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## Characterization of the metabolic shift of *Saccharomyces bayanus* var. *uvarum* by continuous aerobic culture

**Abstract** *Saccharomyces bayanus*, being of interest for wine-making, is not as well known as *S. cerevisiae* and, due to many changes in the yeast classification, accurate data concerning its metabolic activity are difficult to find. In order to produce this yeast as an active dry yeast to be used as a starter in wine-making, its sensitivity to glucose was determined as the objective of our work. Using the pulse technique in continuous culture, it was found that growth in a synthetic medium was not limited by vitamins or mineral salts. We determined the critical dilution rate of a continuous culture and performed an aerobic continuous culture, measuring the respiratory quotient on-line in order to observe the metabolic shift from respiratory to fermentative metabolism. The *S. bayanus* var. *uvarum* strain studied was Crabtree-positive (glucose-sensitive) but had a weaker respiratory capacity than *S. cerevisiae* since the dilution rate of the metabolic shift was only 0.15 h<sup>-1</sup>. These new data provide essential information for the biomass production of this yeast strain for wine-making.

### Introduction

*Saccharomyces bayanus* var. *uvarum* is considered as a cryotolerant yeast in wine-making (Kishimoto and Goto 1995; Naumov 1996; Rainieri et al. 1998). Cryotolerant strains possess a number of advantages compared with non-cryotolerant strains. They have been used as starters in wine-making from low-acid musts since they synthesize malic acid and succinic acid (Rainieri et al. 1998), inhibit malolactic fermentation (Caridi and Corte 1997) and produce more glycerol and  $\beta$ -phenylethanol and less acetic acid and ethanol (Bertolini et al. 1996; Castellari et

al. 1994). The use of these yeasts make it possible to decrease the quantity of sulfites added to stabilize wines (Caridi and Corte 1997), a considerable advantage for sulfite-sensitive consumers. *S. bayanus* var. *uvarum* consequently is of great interest for wine-making and can be used as an active dry yeast to inoculate some grape musts. The industrial production of yeast cells is made generally by fed-batch cultures of glucose-sensitive yeasts. In order to optimize aerobic fed-batch cultures with regard to productivity and cell yield, it is necessary to use feed-back control devices for feeding. The most efficient one is based on respiratory quotient (RQ) measurements, using the fermentor exhaust gas stream (Aiba et al. 1976; Cooney et al. 1977). Thanks to the RQ value, it is possible to know the active metabolic state of cells and to limit the yeast to respiratory metabolism by a fitted feeding of the reactor according to this value. Thereby, to optimize the industrial production of yeast cells, their metabolic activity and sensitivity to glucose must be fully understood.

*S. bayanus* var. *uvarum* is already used in wine-making, but its sensitivity to free glucose has never been accurately determined. Molecular finger-printing was the first to show that the species *bayanus* was heterogeneous and could be divided into two groups: *bayanus* and *uvarum* (Rainieri et al. 1999). More recently, the taxon *S. bayanus* var. *uvarum* Naumov G. was established by genetic analysis (Naumov 2000) and so the taxonomic species *S. uvarum* was reinstated at the variety level. Such a lot of changes in classification (Barnett et al. 1990; Kreger-van Rij 1984; Vaughan-Martini and Martini 1998) since the beginning of taxonomic studies has caused great confusion about the name *bayanus* for wine-makers. Due to these problems, it is difficult to know whether data found in literature concern the right species. The strain used in this work is *S. bayanus* var. *uvarum* according to taxonomy but is called *S. uvarum* by wine-makers.

Two main categories of yeast have been differentiated on the basis of their sensitivity to free glucose. *S. cerevisiae*, the best wine yeast, is known to be Crabtree-positive. Some milligrams of glucose (100–500 mg l<sup>-1</sup>;

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Posten and Cooney 1993; Wang et al. 1979) in the medium are sufficient to induce a shift in the metabolism from respiration to fermentation. In fact, respiration is repressed in the presence of small concentrations of free glucose and the yield of biomass is drastically decreased. In contrast, Crabtree-negative yeasts show relatively fast growth and high yield of biomass under unrestricted oxygen supply but do not produce ethanol. They are insensitive to free glucose (Fiechter et al. 1981) and are not used for wine-making.

The main aim of this work was to characterize the sensitivity of *S. bayanus* var. *uvarum* to free glucose and to establish the transition conditions from respiratory metabolism to fermentative metabolism. In a first step, we checked that the growth of *S. bayanus* var. *uvarum* was not limited by the medium used. However, before using the pulse technique, it was necessary to determine the critical dilution rate, which allows a maximal growth rate without cells washing-out. In a second step, we took the advantage of the continuous culture technique to progressively increase the glucose quantity provided to the cells and thus to determine the optimal dilution rate in terms of respiratory metabolism.

## Materials and methods

### Yeast strain

*S. bayanus* var. *uvarum* P3 (CBS 395) was from the culture collection of the Faculty of Enology of Bordeaux. This strain was isolated from fermenting grape must (in 1994) in a winery located in the Val de Loire area.

### Culture media

The synthetic medium contained (per gram of glucose): 300 mg ammonium sulfate, 140 mg  $\text{KH}_2\text{PO}_4$ , 5 mg  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 10 mg NaCl, 120 mg  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  and 20 mg yeast extract. Ammonium sulfate and the other components of the medium were separately heat sterilized (121 °C, 20 min) to avoid the Maillard reaction, then mixed and complemented (per gram of glucose) with 0.2 ml of a sterile vitamin and/or mineral solution. The vitamin solution had the following composition (per liter): 3 mg biotin, 40 mg calcium pantothenate, 250 mg inositol, 50 mg pyridoxine HCl and 100 g thiamine HCl; and the mineral solution contained (per liter): 556 mg  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 576 mg  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 14 mg  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 50 mg  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 50 mg  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 338 mg  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  and ten drops of  $\text{H}_2\text{SO}_4$ . For continuous cultivation, the sterilization of the feeding medium was carried out in 10 l vessels and 22 µl antifoam (Polyglycol P 2000 E; Lambert Rivière, Fontenay sous Bois, France) was added, per liter. Experiments in Erlenmeyer flasks had earlier proved this antifoam concentration had no effect on the yeast growth (data not shown).

### Culture conditions

Precultures were grown to the stationary phase in shake flask cultures on synthetic medium (50 g glucose  $\text{l}^{-1}$ ) at 25 °C, 2500 rpm. These cultures were used to inoculate chemostat cultures which were run in 5 l bioreactors (Inceltech series 210; Toulouse, France) equipped with a condenser to avoid loss of water by evaporation during the culture. A working volume of 1.3 l was maintained by a double peristaltic pump for feeding and drawing off (Minipuls 3;

Gilson, Villiers Le Bel, France), coupled to a volume control device (liquid level). The pH was kept constant at  $3.0 \pm 0.1$  by automatic addition of 1 M NaOH with a pH controller (LH PPV3; Inceltech, Toulouse, France). The reactor was aerated with air at a flow rate of  $80 \text{ l h}^{-1}$ , corresponding to 1 vvm. The dissolved oxygen concentration was monitored with an oxygen electrode (Ingold model 34 10 3003; Mettler Toledo, Zurich, Switzerland) at 20% air saturation, by stirring rate variation. The temperature was kept constant at 25 °C.

### Pulse and shift technique

The pulse technique allowed the evaluation of the effect of a compound in a chemostat. The cells were fed continuously into a medium in which the studied compound was missing or present in a small quantity. When the stationary phase was reached, the compound was injected into the fermentor and the response in terms of the cell growth was observed during one residence time (Kuhn et al. 1979). A residence time is the time required for one reactor volume to pass through the vessel and is equal to the reciprocal of the dilution rate. The dilution rate is expressed in units per hour and is defined as the ratio of the feeding flow rate to the liquid volume in the reactor. The volume of the vitamin and mineral solutions pulsed into the fermentor was equal to  $0.2 \text{ ml g}^{-1}$  initial glucose $^{-1}$ . The addition of the pulse was carried out using a sterile syringe, whose needle was inserted into the reactor, through a membrane (pore size 0.22 µm), into the culture liquid with cells growing in a steady state. Shifts in dilution rate were achieved by increasing the flow rate of the feeding medium. A steady state was considered as reached when at least two volume changes had passed after the last change in flow rate and when the biomass, product (ethanol) and substrate (glucose) concentrations had remained constant (Kuhn et al. 1979). This typically required three to five volume exchanges after each change in the dilution rate.

### Biomass evaluation

Two methods were used to estimate the active biomass growth in the cultures: optical density and dry weight.

For optical density measurements, samples were poured into 1 cm optical length spectrometric cuvettes (Alliance Concept, Toulouse, France); and the optical density of yeast suspensions was measured with a spectrophotometer (Anthelic Advanced; Secoman, Toulouse, France) at 620 nm. A linear regression between optical density and biomass concentration was observed for a measured optical density range of 0.1–0.8. For higher values, samples were diluted with distilled water. We defined the optical density as the measured optical density value multiplied by a dilution factor.

For measuring the dry weight (biomass concentration), 10 ml of culture samples were centrifuged for 10 min at 250 rpm. The cells were washed twice with distilled water to eliminate compounds of the culture medium and then dried using a humidity analyzer (model HA60; Precisa, Zurich, Switzerland). The linear regression of biomass concentration (y) versus optical density (x) was given by the equation:  $y = 0.2741x$ .

### Determination of glucose and ethanol

Glucose was determined using an enzymatic glucose analyzer (2700 Select; YSI, Yellow Springs, Ohio). Ethanol was analyzed by gas chromatography (Chrompack 437A; Chrompack, Delft, The Netherlands). Prior to injection, the cells were removed by centrifugation and a known amount of 1% isopropanol solution was added as an internal standard. The column was a Chrompack Poraplot Q wide bore (0.53 × 25,000 mm), the injector and detector temperatures were both 200 °C and the carrier gas was nitrogen ( $8 \text{ ml min}^{-1}$ ).

## Gas analysis

Gas analysis was performed on line with a gas analyzer (type 4100; Servomex, Crowborough, UK). The exhaust gas was cooled in a condenser, dried with a Hygrostop (Environnement, Paris, France) and subjected to gas analysis in a paramagnetic oxygen and infrared carbon dioxide gas analyzer, sampling every 5 min during the continuous culture. The gas analyzer was calibrated with air and nitrogen and a mixture of 5% carbon dioxide and 95% synthetic air. After reaching the steady state, values for the oxygen consumption rate ( $q_{O_2}$ ,  $g\ l^{-1}\ h^{-1}$ ) and the carbon dioxide production rate ( $q_{CO_2}$ ,  $g\ l^{-1}\ h^{-1}$ ) were calculated in order to obtain the respiratory quotient (the molar ratio of carbon dioxide production to oxygen consumption).

## Results

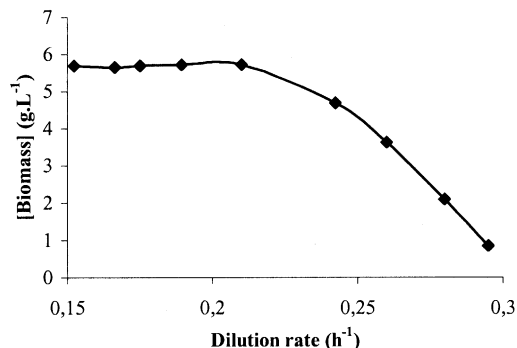
The nutritional requirements of *S. bayanus* var. *uvarum*

For checking the non-limitation of the medium, we used the pulse technique, conducted as described in the Materials and methods section. The pulse of elements had to be done at maximal cell growth, before the critical dilution rate. The critical dilution rate is the value of the dilution rate for which the biomass concentration in the fermentor starts to decrease. Therefore, we first determined this value.

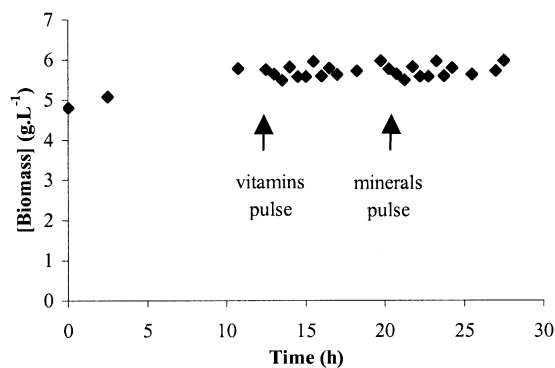
Figure 1 shows the biomass production as a function of the dilution rate during an aerobic continuous culture. The feeding medium contained  $50\ g\ glucose\ l^{-1}$ . We could see that the biomass concentration was maximal and constant until a dilution rate equal to  $0.21\ h^{-1}$ . At higher dilution rates, the biomass concentration decreased drastically; and the washing-out of cells began. So, the critical dilution rate of this strain of *S. bayanus* var. *uvarum* was  $0.21\ h^{-1}$ , under these conditions.

The composition of the synthetic medium was established to fulfill the nutritional requirement of *S. cerevisiae* in a previous study (Aranda Barradas, personal communication). However, the requirement of vitamins and minerals is different for each strain. So we tested the effect of adding supplementary amounts of these two solutions. To obtain a maximal biomass production without carbon limitation, the dilution rate was fixed at  $0.21\ h^{-1}$ , the critical dilution rate. When the steady state was reached, the injection of nutrients was done directly into the fermentor (Fig. 2). The response was obtained during the next 5 h (duration of the residence time). Before the second pulse, this delay was also observed, in order to be sure that the nutrients injected first were consumed by cells or were drawn off from the medium vessel.

After the pulse of each solution, the biomass concentration was constant and the steady state was not modified, so we could conclude that the initial composition of the synthetic medium was not vitamin- or mineral-limited for the growth of this *S. bayanus* var. *uvarum* strain. Thus, this medium had a suitable composition for the study of the metabolic activity of the yeast. Moreover, Larsson et al. (1993) had reported that the



**Fig. 1** Determination of critical dilution rate of *Saccharomyces bayanus* var. *uvarum* growing on glucose in aerobic continuous culture

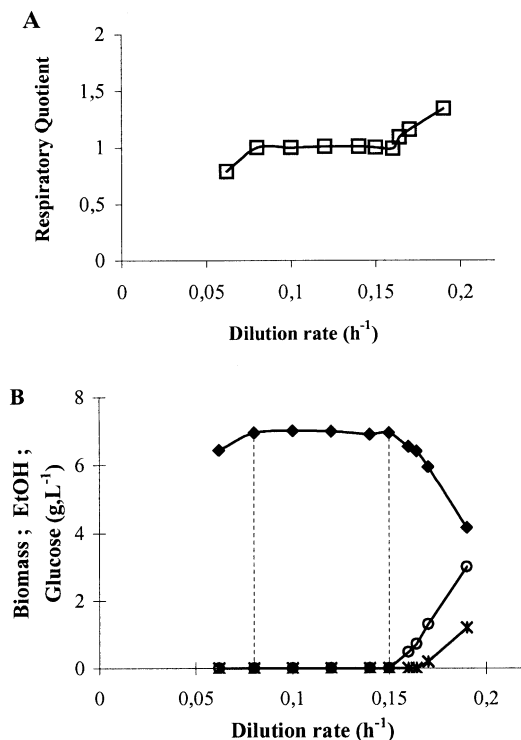


**Fig. 2** Response of *S. bayanus* var. *uvarum* to pulses of vitamin and mineral solutions at the critical dilution rate

value of the critical dilution rate was very much dependent on the C/N ratio of the medium. For a C/N ratio superior to 10, i.e. with a nitrogen limitation, significant amounts of ethanol were produced at a low dilution rate. The respiratory capacity of the cells, i.e. the specific oxygen consumption rate, was decreased by this limitation. So, for a good estimation of the metabolic activity of the yeast, we had to be limited only by glucose. In this synthetic medium, a C/N ratio of 6.3 was regarded as favorable.

### Metabolic activity of *S. bayanus* var. *uvarum* in aerobic continuous culture

Previously, the critical dilution rate corresponding to the washing-out of cells was determined to be  $0.21\ h^{-1}$ . The optimal dilution rate ( $D_{opt}$ ) is the value of the dilution rate for which the metabolic activity of cells shifts from respiratory to fermentative metabolism. This value is commonly close to 75% of the critical dilution rate value (Fiechter et al. 1981). The study of the metabolic activity can be done at a dilution rate between  $0.05\ h^{-1}$  and  $0.2\ h^{-1}$ . Moreover, it is necessary to keep dilution rate shifts increasingly small when approaching  $D_{opt}$ . When



**Fig. 3** Influence of dilution rate on (A) the respiratory quotient and (B) the steady state biomass (black diamonds) and glucose (crosses) and ethanol (EtOH, white circles) concentrations during a continuous culture of *S. bayanus* var. *uvarum* with a feeding glucose concentration of 20 g l<sup>-1</sup>. Dotted lines indicate the metabolic shift

steps larger than 0.01 h<sup>-1</sup> are applied in a dilution rate range close to  $D_{opt}$ , repression is frequently triggered, as indicated by the appearance of ethanol in the culture medium. After a short phase of ethanol production, the cells are able to fully re-establish oxidative growth only at dilution rates below  $D_{opt}$ , but this return to complete oxidative growth can take two or three residence times. This effect could lead to an erroneous identification of  $D_{opt}$  (Rieger et al. 1983).

The studied yeast *S. bayanus* var. *uvarum* showed the typical growth of a glucose-sensitive yeast, according to the Fiechter definition (Fiechter et al. 1981). Up to a dilution rate of 0.15 h<sup>-1</sup>, the glucose was strictly transformed into biomass and carbon dioxide; and glucose and ethanol did not accumulate in the vessel (Fig. 3B). The metabolism was purely oxidative. The RQ value was close to 1.0, indicating a stoichiometric oxidation of glucose (Fig. 3A).

In the first part of the curve (until the dilution rate was equal to 0.08 h<sup>-1</sup>), the biomass concentration and the RQ value were low. This phenomenon was frequently observed during glucose limitation: some glucose was partly consumed for cell maintenance, inducing a decrease in the biomass yield at a low dilution rate (Enfors 1998). Previously, Bailey and Ollis (1986) determined that, for a RQ value of 0.7–0.8, the yeast *Saccharomyces cerevisiae* underwent an endogenous metabolism.

A dilution rate above 0.15 h<sup>-1</sup> was characterized by a decrease in biomass production and an accumulation of ethanol and glucose in the culture medium. The RQ value increased above 1.0, indicating that glucose was only partly oxidized. The metabolism became respiro-fermentative.

## Discussion

In order to characterize the sensitivity of *S. bayanus* var. *uvarum* to free glucose, experiments were carried out in aerobic continuous culture with a progressive increase in the dilution rate. The results presented here show that growth of *S. bayanus* var. *uvarum* in this synthetic medium is not limited by vitamins or by mineral salts. Moreover, this strain is a Crabtree-positive yeast: even in oxygen excess ethanol can be synthesized.

These results are in accordance with the observations made by Fiechter et al. (1981), who classified *Saccharomyces* spp as glucose-sensitive yeast strains, growing on glucose and ethanol, whereas other yeast strains were insensitive (e.g. *Candida* sp.) or glucose-sensitive, growing on glucose but not on ethanol (e.g. *Schizosaccharomyces* sp.).

Consequently, this yeast strain had similar properties to *S. cerevisiae*; but the optimal and critical dilution rate values are different (the optimal dilution rate for *S. cerevisiae* being 0.25 h<sup>-1</sup>; Wang et al. 1979). At the level of pyruvate, respiration competes with alcoholic fermentation via the pyruvate dehydrogenase complex that leads to acetyl coenzyme A and the pyruvate decarboxylase that leads to acetaldehyde. Once formed, acetaldehyde may be oxidized to acetate and then to acetyl coenzyme A, or may be reduced to ethanol. The affinity constant ( $K_m$ ) linked to acetaldehyde oxidation is 6 μM, whereas the  $K_m$  of alcohol dehydrogenase is approximately 100-fold higher (Postma et al. 1989). Thus, once formed via pyruvate decarboxylase, acetaldehyde is preferentially oxidized to acetate. However, these two ways of pyruvate conversion into acetyl coenzyme A produce a bottle-neck. When the respiratory capacity of the yeast is saturated, the excess of pyruvate is used by pyruvate decarboxylase rather than by pyruvate dehydrogenase (Postma et al. 1989) to finally form ethanol via acetaldehyde. Because *S. bayanus* var. *uvarum* shifted from a respiratory metabolism to a fermentative metabolism at a lower dilution rate than *S. cerevisiae*, *S. bayanus* had a respiratory capacity smaller than *S. cerevisiae*. That could explain the lower production of acetic acid (a byproduct of acetate) in vinification by *S. bayanus* var. *uvarum* in comparison with *S. cerevisiae* (respectively 0.1 g l<sup>-1</sup> and 0.3 g l<sup>-1</sup>; Rainieri et al. 1998).

In this work, we established that *S. bayanus* var. *uvarum* is a Crabtree-positive yeast, with a transition from respiratory metabolism to fermentative metabolism occurring at a dilution rate of 0.15 h<sup>-1</sup> with a respiratory quotient value of 1.0 at the shift. These qualitative and quantitative data were not available in the literature.

These characteristics are very important for the industrial production of yeast using a fed-batch process to obtain an optimal growth yield. In our case, the reactor was controlled by measuring the RQ value on-line, to maintain a strictly respiratory metabolism; but very often in industrial conditions, a feeding rate profile is pre-established according to the optimal dilution rate. Moreover, to get active dry yeast, the biomass production step is very important, to keep high viability during the drying step. Our work contributes to the production of starter cultures of *S. bayanus* var. *uvarum* which is becoming more and more interesting in wine-making.

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