ISSN 1330-9862
(FTB-1707)

original scientific paper

Effect of Continuous Fermentation of High-Sugar Fruit Must on the Viability and Morphology of Immobilized Yeast on White Foam Glass

Sylwia Bonin and Joanna Skwira*

Department of Food Biotechnology and Microbiology, Faculty of Food Sciences, Warsaw Agricultural University, ul. Nowoursynowska 169C, PL-02767 Warsaw, Poland

Received: April 29, 2006
Revised version: July 11, 2007
Accepted: September 25, 2007

Summary

We studied the effect of continuous fermentation of high-sugar fruit must (containing about 320 g/L of total sugars) on the viability and morphology of yeast cells. The process was carried out for 2.5 months in a 4-column bioreactor at 22 °C, using the *Saccharomyces bayanus* S.o./1AD wine yeast strain, which was immobilized on cubes of white foam glass. During the time of continuous fermentation, the apple wine contained 11.4–16.8 % (by volume) of alcohol and a total sugar concentration of 49.2–115.4 g/L. Yeast cells isolated from the carrier at the end of continuous fermentation were bigger than the cells before immobilization and were characterized by various shapes, *e.g.* they were elongated, large and round or pear-shaped. Some cells were connected to other cells in the form of aggregates. Some yeast cells from the second, third and fourth columns showed a substantial number of wrinkles or folds. Moreover, it was observed that yeast from the carrier in the first column was characterized by the highest viability, 70 %. In the fourth column, the percentage of viable cells was only 11 %.

Key words: continuous fermentation, immobilization, yeast, morphology

Introduction

During continuous fermentation of a high-sugar medium, yeast cells are subjected to osmotic and ethanol stresses. Osmotic stress is due to a high sugar content (around 200 g/L) in the must (*1*). Increasing the initial sugar concentration from 200 to 300 g/L results in a significant decrease of fermentation efficiency and yeast viability (*2*). In the absence of additional nutrients to support growth, incubation in sugar causes sugar-induced cell death (SICD), characterized by rapid production of reactive oxygen species, RNA and DNA degradation, membrane damage, nucleus fragmentation, and cell shrinkage (*3*). A major product of yeast fermentation is ethanol, which has an adverse effect on yeast cells. Ethanol interferes with the hydrogen bonding within and between hydrated cell components, ultimately disrupting enzyme and membrane structure and function (*4*). The sites of ethanol action in yeast are the plasma membrane, hydrophobic proteins of the cell and mitochondrial membranes, nuclear membrane, vacuolar membrane, endoplasmic reticulum and hydrophilic proteins of the cytoplasm. Negative effects of ethanol on yeast include the inhibition of the metabolic activity of the cells, cell growth, division and viability (*5*).

In our previous research we concluded that the continuous fermentation of fruit must containing about 320 g/L of sugar is possible (*6,7*). In this previous process, wine yeast strains *Saccharomyces bayanus* S.o./1 and S.o./1AD were used. Fermentation was carried out for 3.5 months at 22 °C and the yeasts were immobilized on

*Corresponding author; Phone: ++48 600 160 834; Fax: ++48 22 593 7681; E-mail: sylwia.bon@gmail.com

white foam glass. During the continuous fermentation, ethanol concentration in the fruit wine was 11.7–16.8 % by volume. From the second week until the end of the process, the fermentation yield was 93–96 % (for *S. bayanus* S.o./1AD) and 91–94 % (for *S. bayanus* S.o./1). Furthermore, after the fermentation, the yeast showed morphological and intracellular changes in comparison with the yeast before the fermentation.

Magnesium protects yeast cells that are under ethanol stress. Mg^{2+} maintains the structural integrity of membranes, reduces the extent of cell-wall disruption and prevents ethanol stress protein synthesis. During fermentation of a high-sugar medium, magnesium increases ethanol production (*5*,*8*).

The aim of this work is to study the effect of continuous fermentation of high-sugar fruit must, supplemented with magnesium ions, on the viability and morphology of yeast immobilized on white foam glass.

Materials and Methods

Yeast strain and cultivation conditions

For the current experiment, we used an alcohol- and sugar-resistant wine yeast strain (*Saccharomyces bayanus* S.o./1AD) from the collection of pure cultures at the Department of Biotechnology and Microbiology of Warsaw Agricultural University. Before starting the process of continuous fermentation, the yeast was subjected to high sugar and SO_2 content by being grown in media containing higher and higher concentrations of sugar (200––320 g/L) and SO_2 (20–80 mg/L). These media were prepared similarly to the fruit must, but they were sterilized at 117 °C for 10 min. After sterilization, SO_2 in the form of $K_2S_2O_5$ was added. The yeast was incubated by being shaken at 200 rpm and 28 °C for 48 h. After incubation in a medium containing 320 g/L of sugar and 80 mg/L of SO_2, the yeast was added to the columns of the bioreactor, which had been filled with the carrier.

Fruit must

Must was prepared from the apple juice (density 5.7 °Bé), which was obtained through the dilution of the concentrate (37.6 °Bé) with deionized water. The fraction of juice in the must was 70 %. Sugar (in the form of sucrose) was added in order to obtain a high-sugar concentration (approx. 320 g/L). Generally, apple must contains a low concentration of nitrogenous compounds, therefore the medium was enriched with ammonium salts $(NH_4)_2HPO_4$, 0.3 g/L and $(NH_4)_2SO_4$, 0.2 g/L. The must was supplemented with 20 mM magnesium in the form of $MgSO_4{\cdot}7H_2O$. To prevent the development of harmful microflora, SO_2 in the form of $K_2S_2O_5$ was added. The total SO_2 concentration in fruit must was about 80 mg/L.

Continuous fermentation

Continuous fermentation was carried out in the 4-column glass bioreactor (each column was 75 cm high, 5.2 cm i.d.). In each column, cubes of white foam glass (each about 1 cm wide) were placed. White foam glass (also called glass pumice) is an inorganic porous material produced from glass powder with the addition of foaming agents, for example $CaCO_3$. In our experiments, the carrier was used after being washed with 3 % citric acid. Yeast was added every 2 days to each of the columns of the bioreactor (beginning with the fourth). This interval was necessary so that the yeast would become immobilized on the carrier. Must was fed into the bioreactor using a peristaltic pump (Zalimp PP1B-05). The flow rate was about 550 cm^3/day and the total time of flow through the bioreactor was 5 days. This period of flow was adequate for fruit wine fermentation. The reactor operated for a total of 2.5 months at (22±1) °C.

Analytical methods

Ethanol concentration was expressed as alcohol degrees (cm^3 of ethanol/100 cm^3 of wine) after the distillation of samples (*9*). Residual sugars were analyzed according to the Luffa-Schoorla method (*9*), after the hydrolysis of sugars in 0.7 M HCl for 10 min at 70–72 °C and neutralization with 30 % NaOH.

Number and viability of yeast on foam glass

At the end of continuous fermentation, 2 cubes of the carrier from the top, middle and bottom of each column were placed in Erlenmeyer flasks with 100 cm^3 of sterile 0.8 % NaCl solution. The flasks were shaken at 200 rpm at 28 °C for 2 h to remove the yeast from the carrier. The total number of cells was estimated by direct microscopic counting using a haemocytometer. The number of viable cells was determined by methylene blue staining and by plate technique in a malt extract broth after incubation at 28 °C for 48 h. The number of yeast cells was calculated on the surface of 1 cm^2 of foam glass.

Yeast morphology

The cells before immobilization and those removed from the carrier after the process of continuous fermentation were examined under either a light microscope (Carl Zeiss Axiostar Plus) or a scanning electron microscope (Joel JSM-35). For the scanning electron microscope, yeast cells were fixed in 2 % glutaraldehyde, dehydrated in a graded series of ethanol concentration, dried to the critical point and sputter-coated with gold. The size of the cells was characterized by image analysis in Zeiss LSM Image Browser program. The length and width of 100 cells were measured.

Results

The concentration of ethanol and residual sugars in the must during continuous fermentation is presented in Fig. 1. The initial concentration of sugars in fruit must was sufficient to obtain 18.5 % (by volume) of alcohol. In the first 5 weeks of continuous fermentation, alcohol fraction in the apple wine rose from 11.4 to 16.8 % (by volume). Afterwards, there was a gradual decrease to 14.7 % (by volume) until the 73rd day. The concentration of residual sugars was relatively high and it correlated with alcohol content. After 3 days of continuous fermentation, the concentration of residual sugars in the fruit wine was 115.4 g/L. This concentration decreased over 5 weeks to 49.2 g/L, then increased towards the end of fermentation.

After the fermentation was completed, yeast was isolated from the carrier. The total number of cells on the surface of white foam glass and the number of viable cells were determined. The average results were obtained from 3 levels (top, middle and bottom) of each column. Both the total number of cells and the number of viable cells were the highest in the first column, and both values decreased with each subsequent column (Table 1). We determined that there were $2.5 \cdot 10^7$ cells/cm^2 per carrier in the first column and $9.6 \cdot 10^6$ cells/cm^2 in the fourth column (direct counting). The number of viable cells was smaller, from $7.1 \cdot 10^6$ CFU/cm^2 of foam glass in the first column to $6.0 \cdot 10^4$ CFU/cm^2 in the last column. The percentage of viable cells was determined by methylene blue staining. Yeast isolated from the carrier from the first column was characterized by the highest part of viable cells – an average of 70 % across the three levels. In comparison, this number was 47 % in the second column, 17 % in third and only 11 % in the last (Table 2). These differences were statistically significant (Tukey's HSD (honestly significant difference)= =15.6). The number of cells and their viability were determined only from the yeast cells that were isolated from the carrier. After 2 hours of shaking in the solution and all the subsequent procedures of preparation, we examined crushed pieces of carrier with yeast under an electron-scanning microscope (Fig. 2). Actually, the number of cells on the carrier was higher and the viability was probably higher as well.

Yeast cells before immobilization were ovoid, single or budding (Fig. 3). The average cell was 6.7 μm long and 5.2 μm wide. Most cells were 6–7 μm long (42 %) and 5–6 μm wide (49 %) (Table 3). At the end of fermentation, the cells were characterized by various shapes

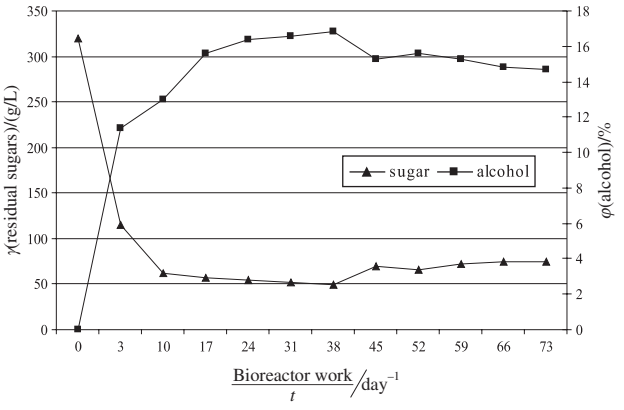

Fig 1. Effect of fermentation time on alcohol fraction and residual sugars in fruit wine

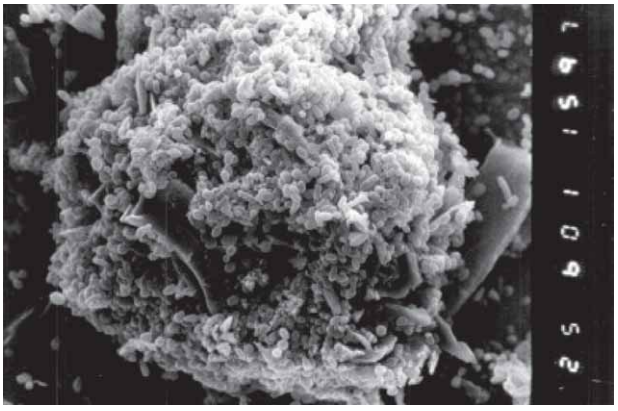

Fig. 2. White foam glass with the yeast after 2 h of shaking, magnification ×600. Figure is reproduced at 80 % of the original

Table 1. Number of yeast cells isolated from the carrier after the end of fermentation: direct counting (cells/cm^2 of white foam glass), plate methods (CFU/cm^2 of white foam glass)

Level of the column	First column		Second column		Third column		Fourth column	
	direct counting	plate methods	direct counting	plate methods	direct counting	plate methods	direct counting	plate methods
	cells/cm^2	CFU/cm^2	cells/cm^2	CFU/cm^2	cells/cm^2	CFU/cm^2	cells/cm^2	CFU/cm^2
Top	$3.1 \cdot 10^7$	$6.8 \cdot 10^6$	$1.4 \cdot 10^7$	$5.9 \cdot 10^6$	$1.5 \cdot 10^7$	$9.0 \cdot 10^5$	$9.9 \cdot 10^6$	$1.1 \cdot 10^5$
Middle	$2.5 \cdot 10^7$	$7.9\ 10^6$	$1.5 \cdot 10^7$	$8.0 \cdot 10^6$	$1.3 \cdot 10^7$	$7.0 \cdot 10^5$	$9.5 \cdot 10^6$	$4.0 \cdot 10^4$
Bottom	$1.9 \cdot 10^7$	$6.7 \cdot 10^6$	$1.9 \cdot 10^6$	$9.9 \cdot 10^5$	$8.1 \cdot 10^6$	$2.0 \cdot 10^5$	$9.4 \cdot 10^6$	$2.0 \cdot 10^4$
Average	$2.5 \cdot 10^7$	$7.1 \cdot 10^6$	$1.3 \cdot 10^7$	$5.0 \cdot 10^6$	$1.2 \cdot 10^7$	$6.0 \cdot 10^5$	$9.6 \cdot 10^6$	$6.0 \cdot 10^4$

Table 2. Viable cells isolated from the carrier after the end of fermentation, direct counting with methylene blue

Level of the column	First column/%	Second column/%	Third column/%	Fourth column/%
Top	77	58	17	12
Middle	68	46	16	11
Bottom	63	36	19	10
Average	70	47	17	11

Tukey's HSD (honestly significant differences) between columns=15.6

and sizes, in comparison with cells before immobilization. Yeast cells from the carrier from the first, second and third columns were longer, but their average width was smaller than of the cells isolated before continuous fermentation (Table 4). The longest cells were in the first column, where they had an average length of 7.7 μm. We observed that the average length decreased with the column number and cells from the last column were the shortest (6.4 μm). Generally, cells from this column were the smallest, because they also had the smallest average width (4.3 μm). In the first column 33 % of the cells were 8–10 μm long and 7 % were 10–12 μm long. In the sub-

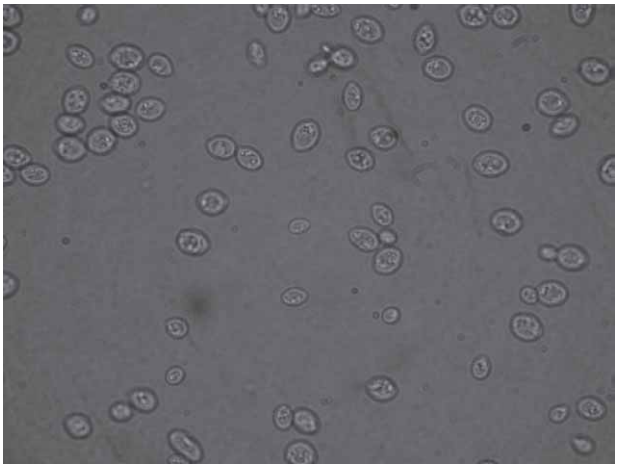

Fig. 3. Yeast cells before immobilization, magnification ×1200. Figure is reproduced at 80 % of the original

sequent columns, the percentage of yeast cells in these dimensions decreased, and in the last column, 11 % of the cells were 8–10 μm long, while there were no cells that were 10–12 μm long. In the first column, the presence of the cells that were 8–12 μm long is due to their elongated and rod-like shape (Fig. 4a).

We observed that morphological changes of yeast isolated from the carrier after continuous fermentation varied in each of the columns. In the first column, some cells had normal shapes: ovoid, single or budded. These cells also had bud scars. Certain cells did not have normal shapes (Fig. 4a). As we mentioned above, some cells were elongated, in the form of longer or shorter rods. Other forms of yeast had a pear-like morphology. These

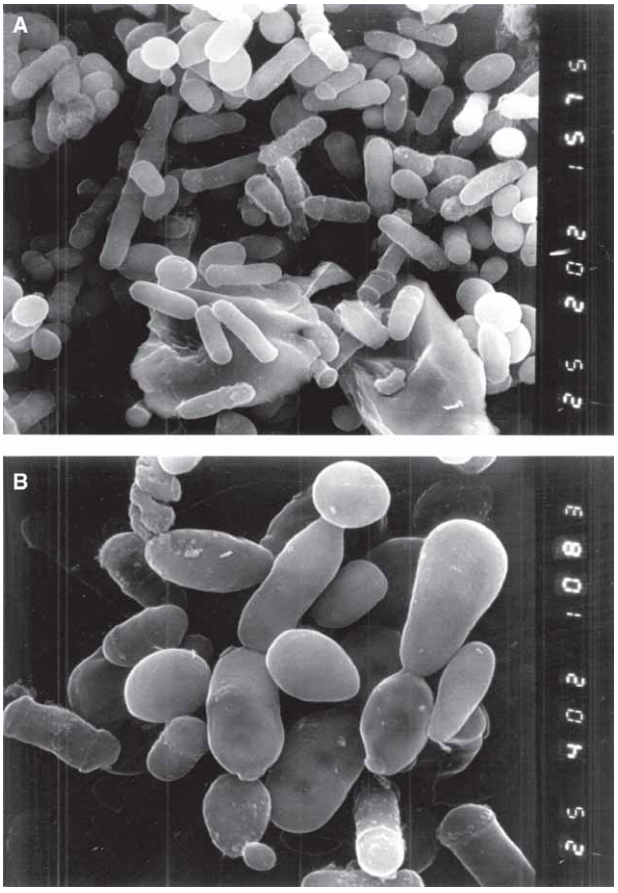

Fig. 4. Yeast cells isolated from the carrier from the first column, magnification ×2000 (A) and ×4000 (B). Figures are reproduced at 80 % of the original

Table 3. Percentage of the cells in the given range of sizes and average size of cells (the yeasts before fermentation)

Parameter	Dimension/μm							Average size/μm	Standard deviation
	3–4	4–5	5–6	6–7	7–8	8–9	9–10		
	Cells/%								
Length	0	6	12	28	42	11	1	6.7	1.6
Width	12	26	49	13	0	0	0	5.2	0.8

Table 4. Percentage of the cells in the given range of sizes and average size of cells (the yeasts isolated from the carrier after the end of fermentation)

Column number	Length/μm					Average size/μm	Standard deviation
	2–4	4–6	6–8	8–10	10–12		
	Cells/%						
I	0	14	46	33	7	7.7	1.6
II	0	15	61	20	4	7.4	1.5
III	3	29	49	19	0	7.0	1.5
IV	9	34	46	11	0	6.4	1.9
Column number	Width/μm					Average size/μm	Standard deviation
	2–4	4–6	6–8	8–10			
	Cells/%						
I	26	55	19	0		4.9	1.2
II	19	61	20	0		5.1	1.1
III	44	53	2	1		4.7	1.0
IV	12	75	13	0		4.3	1.1

pear-like cells were firmly bound to either other pear--like cells or to cells with normal shapes. This resulted in forms of small (3–6 cells) pseudohypha (Figs. 4b and 5). In the second column, we observed budding cells and cells with bud scars (Fig. 6A). Sometimes we found wrinkled or folded cells (Fig. 6B), while some yeast cells occurred in the form of few connected cells, had a pear--like morphology, but were less elongated (Fig. 7). In the third column, budding cells were rarely observed. These cells had shapes similar to the shapes of the yeast cells

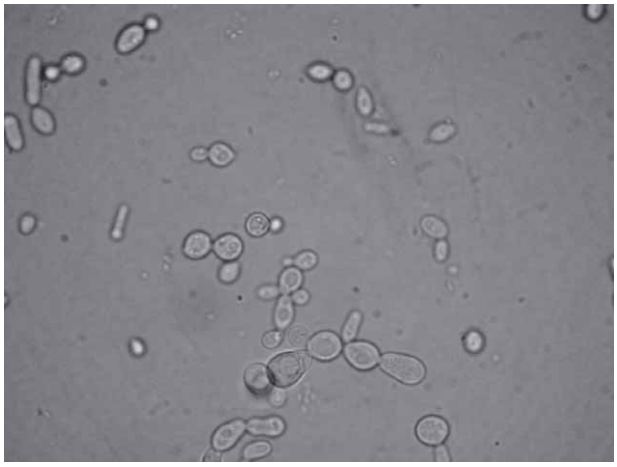

Fig. 5. Yeast cells isolated from the carrier from the first column, magnification ×1200. Figure is reproduced at 80 % of the original

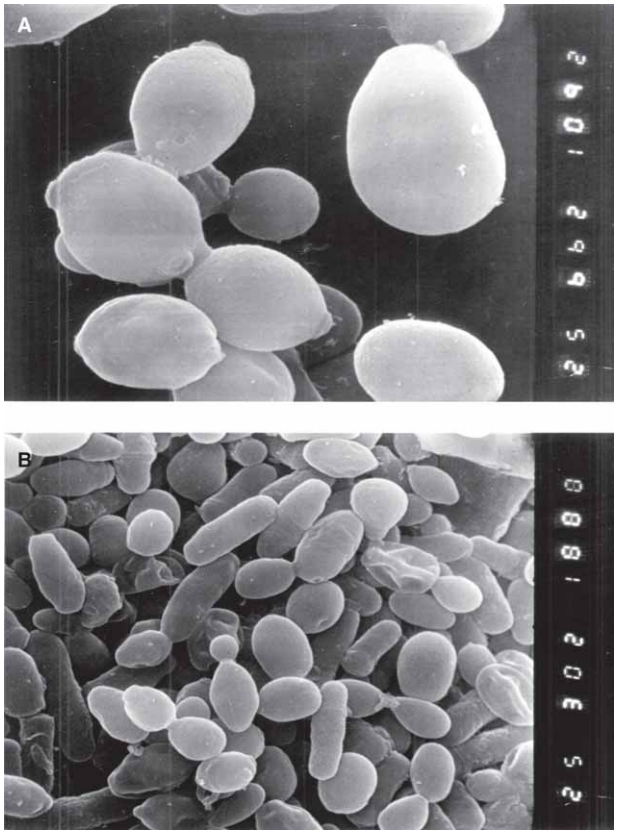

Fig. 6. Yeast cells isolated from the carrier from the second column, magnification ×6600 (A) and ×3000 (B). Figures are reproduced at 80 % of the original

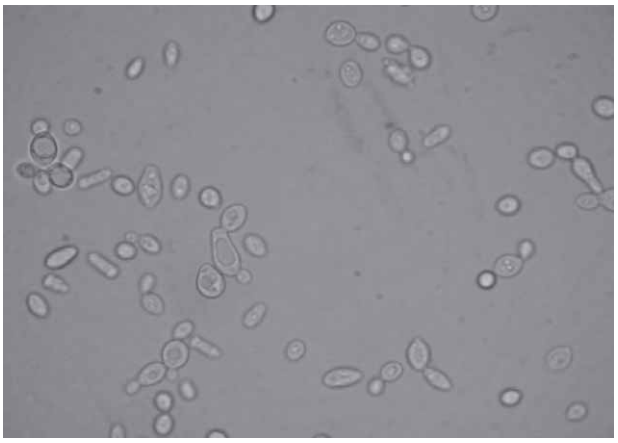

Fig. 7. Yeast cells isolated from the carrier from the second column, magnification ×1200. Figure is reproduced at 80 % of the original

in the second column. Furthermore, certain cells were more distorted and we found large, round cells (Fig. 8). In the fourth column, cells with a rod-like morphology were scarce. The majority of cells in this column were large and round (Figs. 9 and 10), distorted and with a

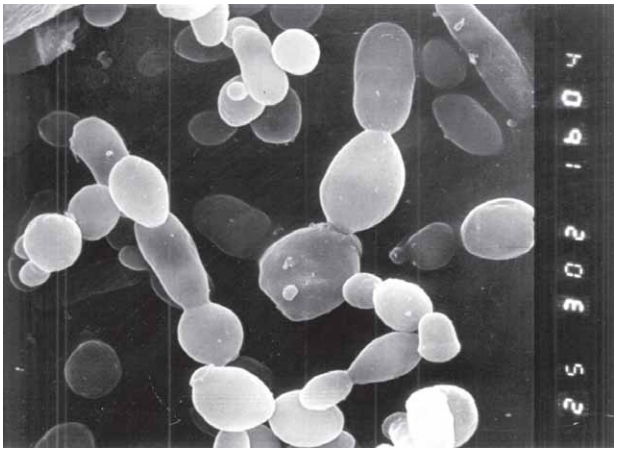

Fig. 8. Yeast cells isolated from the carrier from the third column, magnification ×3000. Figure is reproduced at 80 % of the original

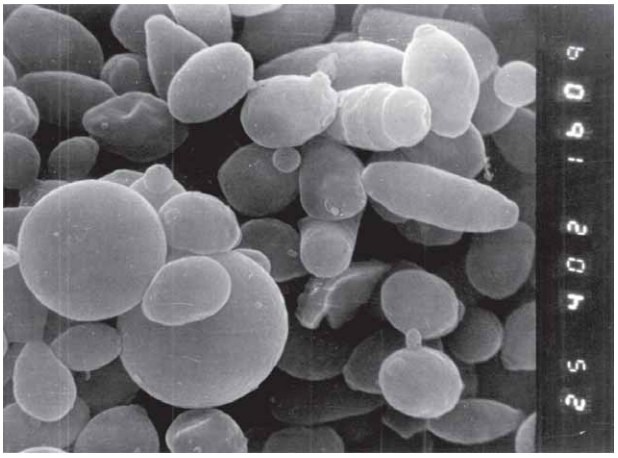

Fig. 9. Yeast cells isolated from the carrier from the fourth column, magnification ×4000. Figure is reproduced at 80 % of the original

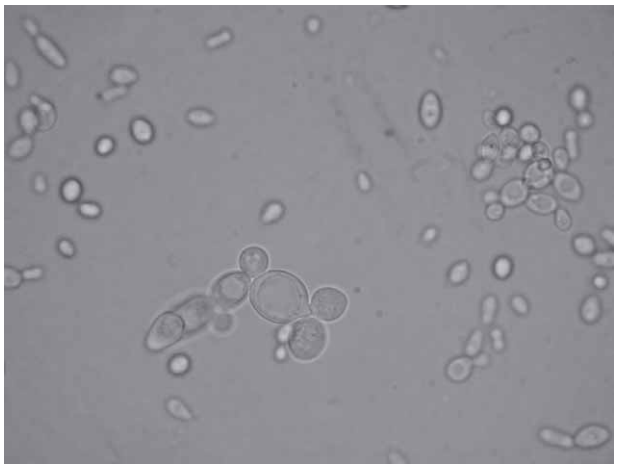

Fig. 10. Yeast cells isolated from the carrier from the fourth column, magnification ×1200. Figure is reproduced at 80 % of the original

large number of wrinkles or folds. These large and round cells were observed more often under the light microscope than under the scanning electron microscope.

Discussion

During the whole experiment, the yeast was subjected to high ethanol and sugar concentrations. An ethanol fraction of about 15.9 % (by volume) is the growth limit for most *Saccharomyces cerevisiae* strains (*4*), but sake yeast and some wine yeasts are known to produce about 20 % ethanol (*10*). Carrasco *et al.* (*1*) found that resistance to ethanol and osmotic stress are dependent on the wine yeast strain, and the most ethanol-resistant commercial wine strain was able to grow at 15 % ethanol. The yeast strain *Saccharomyces bayanus* S.o./1AD used in our experiment was obtained in our department from the *S. bayanus* S.o./1 strain by a long-time adaptation to high sugar concentrations and was able to produce about 18 % (by volume) of ethanol (*11*). Ogawa *et al.* (*12*) found that in the ethanol-tolerant mutant the expression level of stress responsive genes was further increased after exposure to ethanol. The ethanol-tolerant mutant also exhibited resistance to other stresses including heat, high osmolarity and oxidative stress in addition to ethanol tolerance.

In our previous work (*13*) fruit must was prepared using tap water, without a magnesium supplement. At the completion of 3.5 months of continuous fermentation, we determined that the number of viable *S. bayanus* S.o./1AD cells on the surface of the carrier was higher than that of *S. bayanus* S.o./1 cells. The percentage of viable *S. bayanus* S.o./1AD cells was the highest in the first column (43 %), and it then decreased with each subsequent column. Yeasts of *S. bayanus* S.o./1 isolated from the carrier from the first and second columns were characterized by a similar viability (29 and 30 %). Analysis of both strains in the third and fourth columns showed the same percentage of viable cells (20 and 10 %).

In this experiment, fruit must was prepared using deionized water supplemented with 20 mM of magnesium. Continuous fermentation lasted only 2.5 months, one month less than in our previous research. Yeast cells isolated from the carrier from the first column were characterized by the highest viability (70 %), while in the third and fourth columns, the percentage of viable cells was similar to the results in our previous work (17 and 11 %).

When examined under the scanning electron microscope, some yeast cells from the second, third and fourth columns had wrinkles or folds on their cell walls. Similar cells were found in our previous research. In the case of *S. bayanus* S.o./1AD strain, these morphologically changed cells were found in the second, third and fourth columns, but in the case of *S. bayanus* S.o./1 strain, the morphologically changed cells were in all columns (*6,13*). This cell deformation correlates with the loss of cell viability in the columns.

When yeast cells are stored in wine for a long period (for example in the production of sparkling wine), autolysis of the yeast takes place. Autolysis depends on the culture, age, and physiological conditions of the cell. The first stage of autolysis is the degradation of cell endostructures. Turgor is absent, the cell wall thickens and the cell diameter decreases almost 1.5-fold. The second stage of autolysis involves hydrolysis of intracellular biopolymers, which leads to the diffusion of the hydrolysis products into the extracellular medium (*14*). Kollár *et al.* (*15*) reported that after 12 hours of model autolysis, cells are smaller in size and the surface of the cells is rough. After 24 hours, the cytoplasmic content of the cells is released and the cells are transformed into ghosts. Similarly, Martínez-Rodríguez *et al.* (*16*) observed empty yeast cells with wrinkles or folds on the walls after 24 h of induced autolysis in a model wine system. Cell shrinkage occurs not only in wine or ethanol, but also in glucose (*3*).

Apart from the above mentioned wrinkled yeast cells, other yeast cells isolated from the carrier were characterized by various shapes and occurred in the form of a few connected cells. Morphological changes varied according to the column number.

During the exponential growth, bud cells are dominant, frequently in the form of four-cell aggregates. In the stationary phase, the majority of the cells are in single form (*17*). Singh *et al.* (*18*) observed that cells synchronized at the beginning of the S-phase showed a rapid increase in multiple budding. This resulted in a pseudohypha of 5 or 6 cells when both mother and daughter cells produce buds, which were firmly attached together by their cell walls. Sometimes on the doubly budded mother cells, the daughter buds were of a different size, indicating that they were developing at different rates.

At a very low glucose concentration, newly budded cells have an elliptical shape. By elongating, the cells greatly increase their surface area to volume ratio, thus making it easier for them to find glucose molecules (*19*). Throughout our experiment, yeast had a sufficient amount of glucose in all the columns. In the first column, with the highest glucose concentration, many yeast cells were

elongated, with a rod-like morphology. Such changes were not observed in our previous experiments (*6*,*13*).

During alcoholic fermentation, higher alcohols and other metabolic products are also produced. Those products have inhibitory effects on the yeast at the level of the cell membrane, leading to passive proton influx or interfering directly with cellular transport processes (*20*). Fermentation by-products have an adverse effect on yeast morphology. When exposed to acetic acid, yeast cells become elongated and irregular, and daughter cells do not separate from mother cells after cell division. In a similar manner, 1-propanol and 2-methyl-1-butanol affect pseudomycelia. Acetaldehyde affects yeast cell morphology by increasing their cell size to over twice their normal diameter (*21*).

Conclusions

The period of 2.5 months of continuous fermentation of a high-sugar fruit must affected the number, viability, and morphology of immobilized yeast cells. These changes varied according to each bioreactor column. The most favourable conditions for the yeast were in the first column; this column contained a high sugar concentration and a small fraction of ethanol. In the subsequent columns, the fraction of ethanol increased and the concentration of sugar decreased. The worst conditions for the yeast were in the fourth column. There we found the smallest number of cells, the lowest yeast viability, as well as many distorted cells.

Acknowledgement

The authors would like to express sincere thanks to Joanna Leszczynska from the Department of Environmental Medicine, New York University for proofreading the paper. We thank Maria Gromadka for her assistance in the experimental part of the electron microscope studies, and Chris Leszczynski for all his help.

References

1. P. Carrasco, A. Querol, M. Olmo, Analysis of the stress resistance of comercial wine yeast strains, *Arch. Microbiol. 175* (2001) 450–457.
2. T. Roukas, Ethanol production from non-sterilized beet molasses by free and immobilized *Saccharomyces cerevisiae* cells using fed-batch culture, *J. Food Eng. 27* (1996) 87–96.
3. D. Granot, A. Levine, E. Dor-Hefetz, Sugar-induced apoptosis in yeast cells, *FEMS Yeast Res. 4* (2003) 7–13.
4. J. Hallsworth, Ethanol-induced water stress in yeast, *J. Ferment. Bioeng. 85* (1998) 125–137.
5. G.M. Walker: *Yeast Physiology and Biotechnology*, John Wiley & Sons, Ltd., Chichester, UK (2000) pp. 163–168.
6. S. Bonin, W. Wzorek, Influence of long-time continuous wine fermentation on morphological changes of yeast immobilized on foam glass, *Biotechnologia, 63* (2003) 167–181 (in Polish).
7. S. Bonin, W. Wzorek, Comparison of continuous wine fermentation with two yeast strains immobilized on foam glass, *Acta Sci. Pol. Technol. Aliment. 3* (2004) 83–93 (in Polish).
8. R.M. Birch, G.M. Walker, Influence of magnesium ions on heat shock and ethanol stress responses of *Saccharomyces cerevisiae*, *Enzyme Microb. Technol. 26* (2000) 678–687.
9. Community Methods for the Analysis of Wines, Commision Regulation (EEC) No. 2676/90, OJ L272 (1990).
10. Y.H. Hui, G.G. Khachatourians: *Food Biotechnology: Microorganisms*, Wiley-VCH, Inc., New York, USA (1995) pp. 876–877.
11. A. Bugajewska, W. Wzorek, Improvement of technological properties of wine yeast, *Workshop: Biotechnology for High Quality Products*, Bonn, Germany (2000) pp. 4–11.
12. Y. Ogawa, A. Nitta, H. Uchiyama, T. Imamura, H. Shimoi, K. Ito, Tolerance mechanism of the ethanol-tolerant mutant of sake yeast, *J. Biosci. Bioeng. 90* (2000) 313–320.
13. S. Bonin, W. Wzorek, L. Koper, Influence of long-time continuous wine fermentation on yeast immobilized on foam glass, *Acta Sci. Pol. Technol. Aliment. 5* (2006) 25–38 (in Polish).
14. T.L. Babayan, M.G. Bezrukov, Autolysis in yeasts, *Acta Biotechnol. 5* (1985) 129–136.
15. R. Kollár, J. Voříšek, E. Šturdík, Biochemical, morphological and cytochemical studies of enhanced autolysis of *Saccharomyces cerevisiae*, *Folia Microbiol. 38* (1993) 479–485.
16. A.J. Martínez-Rodríguez, M.C. Polo, A.V. Carrascosa, Structural and ultrastructural changes in yeast cells during autolysis in a model wine system and in sparkling wines, *Int. J. Food Microbiol. 71* (2001) 45–51.
17. K. Zalewski, R. Buchholz, Morphological analysis of yeast cells using an automated image processing system, *J. Biotechnol. 48* (1996) 43–49.
18. V.K. Singh, D.A. McCracken, M.J. Nadakavukaren, R.K. Jayaswal, Unique morphogenesis in *Saccharomyces cerevisiae* strain GS1731, *FEMS Microbiol. Lett. 166* (1998) 95–101.
19. G.A. Hill, C.W. Robinson, Morphological behaviour of *Saccharomyces cerevisiae* during continuous fermentation, *Biotechnol. Lett. 10* (1988) 815–820.
20. A.R. Barber, F. Vriesekoop, N.B. Pamment, Effects of acetaldehyde on *Saccharomyces cerevisiae* exposed to a range of chemical and enviromental stresses, *Enzyme Microb. Technol. 30* (2002) 240–250.
21. B. Maiorella, H.W. Blanch, C.R. Wilke, By-product inhibition effects on ethanolic fermentation by *Saccharomyces cerevisiae*, *Biotechnol. Bioeng. 25* (1983) 103–121.