ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 7 (3): 493-498, 2008
ISSN 1682-296X

Selection of *Saccharomyces cerevisiae* and Investigation of its Performance for Very High Gravity Ethanol Fermentation

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Abstract: This research aims to select and evaluate the performance of three high-ethanol-producing strains of *Saccharomyces cerevisiae* (TISTR 5048, TISTR 5339 and NP 01) in very high gravity (VHG) ethanol fermentation. The maximum specific growth rates (μ_{max}) of TISTR 5048 and NP 01 grown in yeast extract malt extract broth containing 150 g glucose L^{-1} were 0.49 and 0.46 h, respectively while μ_{max} of TISTR 5339 could not be determined due to cell flocculation. The ethanol production by TISTR 5048 and NP 01 was further carried out in batch mode at 30°C under normal gravity fermentation (240 g glucose L^{-1}) and VHG fermentation (280 and 320 g glucose L^{-1}) and the initial cell concentration was 1×10^{8} cells mL^{-1}. The results showed that TISTR 5048 cultured in 240 and 280 g glucose L^{-1} gave the maximum ethanol concentration (P) with the value of 99.58 g L^{-1}, while NP 01 cultured in 280 and 320 g glucose L^{-1} gave the maximum P with the value of 104.68 g L^{-1}. Ethanol productivities (Q_p) of NP 01 were slightly higher than those of TISTR 5048 at all conditions tested.

Key words: Ethanol, fermentation, normal gravity, very high gravity, *Saccharomyces cerevisiae*

INTRODUCTION

Very-high-gravity (VHG) ethanol fermentation is one of process improvements for the fuel ethanol production. It aims at increasing both ethanol concentration and fermentation rate. It can reduce capital costs, energy costs per litre of alcohol as well as the risk of bacterial contamination (Thomas *et al*., 1996; Bvochora *et al*., 2000; Narendranath and Power, 2005). The VHG process involves preparation and fermentation of mashes containing at least 27 g of dissolved solids per 100 g mash (Bafrncová *et al*., 1999; Bayrock and Ingledew, 2001; Bai *et al*., 2004a, b). Under normal gravity, dissolved solid concentrations of 20-24 g per 100 g mash (Narendranath and Power, 2005) and suitable environmental parameters, the higher initial sugar concentration is used, the higher ethanol concentration is produced. However, ethanol tolerance and the ability to accumulate high ethanol concentrations are strain-dependent characteristics (Kosaric and Vardar-Sukan, 2001) especially under VHG conditions. In addition, environmental parameters such as temperature, osmotic pressure and carbon dioxide levels may directly affect yeast growth and ethanol productivity (Jones *et al*., 1981 cited in Nagashima, 1990).

Saccharomyces cerevisiae is one of ethanol-producing organisms used in industrial processes. Under VHG conditions if appropriate environment and all required nutrients in adequate amounts were provided, *S. cerevisiae* could ferment increased amount of sugars in the medium (Reddy and Reddy, 2005, 2006). In addition, it could produce and tolerate high ethanol concentrations (Thomas *et al*., 1996; Bafrncová *et al*., 1999). Successful VHG fermentation is therefore dependent not only on the optimal composition of a fermentation medium, but also on the yeast strain.

In Thailand, *S. cerevisiae* TISTR 5048 and TISTR 5339 are recommended as high ethanol producing strains under the normal gravity conditions (Arunpairojana *et al*., 2000) and *S. cerevisiae* NP01 was found to be a high ethanol producer in a Thai rice wine (Rittiplang, 2006). However, none has studied ethanol production using those strains under the VHG conditions. Thus, this research aims to select and evaluate the performance of the three high-ethanol-producing strains of *S. cerevisiae* in VHG ethanol fermentation in a synthetic ethanol production medium and to raise the final ethanol concentration in a batch system.

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MATERIALS AND METHODS

Microorganisms and growth conditions: *S. cerevisiae* TISTR 5048 and TISTR 5339 were obtained from MIRCEN, Bangkok, Thailand and *S. cerevisiae* NP01 was isolated from Loog-pang (Chinese yeast cake) for Sato (Thai rice wine) making (Rittiplang, 2006). The yeasts were grown in yeast extract malt extract (YM) broth containing 10, 150 and 240 g glucose L^{-1} on a rotating shaker at 100 rpm, 30°C. Maximum specific growth rate (μ_{max}) of the yeasts was calculated by determining viable cells using methylene blue staining technique (Zoecklien *et al*., 1995). The yeast cells in log phase grown in the YM broth giving μ_{max} were harvested and used as inoculum for ethanol production.

Ethanol production medium: Ethanol production medium (EP) consisted of (g L^{-1}) yeast extract, 3; peptone, 5; $MgSO_4.7H_2O$, 0.025; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1; $(NH_4)_2PO_4$, 1; $MnSO_4.6H_2O$, 0.5; $Zn(NO_3)_2$, 0.2 and glucose, 240, 280 or 320. The EP medium was transferred into a 500 mL air-locked Erlenmeyer flask with a final working volume of 400 mL and autoclaved at 110°C for 15 min.

Ethanol fermentation: The fermentation was carried out in batch mode under static condition at 30°C. The sterile EP medium at various initial glucose concentrations was inoculated with the *S. cerevisiae* strains to give the final cell concentrations of approximately 1×10^8 cells mL^{-1}. The samples were collected at time intervals for further analyses.

Analytical methods: The cell numbers of the fermentation broth were determined by direct counting method using heamacytometer (Zoecklien *et al*., 1995). The biomass yield (Y_{xs}) was calculated as the actual viable cells produced and expressed as cells per g sugar utilized (cells g $glucose^{-1}$). The fermentation broth was centrifuged at 13,000 rpm for 10 min. The supernatant was then determined for total residual sugars by a phenol sulfuric acid method (Mecozzi, 2005). Percentage of glucose utilization was calculated as the ratio of the consumed mass of glucose to the initial mass of glucose. Ethanol concentration was analyzed by gas chromatography (Shimadzu GC-14B, Japan, Solid phase: polyethylene glycol (PEG-20M), carrier gas: nitrogen, 90°C isothermal packed column, injection temperature 160°C, flame ionization detector temperature 230°C; C-R7 Ae plus Chromatopac Data Processor) and isopropanol was used as an internal standard (Laopaiboon *et al*., 2007). The ethanol yield (Y_{ps}) was calculated as the actual ethanol produced and expressed as g ethanol per g glucose utilized (g g^{-1}). The volumetric ethanol productivity (Q_p) and the percentage of conversion efficiency or yield efficiency (E_y) were calculated by the following equations:

$$Q_p = \frac{P}{t} \qquad \text{and} \qquad E_y = \frac{Y_{ps}\times 100}{0.51}$$

where, P is the actual ethanol concentration produced (g L^{-1}), t is the fermentation time (h) giving the highest ethanol concentration and 0.51 is the maximum theoretical ethanol yield of glucose consumption.

All the experiments were performed in duplicate and the results were expressed as mean±SD of the duplicated experiments. Statistical analysis was carried out using SPSS 15.0 for Windows.

RESULTS AND DISCUSSION

Effects of glucose on cell growth: Microbial growth patterns of the three high-ethanol-producing strains of *S. cerevisiae*; TISTR 5048, TISTR 5339 and NP01, were investigated under various initial glucose concentrations. Figure 1 shows the growth curve of the yeasts in the YM broth containing glucose at concentrations of 10, 150 and 240 g L^{-1}. No lag phase was observed after the yeast cells were inoculated into the YM broth at all sugar concentrations and stationary phase occurred at 12 to 15 h of the cultivation except for TISTR 5339. Cell concentrations of TISTR 5339 grown under glucose concentrations of 150 and 240 g L^{-1} did not increase after 3 h of the experiments due to cell flocculation. Consequently, the growth rate of this strain under both conditions could not be determined.

Main growth kinetic parameters (μ_{max}, glucose utilization and biomass yield) of the three strains are shown in Table 1 and 2. When μ_{max} of the three strains were compared, the strain giving the highest μ_{max} under glucose concentration of 10, 150 and 240 g L^{-1} was TISTR 5339, TISTR 5048 and NP 01, respectively (Table 1). Under 10 g L^{-1} of glucose, the sugar was almost utilized and the maximum biomass yields were obtained. Even though YM broth containing 10 g glucose L^{-1} is used as a standard medium for yeast inoculum preparation, in this experiment it is not suitable for inoculum preparation. The total biomass produced under 10 g glucose L^{-1} was significantly ($p\leq0.05$) lower than those produced under the other two glucose concentrations (Table 2). If 10 g L^{-1} of glucose is used for inoculum preparation, massive volume of culture medium is needed to obtain high biomass. Another important reason is that the inoculum or cells prepared under a high initial glucose concentration will be acclimatized under high sugar concentrations, which then be useful for ethanol fermentation under VHG condition. Therefore, TISTR 5339 was not selected for further studies because it was

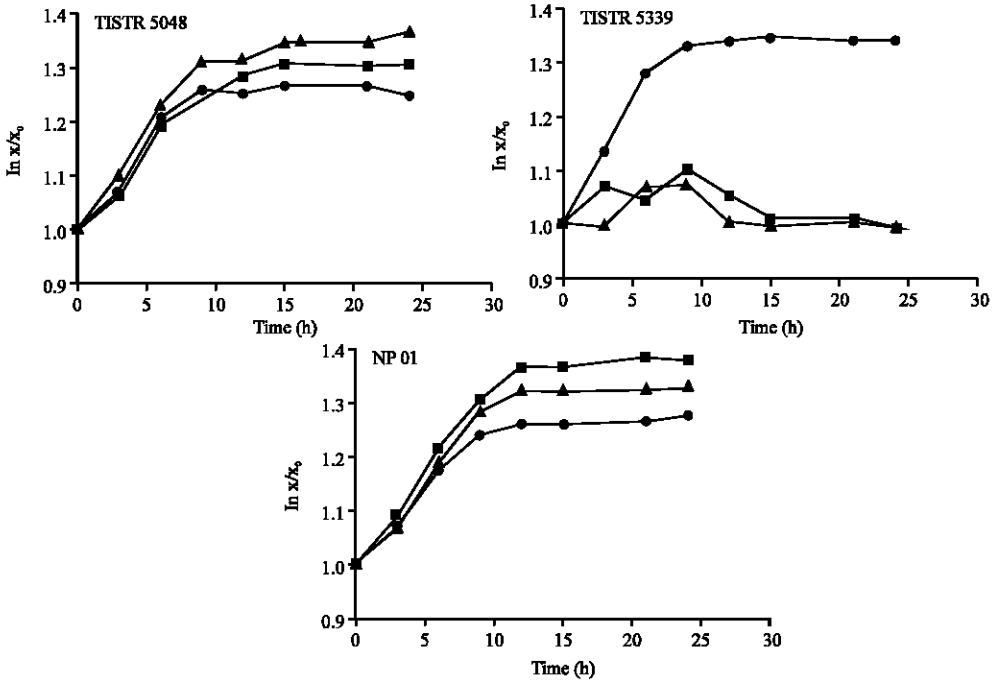

Fig. 1: Growth curves of *S. cerevisiae* TISTR 5048, *S. cerevisiae* TISTR 5339 and *S. cerevisiae* NP 01 at 30°C, 100 rpm in YM medium containing various glucose concentrations: (●) 10, (▲), 150 and (■) 240 g L^{-1}

Table 1: Maximum specific growth rate (μ_{max}) of the *S. cerevisiae* strains grown in YM medium containing various glucose concentrations at 30°C, 100 rpm

	μ_{max} (h^{-1}) (mean±SD)		
Strains	10 g glucose L^{-1}	150 g glucose L^{-1}	240 g glucose L^{-1}
TISTR 5048	0.43±0.03	0.49±0.03	0.45±0.02
TISTR 5339	0.62±0.02	-	-
NP 01	0.39±0.04	0.46±0.01	0.48±0.01

*The experiments were performed in duplicate

Table 2: Glucose utilization and biomass yield of the *S. cerevisiae* strains in YM medium containing various glucose concentrations

Strains	Glucose concentration (g L^{-1})	Initial cell concentration (cells mL^{-1})[a]	Glucose utilized (%)[a]	Final cell concentration (cells mL^{-1})[a]	Y_{xs}[b]
TISTR 5048	10	(1.30±0.28)×10^6	94.65±0.21	(5.50±0.42)×10^7	5.67×10^6
	150	(1.08±0.25)×10^6	91.13±0.17	(1.32±0.05)×10^8	9.58×10^5
	240	(1.25±0.00)×10^6	45.04±1.29	(8.55±1.91)×10^7	7.80×10^5
TISTR 5339	10	(0.80±0.10)×10^6	94.39±0.02	(7.40±1.13)×10^7	7.76×10^6
	150	(2.00±0.14)×10^6	-	-	-
	240	(1.68±0.25)×10^6	-	-	-
NP 01	10	(1.80±0.71)×10^6	96.50±0.00	(7.40±0.43)×10^7	7.48×10^6
	150	(1.85±0.01)×10^6	81.33±0.12	(1.97±0.00)×10^8	1.60×10^6
	240	(0.95±0.14)×10^6	49.52±0.44	(1.80±0.13)×10^8	1.51×10^6

[a]The results were expressed as mean±SD of the two replication. [b]Biomass yield (cells produced per g glucose utilized)

unable to grow under high glucose concentrations. Biomass yields of both TISTR 5048 and NP 01 under 150 g glucose L^{-1} were similar to those under 240 g glucose L^{-1}. However, glucose utilization under 240 g L^{-1} was relatively low at only 45-50%, whereas glucose utilized under 150 g L^{-1} was quite high, at approximately 81-91% depending on the yeast strains. In addition, the biomass yields of the two strains grown under 150 g glucose L^{-1} were similar. Therefore, both TISTR 5048 and NP 01 were further investigated for VHG ethanol fermentation and the YM broth containing 150 g L^{-1} of glucose would be used for inoculum preparation for the subsequent experiments.

Normal gravity and VHG ethanol fermentations: The time profiles of total residual sugar, ethanol and cell numbers of the fermentation broth during normal gravity and VHG fermentations by TISTR 5048 and NP 01 are shown in Fig. 2. At all experimental conditions, the cell concentrations were relatively constant throughout 40 h

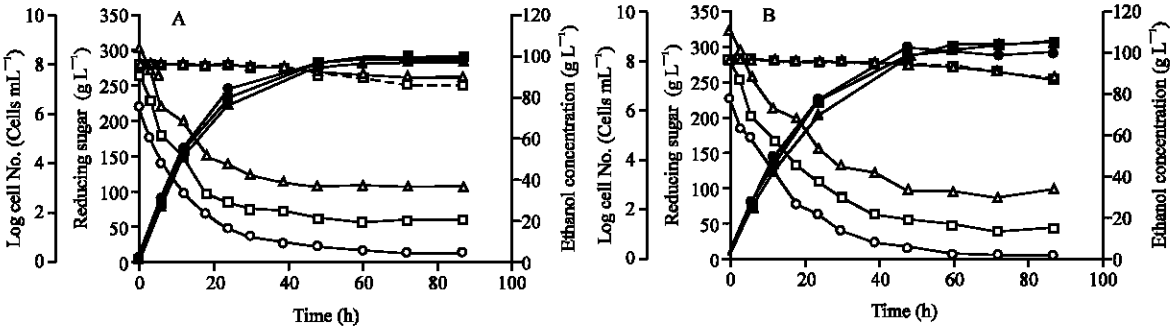

Fig. 2: Effects of initial glucose concentration on cell growth, sugar utilization and ethanol production during batch ethanol fermentation by *S. cerevisiae* TISTR 5048 (A) and *S. cerevisiae* NP 01 (B) : glucose 240 g L^{-1} (●,○), glucose 280 g L^{-1} (■,□), glucose 320 g L^{-1}(▲,Δ); cell numbers (open symbol, _ _ _), reducing sugar (open symbol, ___) and ethanol (close symbol)

Table 3: Fermentation kinetic parameters of ethanol production at various initial glucose concentrations by *S. cerevisiae* TISTR 5048 and *S. cerevisiae* NP 01

Strains	Glucose concentrations (g L^{-1})	Parameters (mean±SD)[a]				
		P (g L^{-1})	Q_p (g L^{-1} h^{-1})	Y_{ps} (g g^{-1})	E_y (%)	t (h)
TISTR 5048	240	99.58±1.06[b]	1.66±0.02	0.50±0.01	98	60
	280	99.42±1.40	1.66±0.03	0.49±0.01	96	60
	320	97.01±0.48	1.61±0.01	0.50±0.01	98	60
NP 01	240	101.95±0.50	2.12±0.01	0.48±0.02	94	48
	280	104.68±0.11	1.75±0.00	0.44±0.00	86	60
	320	104.68±0.00	1.75±0.00	0.44±0.01	86	60

[a]P Ethanol concentration produced; Q_p Volumetric ethanol productivity; Y_{ps} ethanol yield; E_y yield efficiency and t fermentation time, [b]The experiments were performed in duplicate

of the fermentations and they were less than one log reduction at the end of the experiments. The sugars were almost completely consumed under normal gravity fermentation (240 g glucose L^{-1}). Under VHG conditions at the initial sugar concentrations of 280 and 320 g L^{-1}, stuck fermentation was observed with approximately 42 to 60 and 96 to 107 g L^{-1} of reducing sugar remaining in the fermentation broth, respectively. In addition, the results showed that the sugars remaining in the fermentation broth using NP 01 were 3 to 7% lower than those using TISTR 5048 at all initial sugar concentrations. This indicated that the sugar utilization was strain-dependent and NP 01 had a better capability of glucose utilization than TISTR 5048. Nagashima (1990) reported that as the ethanol concentration increased, a decrease in growth rate was the first incident observed. However, the results obtained from this study revealed that both TISTR 5048 and NP 01 could tolerate high ethanol concentrations up to approximately 100 g L^{-1} before some yeast cells died.

Table 3 shows the important fermentation kinetic parameters at various initial glucose concentrations by TISTR 5048 and NP 01. When fermentation kinetic parameters were compared, the ethanol concentration produced, P, using TISTR 5048 under the normal gravity (99.58 g L^{-1}) was not significantly different ($p \leq 0.05$) from that under the VHG at 280 g glucose L^{-1} (99.42 g L^{-1}). The results suggested that this strain was suitable for ethanol fermentation under normal gravity more than under VGH condition. When NP 01 was used, the VHG fermentations gave higher ethanol concentration than under normal gravity fermentation. However, further increase in glucose concentration from 280 to 320 g glucose L^{-1} did not lead to an increase in ethanol concentration. The P at 280 and 320 g glucose L^{-1} were almost the same, implying that NP 01 was a suitable strain for VHG fermentation at the initial glucose concentration not exceeding 280 g L^{-1}. When the main fermentation kinetic parameters of the two strains were compared, the results showed that P and Q_p of NP 01 were higher than those of TISTR 5048 under both normal gravity and VHG fermentations, while Y_{ps} of both strains were similar under the normal gravity condition. Under VHG conditions, however, ethanol yield efficiencies (E_y) of TISTR 5048 was about 10% higher than those of NP 01. The results implied that additional by-products such as glycerol, succinate, alpha-ketoglutarate, butanediol and diacetyl (Zoecklein *et al*., 1995) might be produced by NP 01 during the fermentations. TISTR 5048 gave lower P than NP 01 suggesting that TISTR 5048 might be less ethanol tolerant.

As approximately 15% of initial sugar concentration still remained at the end of the VHG fermentation at 280 g glucose L^{-1} by NP 01, complete sugar utilization may be achieved by optimization of aeration rate, agitation rate and nutrient supplementations (Bafrncová *et al*., 1999; Alfenore *et al*., 2004).

CONCLUSION

The results obtained from this study have demonstrated that among the three *S. cerevisiae* strains, NP 01 was found to be the most suitable strain for ethanol production under VHG fermentation. At total sugar concentration of 280 g L^{-1}, P, Q_p and Y_{ps} were 104.68 g L^{-1}, 1.75 g L^{-1} h^{-1} and 0.44 g g^{-1}, respectively. To achieve the goals of VHG fermentation, which are the improvement of ethanol production efficiency and complete sugar utilization, environmental parameters such as aeration rate and stirring speed during VHG fermentation as well as nutrient supplementation should be further studied.

ACKNOWLEDGMENTS

The authors would like to thank Assistant Prof. Dr. Paiboon Danviruthai, Faculty of Technology, Khon Kaen University for providing the NP 01 strain and the Thailand Research Fund (TRF) for financial support. We also gratefully acknowledge the Royal Bangkok Sports Club (RBSC), Bangkok, Thailand and the Fermentation Research Center for Value Added Agricultural Products (FerVAAP) for financial support for Mr. Sunan Nuanpeng.

REFERENCES

Alfenore, S., X. Cameleyre, L. Benbadis, C. Bideaux, J.L. Uribelarrea, G. Goma, C. Molina-Jouve and S.E. Guillouet, 2004. Aeration strategy: A need for very high ethanol performance in *Saccharomyces cerevisiae* fed-batch process. Applied Microbiol. Biotechnol., 63 (5): 537-542.

Arunpairojana, V., C. Sawannachart, L. Chatanon and P. Budhaka, 2000. TISTR Culture Collection: List of Cultures. 6th Edn. Bangkok MERSEN, Bangkok.

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

Bai, F.W., L.J. Chen, W.A. Anderson and M. Moo-Young, 2004a. Parameter oscillations in very high gravity continuous ethanol fermentation and their attenuation on multi-stage packed column bioreactor system. Biotechnol. Bioeng., 88 (5): 558-566.

Bai, F.W., L.J. Chen, Z. Zhang, W.A. Anderson and M. Moo-Young, 2004b. Continuous ethanol production and evaluation of yeast cell lysis and viability loss under very high gravity medium condition. J. Biotechnol., 110 (3): 287-293.

Bayrock, D.P. and W.M. Ingledew, 2001. Application of multistage continuous fermentation for production of fuel alcohol by very-high-gravity fermentation technology. J. Ind. Microbiol. Biotechnol., 27 (2): 87-93.

Bvochora, J.M., J.S. Read and R. Zvauya, 2000. Application of very high gravity technology to the cofermentation of sweet stem sorghum juice and sorghum grain. Ind. Crop Prod., 11 (1): 11-17.

Kosaric, N. and F. Vardar-Sukan, 2001. Potential Source of Energy and Chemical Products. In: The Biotechnology of Ethanol: Classical and Future Applications, Roehr, M. (Ed.). Wiley-VCH, Weinheim.

Laopaiboon, L., P. Thanonkeo, P. Jaisil and P. Laopaiboon, 2007. Ethanol production from sweet sorghum juice in batch and fed-batch fermentations by *Saccharomyces cerevisiae*. World J. Microbiol. Biotechnol., 23 (10): 1497-1501.

Mecozzi, M., 2005. Estimation of total carbohydrate amount in environmental samples by the phenol-sulphuric acid method assisted by multivariate calibration. Chemom. Intel. Lab. Syst., 79 (1-2): 84-90.

Nagashima, M., 1990. Progress in Ethanol Production with Yeasts. In: Yeast: Biotechnology and Biocatalysis, Hubert, V. and De M. Rene (Eds.). Marcel Dekker, Inc., New York.

Narendranath, N.V. and R. Power, 2005. Relationship between pH and medium dissolved solids in terms of growth and metabolism of Lactobacilli and *Saccharomyces cerevisiae* during ethanol production. Applied Environ. Microbiol., 71 (5): 2239-2243.

Reddy, L.V.A. and O.V.S. Reddy, 2005. Improvement of ethanol production in very high gravity fermentation by horse gram (*Dolichos biflorus*) flour supplementation. Lett. Applied Microbiol., 41 (5): 440-444.

Reddy, L.V.A. and O.V.S. Reddy, 2006. Rapid and enhanced production of ethanol in very high gravity (VHG) sugar fermentations by *Saccharomyces cerevisiae*: Role of finger millet (*Eleusine coracana* L.) flour. Process Biochem., 41 (3): 726-729.

Rittiplang, J., 2006. Specific selection of microorganisms for high efficiency loog-pang preparation for quality rice wine production. M.Sc. Thesis, Khon Kaen University, Thailand.

Thomas, K.C., S.H. Hynes and W.M. Ingledew, 1996. Practical and theoretical considerations in the production of high concentrations of alcohol by fermentation. Process Biochem., 31 (4): 321-331.

Zoecklien, B.W., K.C. Fugelsang, B.H. Gump and F.S. Nury, 1995. Laboratory Procedures. In: Wine Analysis and Production. Chapman and Hall, New York.