PRODUCTION OF SPARKLING WINE WITH IMMOBILIZED YEAST FERMENTATION.

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Key words: Yeast Immobilization, Sparkling Wine, *Debina, Bekari, Vlachico*

Abstract

The use of immobilized yeast technology and its advantages in sparkling wine production has been used for rose and white sparkling wines. The aim of this work is to study and develop the method for the production of sparkling wines using the yeast double immobilization technique. The developed method was applied in the production of Rose sparkling wine successfully. The wine was produced from a local grape variety Debina, cultivated in the Zitsa region, Ioannina, Greece. For the double immobilization technique were employed different entrapment matrices such as sodium alginate, carrageenan, and glycerin, for the formation of the internal core. The results indicated that with 2 % concentration of sodium alginate loaded with 2.10^9 cells/g of gel and 2,65 g of beads in each bottle no cell release was observed and the wine remained absolutely free of yeast cells even six month after the completion of the fermentation. Sensory properties of the produced sparkling wines, compared to sparkling wine produced with free yeast did not show any significant differences.

Introduction

In the oenological region of the Zitsa municipality, in North-western Greece, a ancient grape white variety *Debina*, is cultivated. Since 1971, with the establishment of the Greek system of quality wines (V.Q.P.R.D.), this variety is used for the production of sparkling and semi-sparkling white wines. In the area traditionally, rose sparkling wines are produced from the red varieties *Vlachico* and *Bekari*, in combination with the variety *Debina* and other white local varieties. This wine even though is preferred by the locals, has not yet found its place in the market mainly due to problems related to the technological disadvantages namely the duration and the high cost of the traditional production.

A technology that after many years of development has come to industrial practice is the use of immobilized cells for the second fermentation in champagne production (Silva *et al.,* 2002a; 2002b; 2002c; 2004; Strehaiano *et al.,* 2002; Teixeira da Silva *et al.,* 1998). This technology preserves the traditional champenoise method in contrast to the “cuvee” and “transfer” methods that are consider to produce lower quality sparkling wines, and in the same time “eliminates” the “remuage” or riddling step, which is a time consuming and delicate step that adds to the production cost requiring flocculent yeasts. During riddling, the yeast are removed from the main

wine body and deposited on the bottleneck by storing and turning the bottles in a inclined position. Next is the freezing of the bottlenecks and the removal of yeast sediment.

During this stage, yeast autolysis also occurs, liberating a large number of substances modifying the wine aroma and flavor. This seems to be affected more by the duration of the ageing process (Pozo-Bayon *et al.,* 2003) rather than the yeast strain. Even though automatic rotating palettes have substituted manual labor, the riddling step has to be treated delicately and demands the use of a freezing line, which results in an additional considerable cost for the producer. Further more several winemakers have observed lower aromatic properties in wine treated with automatic rotating palettes compared to those treated by hand.

Immobilized yeast omits the riddling stage reducing the total duration of yeast sedimentation to a few seconds. A major parameter to promote the sedimentation rate of the beads is the size.

It has also been established that yeast immobilization does not prevent yeast autolysis and wine ageing (Divies, 1989; Yokotsuka *et al*., 1997). Free wine amino acids initialy are absorbed by the yeast cells and in a later stage are released in the wine body over a five monthly period of ageing.

The immobilized yeast technology is a relatively new, for the sparkling wine production and brand development of a traditional local sparkling wine. This technology, compared with the traditional "Champenoise" method, presents several advantages related to the cost, the ability to control the fermentation and minimize its duration as a result of the riddling and disgorging steps elimination and less storage room in the winery. It also facilitates yeast fermentation under CO_2 pressure with the yeast-cells partially protected from the toxicity of ethanol. The critical factor in using immobilized yeast in sparkling wine production is the leakage of cells from the gel beads in the bottle, which leaves the wine with free cells thus negating the previous mentioned advantages. Previous studies by Fumi *et al.* (1987; 1998) have shown that yeast immobilized in a single layer gel result in cell leakage in the wine produced. The way to avoid this is the double immobilization by coating the beads with a gel layer without microorganisms (Klein *et al.,* 1986; Duteurtre *et al.,* 1987;). Another technique used by Martynenko *et al.,* (2004) is the addition of the factor d_1 an aromatic alcohol, 2-(4-hydroxyphenyl)-ethanol which inhibits growth and proliferation processes in yeast cells, without affecting the activity of fermentation.

Several studies have been undertaken on cell leakage, but complete prevention has not been obtained yet in an industrial scale (Bajpai *et al.,* 1985; Robinson *et al.,* 1985, Yokotsuka *et al.,* 1997). The leakage of cells from the bead occurs due to low yeast concentration in the bead which promotes the growth rate resulting in a massive release of CO_2 with a catastrophic eruption of the bead. (Godia *et al.,* 1991) A significant effect on the bead disruption has been occurred when low number of beads have been added to the bottle with a dramatic reduction of cells released occurred when high number of beads were used. The temperature also presents a significant factor on the bead disruption. That is due to cell proliferation in order for the yeast to ferment the residual sugars normally added for the second fermentation. Recently a number of researchers have reported minimal or no cell release when using high concentration of immobilized inoculum (Tanaka *et al.,* 1989; Godia *et al.,* 1991).

Other techniques for avoiding cell leakage have also been reported by Park and Chang, (2000). Immobilized yeast in 2 mm alginate beads with 2.10^9 cells/mL of gel gives satisfying results.
Similar results have been obtained with continuous fermentations by Divies and Deschamps (1986) and by Nerantzis *et al. (1995)* and Nerantzis and Logothetis (2001) with the use of the WITY system, a cascade continuous fermentation system – metabolites.

A synopsis of the work on the secondary fermentation or the fermentation in the bottle for sparkling wines production, shows that typically the bottle fermentation step starts with the addition of yeast and 25 g/L of sugars, which lead to the production of CO_2 and ethanol, however this amount of sugar is relatively low to affect changes of secondary metabolites in the wine significantly.
A large number of researchers have also reported the difference on olfactory characteristics on the sparkling wines produces by free or immobilized yeast is not significant.
The technique has also been used for sparkling wine production of fruits other than grapes as for example cider, pineapple, (Divies-Deschamps 1986), kiwi, (Nerantzis et al., 2004 unpublished data) or of hydro alcoholic infusions of those fruits (Lezni-Cavin, 1985) as well as for brewing (White and Portno, 1978; Ryter, 1985; Masschelein, 1985; Onaka et al., 1985).

Sodium alginate is a GRAS substance deriving from brown algae that has been used as thickener, emulsifier, stabilizer and gelling agent in food production (Gemeiner et al, 1994). It was also one of the first polymers to be used for microbial cell entrapment, but not enzymes since the pore size of (but not for food products) the produced gel is large enough to permit leakage (Scher, 1977; Bucke, 1987). Sodium alginate reacts with Ca^{++} ions (Ba^{++}, Sr^{++}, Mg^{++} is also used but not in food production) forming networks (gels), which are not thermo-reversible. High concentration of K^+, Mg^{++} ions, phosphate, sodium citrate and other chelating agents destroy the formatted gel matrix.

The gelling properties of alginate depend on the ratio of the two monasaccharides 1,4-linked β-D-mannuronic acid (M) and 1,4-linked a-L-guluronic acid (G) as well as the blocks of MM, GG and irregular M and G sequences, their block length and arrangement. High guluronic acid (G) alginates have enhanced ability to form gels (Skjak-Braek *et al.*, 1989). This may be because Ca^{++} ions appear to bind in preference to (G) blocks (Morris, 1986). Guluronic acid (G) blocks may rise up to 70% (Bucke, 1987). Alginate with more than 70 % (G) blocks has the highest mechanical strength, porosity and stability towards monovalent cations as well as the lowest shrinkage.

The most common form of alginate cells is the bead form with 0,5 mm to 3,5 mm diameter. This occurs when a drop of a sodium alginate solution enters a $CaCl_2$ solution. The diameter depends mostly on the surface tension of the alginate solution and not drop (needle) diameter. Smaller beads permit increased cell loads and smaller distances of the cells to the medium thus better substrate and oxygen diffusion. Several methods have been used for that purpose, such as the passing of an air stream concentric to the inner needle (Klein et al, 1993), (Rehg *et al*., 1986, Matulovic *et al*.,

1986) together with the use of surfactants, the use of electrical current (Poncelet *et al.,* 1994; Nedovic et al., 2001; Klokk *et al.,* 2002), controlled drying (Klein et al 1979, 1983) and needle nozzle resonance (Hulst et al, 1985; Woodward, 1988).

As beads disruption risk is high in the presence of substance capable of chelating calcium, several techniques may be used to make them more resistant (Bucke, 1987). Graft polymerization by chemical modification with the use of propylene glycol esters, polyethelenine (PEI) (Veliky and Williams, 1981, Young et al, 1987; Birnbaum et al, 1982, Tanaka et al, 1984) or composite materials (colloidal silica) permit gel "hardening" (Fukushima et al, 1988). It has also been shown that partial drying or other agents, which promotes shrinking, increases mechanical stability of the alginate gels (Klein et al, 1979).

Carrageenans have similar properties to alginates. From the three types of carrageenans (lambda, kappa and iota) the kappa is considered to be most suitable for cell immobilization.

In comparison to calcium alginate, k-carrageenan develops less firm and less elastic gel than that of alginate. Pellets produced with carrageenan deteriorate in flakes faster during fermentation (Munoz et al, 1986; Fiszman et al, 1985). K-carrageenan beads produced by the dripping method are less uniform and spherical (Hulst and Tramper, 1989). This, however, may be solved by the use of a resonance nozzle immobilization technique (Buitellar et al, 1988; Woodward 1988). In any case, alginates and carrageenans have to be properly chosen prior to the application in cell immobilization processes.

Entrapped yeast cells compared to free cells seem to produce more glycerol, more esters, and lower amounts of both higher alcohols and acetaldehyde (Divies, 1989).
Also, amino acids assimilation is more efficient. This could explain why more alcohols are produced by immobilized cells. Low acetaldehyde production may be due to the better utilization of reduced coenzymes (Divies, 1989) and the confinement of yeast cells in the gel.
Siess and Divies (1981) have also shown that when growth is limited and amino acids are less used, glycerol accumulates and there is a high accumulation of propanol.
Other researchers have proposed that these differences are due to a more marked effect of water activity within the gel (Hahn-Hagerdal, 1986) or a better utilization of the reduced nucleotides pool (Divies, 1989).
It has also been established that the composition of immobilized yeast has a higher percentage of glycogen, glucan, mannan, and DNA contents. This data have been corroborated by other researchers (Doran and Bailey, 1986).

The scope of this study was dual, first, we examined several materials for yeast immobilization in double layer gel beads, using sodium alginate for the external layer in various concentrations (0,5 %, 1 %, 2 %) and various concentrations of sodium alginate, carrageenan, glycerin, $CaCl_2$ for the internal layer. The influence of the gel composition as well as that of high initial cell loading and high bead concentration per bottle was examined in relation with the cell leakage. And second, we applied the chosen double immobilization technique for the production and

evaluation of white and rose sparking wines produced from local grape varieties of the Zitsa region,

Materials and Methods

Microorganisms

Oenological yeast from ANCHOR (South Africa) *Saccharomyces cerevisiae* strain NT 45. The same yeast was used for the initial fermentation for the production of the base wine and for the immobilization process.

Grape Must

White sparkling wine was produced from *Debina* must with 188 g/L of total sugars concentration, pH 3.11, total acidity 7.25 g/L as tartaric acid. Rose sparkling wine was produced from *Debina*, Vlachico and Bekari must in (16/2/2 ratio) with 182 g/L of sugars, pH 3.17, total acidity 6.22 g/L as tartaric acid. The must were sulfited with 50 mg/L of SO_2 in the form of potassium bisulfite, inoculated with 10 g/hL of yeast and fermented at a temperature between 18°C to 20°C.

Immobilization of yeast cells

Yeasts were grown in a medium containing 4% glucose, 1% peptone, 2 g/L K_2HPO_4, 2 g/L $MgSO_4$ and 0.5% of yeast extract at 25 °C for 48 hours on a reciprocal shaker. Cells were harvested by centrifugation at 4°C at 10000 rpm for 15 minutes, washed sterile distilled water and centrifuged a second time.

Double immobilization was performed using an apparatus described by Yokotsuka *et al.* (1997) (photo 1) and Bejar *et al.* (1989), two similar apparatus were also manufactured for the purpose of this experiment, one from stainless steel and another using a syringe gauge 12 for the internal nozzle and Teflon for the external nozzle. Double layer gel beads were made by simultaneous passing sodium alginate (0.5% or 1% or 2%) through the outer nozzle and various gels containing 2.10^9 yeast cells per gram of gel (sodium alginate 2.5%, $CaCl_2$ at 2% with or without glycerol ranging from 2% to 10% and carrageenan 4% with or without glycerol ranging from 2% to 10%) through the inner nozzle with the aid of a peristaltic pump (Watson-Marlow, U.K) into a 2% calcium chloride solution. The nozzles were kept at 3 cm above the surface of the calcium chloride solution.

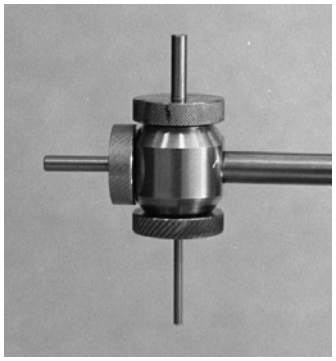

Photo 1. Concentric needle apparatus.

After preliminary studies with various inner and outer gel-solution ratios and flow rates (ranging from 0.28 to 3.2 mL/min), following conditions for bead generation were selected:

Inner nozzle: 2% sodium alginate, flow rate 1.2 mL/min, Outer nozzle 0.5%, 1%, 2%, flow rate 2.6 mL/min. The ratio of outer to inner gels was 2.2 to 1.

All bead production procedures have been previously been described by Yokotsuka *et al.*,(1993). After production the beads were cured for 24 hours in a solution of 2% $CaCl_2$ which hardened the beads and decreased their volume up to 30%. Beads were washed with sterile water and were introduced into the bottles for the second fermentation (photo 2).

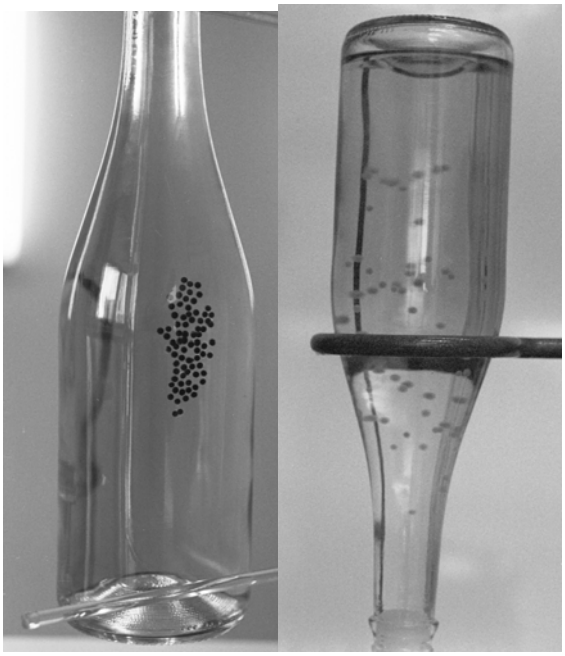

Photo 2. Second fermentation with double immobilized yeast in the bottle.

Second Fermentation

The sugar content of the base wines was adjusted at 24 g/L of sugars, the wines were then filtered through a 0.4 μm membrane (Sartorius) and put into previously steam sterilized bottles. For each experiment 100 bottles of 330 mL and 10 bottles of 750 mL were used. Three bottles from every batch were fitted with a pressure gauge (used for measuring CO_2 pressure in beer bottles) and left at 15 °C for fermentation. Additionally 3 bottles of each batch were periodically opened and samples were analyzed for turbidity changes, optical absorbance at 620 nm, cell number and viability, ethanol and sugar content. All analyses were conducted in triplicate and the average value calculated. At the end of the second fermentation riddling (photo 3) could be done immediately.

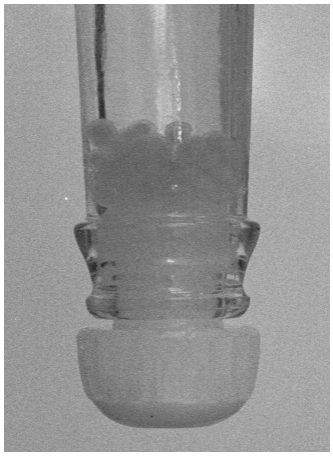

Photo 3. Riddling.

Cell number and viability in beads were determined by dissolving 50 beads in 1% sodium citrate solution, followed by staining with methylene blue as described by Lee *et al.,* (1981). Turbidity was calculated in nephelometric turbidity units (NTU) using a HACH turbidimeter and absorbance at 620 nm using a Hitachi Spectrophotometer. Additionally cell leakage was tested after sterile filtration of hole bottle content and plating on Petri dishes containing Sabouraud agar followed by incubation at 28 °C.

Sugar content was measured with the di-nitrosalicylic acid (DNS) method as described by Miller, (1959).

Volatile acidity and total phenols (expressed as gallic acid), ethanol were analysed as described by Amerine and Ough, (1980), a-amino nitrogen was measured using the method of Dukes and Butzke, (1998).

The volatile substances measured were: acetaldehyde, 1-propanol, isobutanol, ethyl acetate, total amyl alcohols and 2-phenylethanol.

The analysis was performed with a 8500 Perkin Elmer Gas chromatographer, equipped with a Shimadzu C-R3A integrator, a Perkin Elmer 8500μ Head Space and a SGE 25 AQ3/BP 20 0.5 column (25 m x 0.33 mm, particle diameter 0,5 μm). The temperature of the injector and detector was 260 °C. The oven temperature programme was: beginning at 35°C for 2,5 min, augmenting for 20 °C per min, to 200 °C and maintained for 0,8 min. Vector gas nitrogen, pressure 25 PSIG.

Head-Space conditions: vaporisation temperature 60°C, pressurisation time 0,51 min, injection time 0,40 min, sample volume 2 mL. Standard deviation was of 4,2 % for all components except acetaldehyde for which it was 10 %.

Microscopic observation of gel beads was done with an Olympus model CHK2-F-GS microscope.

Results and Discussion

Complete fermentation of sugars for first fermentation took 351 hours for the white must (fig. 1) and 327 hours for the rose, the rose fermentation was faster because of the slightly lower initial sugar concentration. Due to adverse climatic condition of the 2002 harvest, technological maturity of the grapes was late and the acid content was high. The red grapes varieties had a small percentage of mold grapes, which affected their overall quality. The base wines produced had the following composition:

White base wine ethanol 11.25 % vol, residual sugars 0.77 g/L, pH 3.1, total acidity 7.95 g/L as tartaric acid, volatile acidity 0.165 g/L as acetic acid. Rose base wine ethanol 10.11 % vol, residual sugars 0.3 g/L, pH 3.3, total acidity 6.97 g/L as tartaric acid, volatile acidity 0.55 g/L as acetic acid.

Bead production

From the preliminary results regarding bead production, 3 types of beads were chosen (table 1). Beads made by using a solution of 0.5% $CaCl_2$ and with or without glycerin from 2% to 10% for the inner layer produced only strands and not beads. Beads made by using a solution of 4% carrageenan and with or without glycerin from 2% to 10% for the inner layer produced beads with abnormal surface. Only the beads made exclusively with sodium alginate were judged appropriate for usage (normal beads, table 1). Bead diameter was 2 ± 0.2 mm. Photographs of alginate beads (single and double immobilization) can be seen in photo 4.

Table 1. Composition of double layer gel beads

Inner layer	**Outer layer**	**Results**
Sodium alginate 2.5% + yeast	Sodium alginate 2%	Normal beads
Sodium alginate 2.5% + yeast	Sodium alginate 1%	Normal beads
Sodium alginate 2.5% + yeast	Sodium alginate 0.5%	Normal beads
$CaCl_2$ 0.5% +yeast	Sodium alginate 2% or 2.5%	strands
$CaCl_2$ 0.5% + glycerol + yeast	Sodium alginate 2% or 2.5%	strands
Carrageenan + yeast	Sodium alginate 2% or 2.5%	Abnormal beads
Carrageenan + glycerol + yeast	Sodium alginate 2% or 2.5%	Abnormal beads

Photo 4. Single (a) and double (b) immobilization alginate beads.

After immobilization samples of beads were submitted to curing for extended periods and yeast viability was assessed after dissolving the beads in sodium citrate solution. Results (fig. 1) confirmed that immobilization did not have a significant effect on yeast viability.

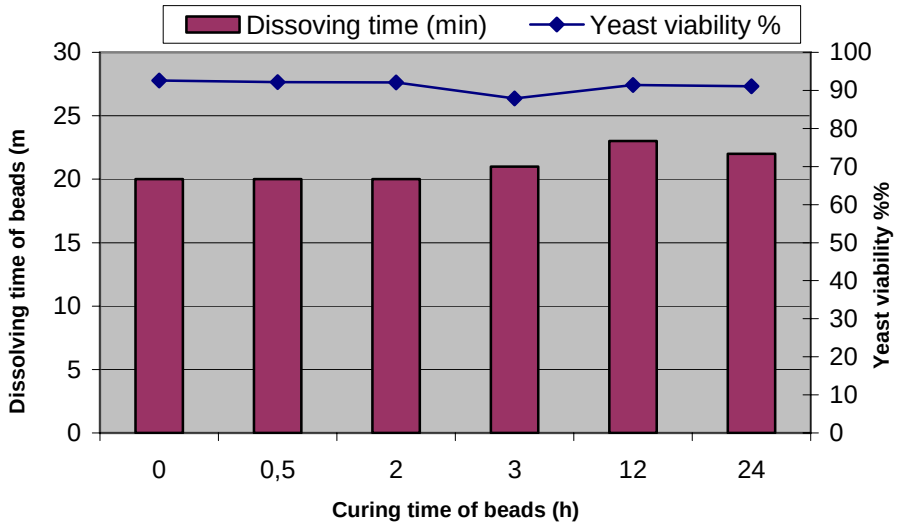

Figure 1. Immobilization effect on yeast viability

Sparkling wine production and fermentation kinetics

For the second fermentation 0,966 g of beads (wet weight) corresponding to approximately 50 beads were aseptically introduced to each 330 mL bottle. This corresponds to 2,22 g/bottle of 750 mL or 115 beads/bottle of 750 mL, which is significantly lower than quantities used previously 6 g/bootle (Godia, *et al.,* 1991) and 220 beads/bottle (Yokotsuka *et al.* (1997), in order to prevent cell leakage.

The second fermentations had a lag phase of 4 days and the actual fermentations were completed in 13 to 15 days for all batches (fig. 2), no significant differences were observed for the different bead gel concentrations. Second fermentation by free yeast cells took 23 days to complete. This does not coincide with the finding of Yokotsuka *et al.* (1997) who found that free cell fermentation took almost 1 month to complete and immobilized yeast fermentation a little longer. Differences may be due to the higher cell loading of the bead in our case as well as temperature and viability differences. However this coincides with data from Divies, (1989) who found that a *S. cerevisiae* strain immobilized in 2 mm beads with 2.10^9 cells/ml of gel had an activity of 0,6 g ethanol/g cells per hour against 0,6 g ethanol/g cells per hour when using free cells. Similar findings have been reported by Navarro and Durand (1977).

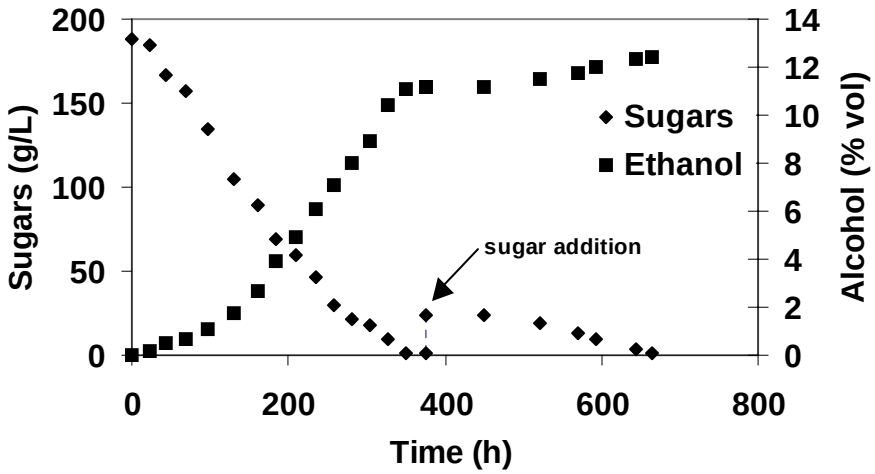

Figure 2. *Debina* white wine fermentation at 18°C to 20°C.

The alcohol content increased by 1.3 degrees % vol. and final sugar content was 1.2 g/L. No significant changes in acidity and pH were observed.
The first and second fermentation of the rose wine (fig. 3) were slightly faster by 1 day but final concentrations of sugars and ethanol were identical to that of the white wine.

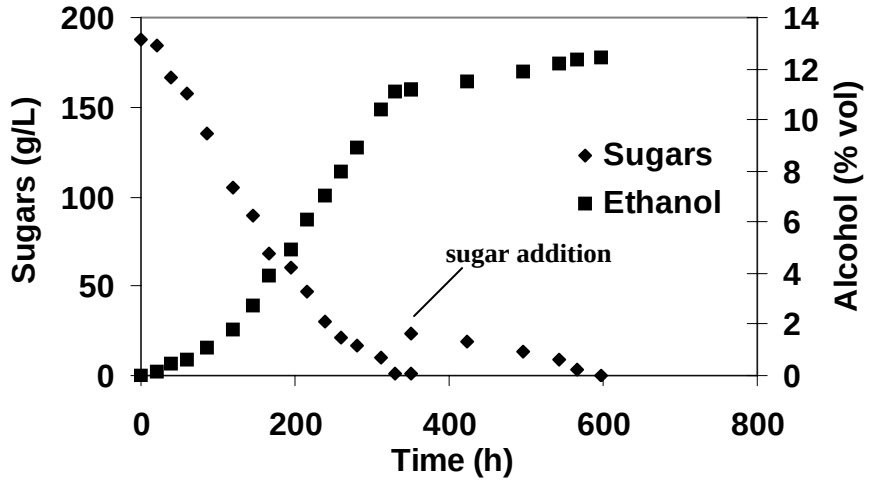

Figure 3. *Debina-Bekari-Vlachico* rose wine fermentation at 18°C to 20°C.

Bottle pressure due to CO_2 at the end of the second fermentation ranged from 4,9 to 5,2 bars. Typical carbon production kinetics during the second fermentation is shown in figure 4. Carbon dioxide production was independent of the inner/outer layer alginate concentration and was the same for all different beads with the exception of 2,5/2 (inner/outer alginate concentration), this is probably due to the slightly lower yeast cell loading of the beads.

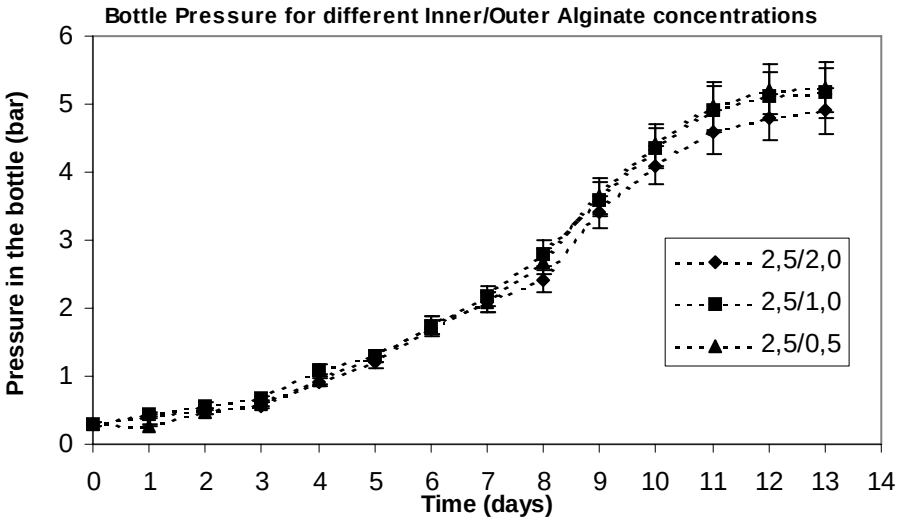

Figure 4. *Debina* white wine CO_2 evolution at 18°C to 20°C.

Special care was given to the examination of free yeast cells in bottles during the second fermentation. Two of the main reasons for free cell leakage are, low initial cell loading of the beads and high initial sugar content. These factors permit yeast proliferation and in some cases bead integrity damage.

In our study we found that cell loading of $2.10^9 \pm 10$ % cell/g of gel, sugar content of less than 24 g/L of sugars and 50 beads per bottle (0.966 g of gel beads per bottle of 330 mL) do not permit significant cell multiplication and no increase in turbidity, absorbance and free cell content inside the bottles. The concentration of the free of yeast external layer ranging from 0.5% to 2% of sodium alginate did not seem to have any influence on fermentation kinetics. These data are in accordance with previous mentioned work by Yokotsuka *et al.,* (1997, 1993) and Godia *et al.,* (1991). Initial cell loading ranging from 10^6 to 10^8 cell/g of gel showed yeast leakage with values of absorbance ranging from 0.001 to 0.06. For the initial cell loading of 2.10^9cell/g of gel, the final absorbance, turbidity and viable free cell number in the final sparkling wine is shown at table 3.

Table 3. End fermentation wine final absorbance and free cell concentration

Inner layer	Outer layer	Absorbance	Viable Free Cell/mL of wine
Sodium alginate 2.5%	Sodium alginate 2%	0.001	0,45
Sodium alginate 2.5%	Sodium alginate 1%	0.001	<0,4
Sodium alginate 2.5%	Sodium alginate 0.5%	0.002	0,65

Thus in our experiments we achieved zero cell leakage even though we used 0,966 g/330 mL bottle of beads corresponding to approximately 50 beads/bottle of 330 mL. This corresponds to 2,22 g/bottle of 750 mL or 115 beads/bottle of 750 mL, which is significantly lower and more economic than quantities used previously 6 g/bootle (Godia, *et al.,* 1991) and 220 beads/bottle (Yokotsuka *et al.* (1997).

In a small number of samples (8%) a sparing number of colonies (3-14 per bottle) were observed after wine filtration through 0.45 nm membranes and

cultivation in Petri dishes containing Sabouraud agar. After identification with API 32 C biochemical tests, 80% of these colonies proved not to be the S. cerevisiae (NT 45 ANCHOR strain was used for this experiment) and since all of them were non-Saccharomyces yeast we concluded that there presence is due to contamination. However, when using double immobilized beads cell leakage can also occur not only from bead damages, but also, through alginate pores bulging when CO_2 bubbles produced inside the bead come trough the gel. This point needs further examination as the gel formation and strength of alginate beads depend on mode of addition and concentration of calcium or other ions and the percentage of L-guluronic acid and L-guluronate residues (Bucke, 1987; Skjak-Braek *et al.,* 1989; Gemeiner *et al.,* 1994). In wine there is an additional risk of calcium alginate gel destruction due to high presence of potassium ions and phosphate and organic acids such as lactic acid (Gemeiner *et al.,* 1994).
At the end of the second fermentation yeast concentration and viability in the beads was found as following (table 4).

Table 4. End fermentation cell loading and viability in double layer gel beads

Inner layer	Outer layer	Cell/g of gel	Viability
Sodium alginate 2.5%	Sodium alginate 2%	$1{,}51\ 10^9$	4 %
Sodium alginate 2.5%	Sodium alginate 1%	$1{,}86\ 10^9$	3 %
Sodium alginate 2.5%	Sodium alginate 0.5%	$1{,}86\ 10^9$	3,5 %

These results show that the initial goal of fermentation without cell proliferation was achieved, but that almost all cells were non viable at the end of the second fermentation. This is not typical of fermentations with immobilized cells, were 90 % viability would be expected. These results would be expected in free cell fermentation (Yokotsuka *et al.,* 1997).

Volatile compounds composition of the wines produced with Immobilized and Free cells was almost identical (table 5). This coincides with data from (Yokotsuka *et al.,* 1997).

Table 5. Volatile compounds composition of the wines

Volatile compounds	Base wine	Free cells	Immobilized cells
Acetaldehyde (mg/L)	37,5	44,0	52
1-Propanol (mg/L)	33,6	35,6	41,5
Isobutanol (mg/L)	38,4	40,2	46,4
Ethyl Acetate (mg/L)	52,3	53,2	57,2
Total Amy Alcohols (mg/L)	245,8	278,3	274,0
2-Phenylethanol*(mg/L)	62,5	59,3	57,2

* 2-Phenylethanol was determined by direct injection.

Differences in volatile acidity, a-amino nitrogen and total phenols 3 months after the end of the second fermentation were similar to those observed by other researcher (Yokotsuka *et al.,* 1997) except that of a-amino nitrogen probably due to

the rapid cell death and subsequent yeast autolysis during the second and third month (table 6).

Table 6. Volatile acidity, a-amino nitrogen and total phenols after 3 months

Compounds	Base wine	Free cells	Immobilized cells
Volatile acidiity (mg/L)	65	62	60
a-amino nitrogen (mg/L)	145	140	158
Total phenols (mg/L)	340	321	333

Organoleptic quality of the sparkling wines produced

The white and rose sparkling wines were submitted to a jury of 16 trained wine experts (enology senior students and academic staff). The wines were also compared to sparkling wines made by free cells with a duo-trio test, two months after fermentation completion, no significant differences was observed (at 95 % significance level), this is in agreement with findings of other researchers (Coulon *et al.,* 1983; Fumi *et al.,* 1987; Godia *et al.,* 1991; Yokotsuka *et al.,* 1997). The white wines are characterized by a high acidity, crispness, a light yellow color and a distinguishable fruity aroma with predominant apple notes together with hints of cinnamon. The rose wine score less than the white one, with less predominant fruity character. This is partially due to the medium quality of the 2002 harvest for the red varieties *Vlachiko* and *Bekari*.

Wines notes on a scale from 1 to 10 (worst to best) according to taste and aroma characteristics are presented on figure 5.

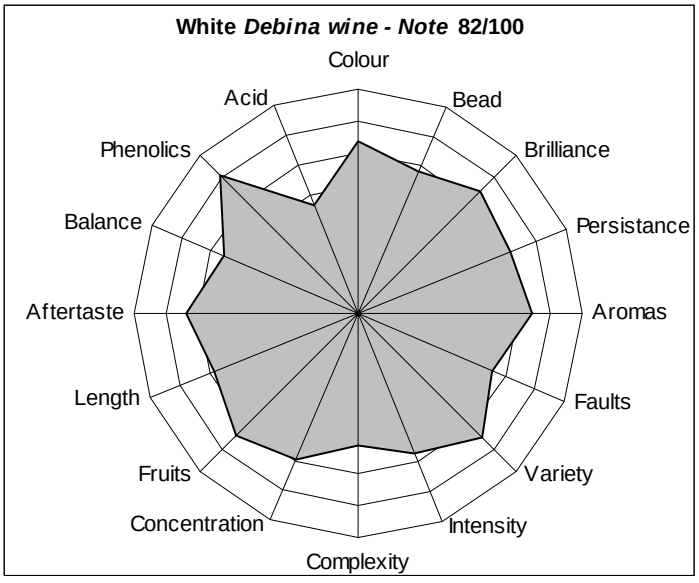

Figure 5. Wine notes of *Debina* white wine.

Conclusion

The use of double immobilized yeast in gel beads is an alternative technology for sparkling wine production. Compared with the traditional "Champenoise" method it

gives clear wine (as long as high cell load per gram of gel is used in beads) without significant differences from traditional sparking wine as far as taste aroma and chemical composition is concerned, but much faster and with lower cost as it is easier to remove the beads (riddling) from the bottles. Thus small or medium wineries can use these advantages for rapid brand development of local V.Q.P.R.D. Zitsa sparkling wines.

Acknowledgements

We thank ANCHOR Biotechnologies S.A. (South Africa) for the kind donation of the yeast strains, Dr Koki Yokotsuka (The Institute of Enology and Viticulture, Yamanashi University, Kofu Japan), for the immobilization device and Professor Athanasios Aravantinos for his expert advise in photography, Dr. Andreas Kombos (Chemical State Laboratory, Ministry of Finance, Athens, Greece) for providing the CO_2 pressure gauge device. This project was financed by the TEI of Athens, (Greece) research program "ATHINA 2004".

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