excretion of ethanol and glycerol by a

brewing yeast strain, *Journal of the Institute of Brewing*, **86**, 207-210 **(1980)**

24. Reddy L.V.A. and Reddy O.V.S., Rapid and enhanced production of ethanol in very high gravity (VHG) sugar fermentation by *Saccharomyces cerevisiae*: Role of finger millet (*Eleusine coracana* L.) flour, *Process Biochemistry*, **41**, 726–729 **(2006)**

25. Thomas K.C., Hynes S.H. and Ingledew W.M., Effects of particulate materials and osmoprotectants on very-high-gravity ethanolic fermentation by *Saccharomyces cerevisiae*, *Applied and Environmental Microbiology*, **60(5)**, 1519-1524 **(1994)**

26. Thomas K.C., Hynes S.H., Jones A.M. and Ingledew W.M., Production of fuel alcohol from wheat by VHG technology, *Applied Biochemistry and Biotechnology*, **43(3)**, 211-226 **(1993)**

27. Piskur J., Rozpedowska E., Polakova S., Merico A. and Compagno C., How did *Saccharomyces* evolve to become a good brewer?, *Trends in Genetics*, **22(4)**, 183-186 **(2006)**

28. Albers E., Larsson C., Lidén G., Niklasson C. and Gustafsson L., Influence of the nitrogen source on *Saccharomyces cerevisiae* anaerobic growth and product formation, *Applied and Environmental Microbiology*, **62(9)**, 3187–3195 **(1996)**

29. Myers D.K., Lawlor D.T.M. and Attfield P., Influence of invertase activity and glycerol synthesis and retention on fermentation of media with a high sugar concentration by *Saccharomyces cerevisiae*, *Applied and Environmental Microbiology*, **63(1)**, 145-150 **(1997)**

30. Ishmayana S., Kennedy U.J. and Learmonth R.P., Further investigation of relationships between membrane fluidity and ethanol tolerance in *Saccharomyces cerevisiae*, *World Journal of Microbiology and Biotechnology*, **33(12)**, 218 **(2017)**

31. Jones R.P. and Greenfield P.F., Ethanol and the fluidity of the yeast plasma membrane, *Yeast*, **3**, 223-232 **(1987)**.