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Role of Oxygen on Acetic Acid Production by *Brettanomyces/Dekkera* in Winemaking

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Abstract: Since the large occurrence of *Brettanomyces* yeasts in strict anaerobiosis environments (sparkling wines) has been found without an increase in acetic acid content, we evaluated the influence of the oxygen concentration on acetic acid production. Results showed that the oxygen concentration exerted a strong influence on both growth and acetic acid production by *Brettanomyces* yeasts in winemaking. Full aerobiosis lead to a large production of acetic acid causing a block of metabolic activity. Semi-aerobiosis resulted in the best condition for alcoholic fermentation (Custers effect) combined with acetic acid production. In anaerobic condition *Brettanomyces* yeasts did not result in high acetic acid production and a pure, even if slow, alcoholic fermentation occurred. The absence of an increase in acetic acid in wines, does not exclude the active presence of *Brettanomyces* yeast since the characteristic 'high acetic producer' in *Brettanomyces* yeast is linked to the presence of oxygen.

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INTRODUCTION

Brettanomyces/Dekkera yeasts have been found in wine fermentation (Peynaud and Domercq 1956; van der Walt and van Kerken 1960; Kunkee and Amerine 1970), in beer fermentation (Andrews and Gilliland 1952; Gilliland 1961; Verachtert *et al* 1989) and in soft drinks (Kolfschoten and Yarrow 1970).

Brettanomyces/Dekkera has been recognised as an overattenuation yeast of special beer (Shantha Kumara *et al* 1993), wine spoilage yeast, imparting undesirable odours and flavours (Heresztyn 1986a, 1986b) and high producer of acetic acid (Sponholz 1993). These yeasts are not among the dominant organisms in grape juice during fermentation but their presence might have a great influence on the final product. *Brettanomyces/Dekkera* yeasts are considered a wine spoilage agents (Sponholz 1993) even if its role in different winemaking condition is not well defined. Their classical habitat in the winemaking environment is the winery and its equipments, particularly barrel-ageing red wines and they were recently isolated also in sherry wines (Ibeas *et al* 1996) where a high acetic acid production was observed.

Brettanomyces/Dekkera yeasts exhibit a particular metabolism. The inhibition of alcoholic fermentation under anaerobic conditions (Custers effect), in fact, has been considered as biochemical characteristic of *Brettanomyces/Dekkera* (Scheffers 1966). This particular behaviour may affect the growth and the fermentation products. Carrascosa *et al* (1981) suggested that the Custers effect was a consequence of a redox inbalance caused by the reduction of NAD^+ during the oxidation of acetaldehyde to acetic acid. Little information is available on the adaptation of *Brettanomyces/Dekkera* during a long period of fermentation in different oxygen concentrations which may affect the final products in the winemaking process. In the present study we have evaluated the occurrence of *Brettanomyces/Dekkera* yeasts in the particular environment of sparkling wine and have verified the influence of the oxygen concentration on acetic acid production and the fermentation behaviour of these yeasts in winemaking conditions.

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EXPERIMENTAL

Isolation

The isolation of *Brettanomyces/Dekkera* strains was carried out from 15 samples of bottle-fermented sparkling wines from a production area of a DOC wine during two consecutive years (1992–1993). The isolation of pure cultures was carried out by direct isolation using traditional streak technique on WL nutrient agar (Unipath Ltd, Basingstoke, UK). Colonies were selected according their macro- and micromorphological aspects, and isolated proportionally to their frequencies. Colonies were picked out after 3 and 7 days of plate incubation. In those sparkling wine samples where no isolates were obtained, 50 ml of wine were filtered through a 0·45 μm pore-size membrane (Millipore Corporation Bedford, MA, USA) and the membranes placed in WL nutrient agar for incubation and subsequent isolation.

The identification of pure cultures was carried out following the procedures of Kreger van Rij (1984). *Brettanomyces* strains were confirmed by the evaluation of the resistance to the cycloeximide (0·004 g litre^{-1}) in malt agar (Difco, Detroit, MI, USA).

Fermentation behaviour

Microorganisms

Brettanomyces spp, strains designated no 1, 9, 58, 63, isolated from sparkling wines, *Brettanomyces bruxellensis* type strain DBVPG 6706 (Industrial Yeasts Collection of Dipartimento di Biologia Vegetale, Università di Perugia) and *Saccharomyces cerevisiae* DBVPG 6663 (Commercial strain Montrachet; Red Star, Milwaukee, Wis. USA), were used to evaluate the fermentation behaviour.

Media

A synthetic grape juice (SGJ) and natural grape juice (NGJ) were used. Each litre of SGJ was formed by three different solutions: acid solution (250 ml), glucose/fructose solution (500 ml) and nutrient solution (250 ml) (Ciani and Ferraro 1996). The three solutions were sterilised separately at 121°C (15 psi) for 20 min and then combined aseptically. Final fermentable sugar was 220 g litre^{-1}.

Natural grape juice (NGJ) from the Trebbiano toscano cultivar (160 g litre^{-1} fermentable sugar, pH 3·01) was added to sucrose (until 240 g litre^{-1} for microfermentations or 200 g litre^{-1} for bench-top tests of fermentable sugar) and heated at 80°C for 15 min.

Fermentation conditions

All fermentations of *Brettanomyces* strains were inoculated with 10% of 5-day-old yeast culture (approximately 1×10^6 cells ml^{-1}) in SGJ or NGJ in static conditions at 25°C.

Fermentations carried out by *S cerevisiae* were inoculated with 5% of 2-day-old yeast culture (approximately 1×10^6 cells ml^{-1}) in the same conditions as *Brettanomyces* tests.

Microfermentations

Duplicate fermentations were carried out in 500-ml flasks, each containing 300 ml of SGJ or NGJ at 25°C. The results of analytical determinations varied no more than $\pm 10\%$.

The fermentations were done under different aeration conditions. In aerobiosis the flasks were fitted with a cotton plug and maintained in agitation on a rotary shaker (150 rev min^{-1}). In semi-aerobiosis, the flasks fitted with cotton plug were maintained in a static condition. Anaerobiosis fermentations were carried out by stoppering the flasks with a glass fermentation trap containing sulphuric acid allowing only the CO_2 to escape the system. The medium was saturated with oxygen-free nitrogen gas for 15 min before and after inoculation.

For several days the microfermentations were followed by weight loss from escaping CO_2 until the end of the fermentation (constant weight for two consecutive days). Final samples were filtered through membranes (GF/C Whatman International, Maidstone, UK) and stored at -20°C until analysis.

Bench-top tests

The fermentations were performed in a 2·5-litre fermentor (Bioindustrie Mantovani, Mantova, Italy) containing 1·8 litres of NGJ 'sterilised' *in situ* at 80°C for 15 min. The fermentations were carried out at $25 \pm 0{\cdot}1$°C. The fermentor was equipped with instrumentation and controllers for the measured parameters: temperature, inlet gas flow rate, agitation speed, pH and dissolved O_2 (probes, Ingold, Zürich, Switzerland). Temperature, pH and dissolved oxygen were continuously recordered. To evaluate the influence of oxygen concentration, three different conditions were tested:

(a) aerobiosis: the dissolved oxygen concentration was kept between 40 and 80% of air saturation by varying the stirrer (400 rev min^{-1}) and air flow rate (0·29 litres litre^{-1} min^{-1});
(b) semi-aerobiosis: the dissolved oxygen concentration was kept between 6·5 and 35% of air saturation by varying the stirrer (300–600 rev min^{-1});
(c) anaerobiosis: the anaerobiosis condition was kept by sparging with ultra-pure nitrogen (0·14 litres litre^{-1} min^{-1}). The stirrer was maintained at 400 rev min^{-1} and before starting the test the medium was saturated with nitrogen for 30 min.

Samples collected at different times of fermentation were submitted to microbiological and chemical analysis. For chemical analysis, samples were filtered

through membranes (GF/C Whatman International, Maidstone, UK) and stored at −20°C.

Analytical methods

Biomass evolution was evaluated by traditional plate count technique in malt agar (Difco, Detroit, MI, USA). Ethanol was measured by gas–liquid chromatographic analyses (AOAC 1990). Acetaldehyde, ethyl acetate and higher alcohols with GC described by Bertuccioli (1982). Acetic acid, glucose and fructose were determined by using specific enzymatic kits (test combination catalogue no 148 261 for acetic acid and no 139 106 for glucose–fructose: Boehringer-Mannheim, Mannheim, Germany).

Protein concentrations were measured by the Lowry method (Lowry *et al* 1951) with bovine serum albumin (Sigma Chemical Co, St Louis, MO, USA) as the standard.

Enzyme assays

Cells were collected by centrifuging at 2000 × *g* for 15 min, washed with 100 mmol litre^{-1} potassium phosphate buffer (pH 7·5) and then stored at −20°C. Cells were lysed with glass beads by the method of Blumer *et al* (1988). Lysate samples were frozen immediately with liquid nitrogen and stored at −70°C until use. Spectrophotometric assays were carried out by a model DU 640 (Beckman, Fullerton, CA, USA). Aldehyde dehydrogenases (NAD^+ and $NADP^+$; EC 1.2.1.5 and EC 1.2.1.4, respectively) were assayed according to Postma *et al* (1989). The assay mixture contained potassium phosphate buffer (pH 8) (100 mmol litre^{-1}), pyrazole (15 mmol litre^{-1}), dithiothreitol (0·4 mmol litre^{-1}), KCl (10 mmol litre^{-1}) and NAD^+ or $NADP^+$ (0·4 mmol litre^{-1}). The reaction was started with 0·1 mmol litre^{-1} of acetaldehyde.

RESULTS

Isolation

Results of isolation from sparkling wines showed a large occurrence of *Brettanomyces* yeasts (Table 1). Forty-nine out of 178 isolated strains (27·5%), belonged to *Brettanomyces* genus. The occurrence of these yeasts during two consecutive years and their exclusive presence in four sparkling wine samples indicates that *Brettanomyces* yeasts are not occasional in this environment. Moreover, the direct isolation confirmed this large occurrence without the use of enrichment methods such as media containing cycloeximide or sorbic acid.

The absence of organoleptic and analytical alterations of sparkling wine samples (data not shown), regarding in particular the production of acetic acid, may be due to the particular fermentation condition such as the strict anaerobiosis. For these reasons we verified the influence of the oxygen concentration on the fermentation behaviour of *Brettanomyces* yeasts.

Fermentation behaviour

Microfermentations

Microfermentations were carried out by four strains of *Brettanomyces* which were isolated from different sparkling wines samples and by the test strains *Br bruxellensis* DBVPG 6706 and *S cerevisiae* DBVPG 6663.

The *Brettanomyces* strains showed a lower fermentation rate in all conditions in comparison with that of *S cerevisiae* test strain. The microfermentations carried out in natural and synthetic grape juice (Tables 2 and 3), showed that the condition of full aerobiosis caused a large production of acetic acid and reduced amounts of ethanol in *Brettanomyces* strains. Semi-aerobiosis was found to produce the most ethanol, achieving, in natural grape juice, similar amounts to that of *S cerevisiae* test strain. In this condition the *Brettanomyces* strains produced higher acetic acid level than that of *S cerevisiae* test strain in NGJ while the contrary was shown in SGJ but no large differences were found. In anaerobic conditions the *Brettanomyces* strains produced the lowest amounts of acetic acid which was very close to that of *S cerevisiae* test strain. Different media (natural or synthetic grape juice) affected the amounts of the final products but it did not vary the effects of the different aeration conditions (Tables 2 and 3). *Saccharomyces cerevisiae* strain produced higher amount of acetic acid in SGJ while *Brettanomyces* strains behaved in the same way in NGJ.

Bench-top tests

Fermentor tests were carried out by the strain no 58 as representative of *Brettanomyces* behaviour since a low variability had been observed between the cultures.

TABLE 1
Distribution of *Brettanomyces* strains isolated from sparkling wine samples[a]

Year	*Sparkling wine samples no*	Brettanomyces *spp strains*	Saccharomyces cerevisiae	*Others*
1992	8	27 (8)	29 (4)	7 (2)
1993	7	22 (4)	77 (7)	16 (4)

[a] The numbers of the samples where the strains were isolated are indicated in parentheses.

TABLE 2
Influence of aeration on acetic acid and ethanol production in synthetic grape juice (SGJ) by *Brettanomyces* strains and *S cerevisiae* control strain (microfermentations)[a]

Strains	*Aerobiosis*		*Semi-aerobiosis*		*Anaerobiosis*	
	Ethanol (*ml litre*$^{-1}$)	*Acetic acid* (*g litre*$^{-1}$)	*Ethanol* (*ml litre*$^{-1}$)	*Acetic acid* (*g litre*$^{-1}$)	*Ethanol* (*ml litre*$^{-1}$)	*Acetic acid* (*g litre*$^{-1}$)
Brettanomyces						
1	13·5	11·19	80·5	0·35	45·0	0·15
9	17·5	8·22	69·4	0·20	45·0	0·14
58	ND	10·45	84·1	0·47	62·0	0·32
63	ND	11·94	82·3	0·43	63·5	0·31
6706[b]	15·0	7·58	82·3	0·39	58·0	0·22
S cerevisiae						
6663[b]	74·4	3·60	139·4	0·73	136·4	0·84

[a] The duration of fermentations: *Brettanomyces* spp: aerobiosis, 35 days; semi-aerobiosis, 55 days; anaerobiosis, 55 days; *S cerevisiae*: 15 days in all conditions.
[b] Accession number of Industrial Yeasts Collection of Dipartimento di Biologia Vegetale, Università di Perugia, Italy.

Under conditions which were more strictly maintained and monitored, fermentor tests confirm the preliminary results of microfermentations. The condition for maximal ethanol production was semi-aerobiosis (oxygen concentration between 6·5 and 35%) (Fig 1b, Table 4). The production of acetic acid was quite high (2·5 g litre^{-1}) and it seems to be formed during the second part of fermentation. Anaerobic conditions, caused a reduction in ethanol and very low amounts of acetic acid (0·7 g litre^{-1}), comparable with those of *S cerevisiae* test strain (Fig 1a, Table 3). On the contrary, full aeration caused a large production of acetic acid and low amounts of ethanol (Fig 1c, Table 4). This high concentration of acetic acid with consequently strong acidification of medium (12 g litre^{-1}, pH 1·91), probably caused a block of metabolic activity (sugar residue 68 g litre^{-1}). Comparing the growth of *Brettanomyces* sp and *S cerevisiae* strains in aerobic and anaerobic conditions (Fig 2) it is possible to note a lower growth rate of *Brettanomyces* strain in all conditions.

The cell growth in *S cerevisiae* was quite similar in both aerobic and anaerobic conditions, achieving a similar amount of cells in the stationary phase. The *Brettanomyces* strain, instead, showed a remarkable difference between aerobic and anaerobic growth. In aerobiosis the growth proceeded relatively quickly but the

TABLE 3
Influence of aeration on acetic acid and ethanol production in natural grape juice (NGJ) by *Brettanomyces* strains and *S cerevisiae* control strain (microfermentations)[a]

	Aerobiosis		*Semi-aerobiosis*		*Anaerobiosis*	
	Ethanol (*ml litre*$^{-1}$)	*Acetic acid* (*g litre*$^{-1}$)	*Ethanol* (*ml litre*$^{-1}$)	*Acetic acid* (*g litre*$^{-1}$)	*Ethanol* (*ml litre*$^{-1}$)	*Acetic acid* (*g litre*$^{-1}$)
Brettanomyces						
1	6·0	12·17	151·8	1·26	92·8	0·97
9	9·3	12·40	139·2	1·06	114·7	0·77
58	7·0	12·07	139·6	0·68	114·2	0·61
63	1·8	13·92	145·4	0·57	108·5	0·64
6706[b]	11·7	10·75	144·2	0·89	111·5	0·78
S cerevisiae						
6663[b]	84·2	3·02	148·0	0·48	142·0	0·41

[a] The duration of fermentations: *Brettanomyces* sp: aerobiosis, 25 days; semi-aerobiosis, 35 days; anaerobiosis, 60 days; *S cerevisiae*: 15 days in all conditions.
[b] Accession number of Industrial Yeasts Collection of Dipartimento di Biologia Vegetale, Università di Perugia, Italy.

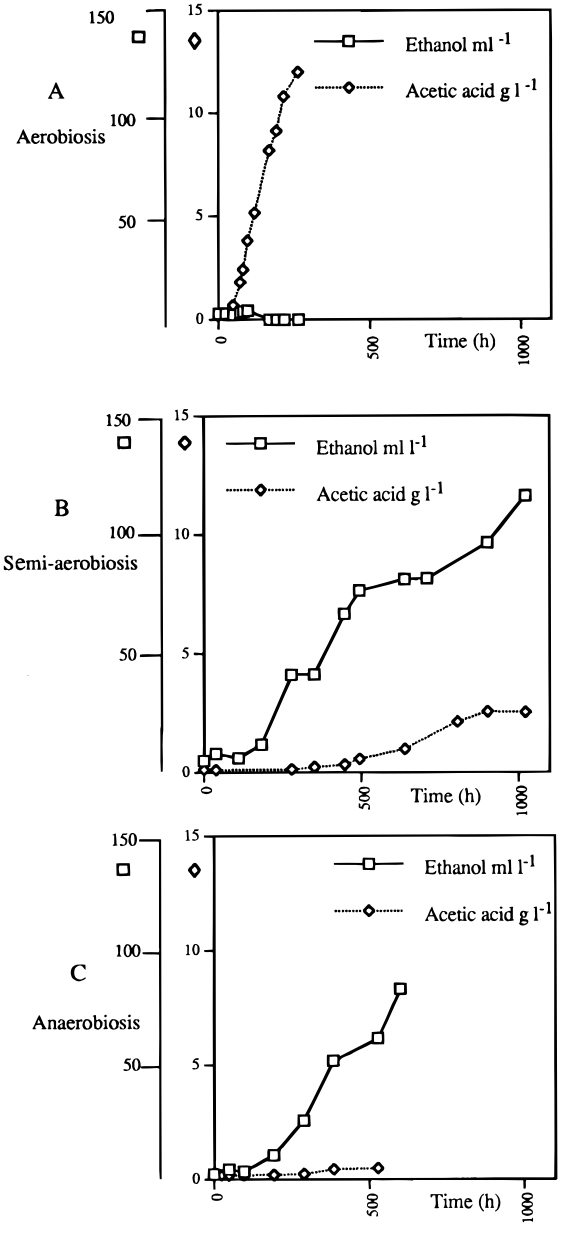

Fig 1. Evolution of acetic acid and ethanol formed by *Brettanomyces* strain no 58 under different oxygen concentrations in NGJ.

large amount of acetic acid caused a loss of viability after 200 h whilst in anaerobic conditions, the growth rate was very low. Moreover, in anaerobiosis the highest cell concentrations were five-fold lower than that in aerobiosis.

To investigate the relationship between the oxygen concentration and the acetic acid production in *Brettanomyces*, the specific activity of NAD^+ and $NADP^+$-linked aldehyde dehydrogenases during aerobic and anaerobic growth were determined. Acetic acid may be produced in yeasts by the oxidation of acetaldehyde by the action of these enzymes (Carrascosa *et al* 1981). Table 5 shows that the specific activities of the aldehyde dehydrogenases in aerobic conditions were much higher (*c* 50 times) than those in anaerobiosis and they appear to be positively correlated to the acetic acid production (Table 4).

The other by-products seem to be correlated to the fermentation activity, resulting in highest levels in semi-aerobiosis then in anaerobiosis and fully aerobiosis (Table 4). In all fermentation conditions acetaldehyde and higher alcohols were lower compared with those of *S cerevisiae*. The amount of ethyl acetate in anaerobic and semi-aerobic conditions was higher than that in full aerobiosis.

DISCUSSION

A large occurrence of *Brettanomyces* strains in sparkling wine samples without organoleptic and analytical alteration, concerning in particular acetic acid, suggested that strict anaerobiosis may affect the fermentation behaviour of these yeasts.

Results of fermentation tests, in fact, show that the different oxygen concentration exerted a strong influence on metabolic activity of *Brettanomyces*.

In the winemaking conditions (high sugar concentration, long fermentation time) semi-aerobiosis was the best condition for alcoholic fermentation associated with low levels acetic acid production whereas the very high acetic acid level in full aerobiosis caused a remarkable death rate and consequently stuck of fermentation. In anaerobic conditions the lower fermentation activity seems to be correlated to the reduced growth due to particular metabolism of this yeast.

In *Brettanomyces* yeasts, the alcoholic fermentation is inhibited under anaerobic conditions (Custers effect) since, in absence of an H-acceptor, the conversion of acetaldehyde in acetic acid will result in a drop in NAD^+/NADH ratio (not restorable by glycerol production) and consequently in a block of the glycolytic flux at level of glyceraldehyde-3-phosphate dehydrogenase. Nevertheless, this inhibition should be transient since the growth and ethanol production resumed after a period of adaptation (Wijsman *et al* 1984). From our results, *Brettanomyces* yeast in anaerobiosis showed, after a long period of adaptation, a slow and reduced alcoholic fermentation producing a limited amount of acetic acid comparable to that produced by *S cerevisiae* strain. The low level of acetic acid and other by-products indicate that a pure, even if slow, alcoholic fermentation occurred. In this condition the tendency to form acetic acid by *Brettanomyces* is strongly limited by the lack or deficiency of H-acceptors.

The positive correlation between the oxygen concentration and the acetic acid production suggests some interesting considerations regarding the influence of these yeasts on wine spoilage. In most of the cases of occurrence of *Brettanomyces* in winemaking environment such as barrel-ageing, oxygen at different levels is

TABLE 4
Analytical profiles of bench-top fermentations carried out at different oxygen concentrations by *Brettanomyces* sp strain no 58 and *S cerevisiae* control strain in NGJ

Aeration conditions	*Species*	*Sugar residue* (*g litre*$^{-1}$)	*Ethanol* (*ml litre*$^{-1}$)	*Acetic acid* (*g litre*$^{-1}$)	*Acetaldehyde* (*mg litre*$^{-1}$)	*Ethyl acetate* (*mg litre*$^{-1}$)	*Total higher alcohols* (*mg litre*$^{-1}$)	*pH*
Aerobiosis[a]	*Brettanomyces*	68·0	1·0	12·00	4·7	27·3	23·1	1·91
	S cerevisiae 6663[b]	ND	88·0	1·44	111·6	12·5	240·9	3·16
Semi-aerobiosis	*Brettanomyces*	ND	117·0	2·50	25·7	130·5	94·2	3·50
Anaerobiosis	*Brettanomyces*	11·8	86·0	0·70	14·6	109·5	78·9	2·80
	S cerevisiae 6663[b]	ND	120·1	0·58	36·6	29·3	120·2	3·20

[a] Aerobiosis: oxygen 40–80% of air saturation; semi-aerobiosis: oxygen 6·5–35% of air saturation; anaerobiosis: oxygen 0% of air saturation.
[b] Accession number of Industrial Yeasts Collection of Dipartimento di Biologia Vegetale, Università di Perugia, Italy.

available and consequently an increase in acetic acid during fermentation or from respiration of ethanol was found. However, in strict anaerobiosis, like in sparkling wine, *Brettanomyces* should carry out a pure fermentation without an consequent increase in acetic acid. Moreover, the level of by-products, did not seem to affect negatively the analytical profiles of the wines.

In conclusion, the results of this work showed that the characteristic 'high acetic producer' in *Brettanomyces* yeast, is linked to the presence of oxygen. The absence of an increase in acetic acid in wine does not exclude the active presence of *Brettanomyces* yeast.

TABLE 5
Specific activity (nmol litre^{-1} x mg protein^{-1} x min^{-1}) of NAD^+ and $NADP^+$-linked aldehyde deydrogenases of *Brettanomyces* sp strain no 58 in aerobic and anaerobic growth condition[a]

Conditions	NAD^+	$NADP^+$
Aerobiosis	12·4	5·0
Anaerobiosis	0·3	0·1

[a] Average of four measurements in the early stationary phase of growth.

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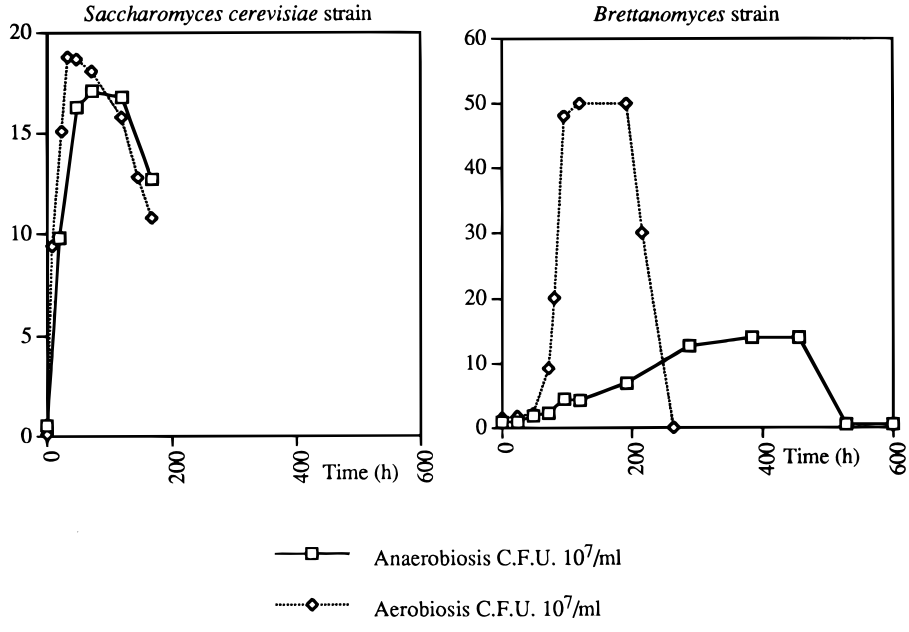

Fig 2. Comparation of the biomass evolution of the *Brettanomyces* sp and *S cerevisiae* strains in aerobic and anaerobic conditions in NGJ.

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