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Study of *Saccharomyces uvarum* CCMI 885 Physiology under Fed-Batch, Chemostat and Accelerostat Cultivation Techniques

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Summary

Physiological studies of the enological yeast strain *Saccharomyces uvarum* CCMI 885 (*Saccharomyces cerevisiae* var. *uvarum*) were carried out under different cultivation techniques, such as chemostat, fed-batch and the accelerostat (A-stat) procedure. Continuous cultivations were carried out on grape must medium, containing a mixture of hexoses (glucose/fructose) and ethanol with a total carbon compounds concentration of 20.6 g/L in the feed. In order to carefully study the yeast behaviour in a wide range of growth rates, the dilution rate (D) in the accelerostat technique (A-stat) varied between an initial value of 0.14 h^{-1} and a final value of 0.41 h^{-1}, with a constant acceleration rate of 0.011 h^{-2}. During this procedure, the shift from purely oxidative to respiro-fermentative metabolism was observed and the critical specific growth rate (μ_{crit}) was found to be 0.21 h^{-1}. Chemostat steady-state cultures were studied at three different dilution rates: 0.10, 0.14 and 0.29 h^{-1}. The chemostat metabolic rates and the growth yields were similar to those obtained under A-stat cultivation. The maximum specific growth rate found for this strain by the wash-out method, 0.37 h^{-1}, was lower than that obtained by A-stat (0.41 h^{-1}). A fed-batch cultivation was performed on the same grape must medium with a feed rate leading to a specific growth rate of 0.19 h^{-1}, with a purely oxidative growth of the yeast culture. The A-stat procedure is, therefore, a powerful technique providing fast and reliable information about yeast physiology.

Key words: yeast physiology, *Saccharomyces uvarum*, critical specific growth rate, accelerostat, fed-batch

Introduction

The utilisation of active dry wine yeasts (ADWY) in wine production is a well established worldwide practice (*1,2*). The main reason for this procedure is related to the necessity of controlling alcoholic fermentation, assuring the desired microbiological conditions during vinification. Furthermore, it is nowadays held by several authors that indigenous wine yeast strains are, to a considerable extent, responsible for the typical characteristics of certain regional wines (*3–5*).

In order to effectively produce this strain as ADWY, it is important to have some knowledge about its physiology and growth parameters, in particular, the critical specific growth rate for purely oxidative metabolism (μ_{crit}). This parameter is especially important from the industrial point of view, since yeast biomass production must be controlled at a growth rate close to the μ_{crit} for optimal productivity, which is usually attained in classical baker's yeast plants using fed-batch cultivation (*6*). Beet

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or sugarcane molasses are generally used as C-source for yeast growth in baker's and wine yeast production (*7*). For wine yeasts, and particulary for *Saccharomyces uvarum* CCMI 885, previous results have shown that grape must based medium is more suitable for biomass production, since it promotes yeast metabolism adaptation to the final substrate (*8*).

The accelerostat technique (A-stat), developed by Paalme *et al.* (*9*), consists of a computer-controlled continuos cultivation procedure with a smooth change of dilution rate. In this continuous cultivation system, dilution rate increases linearly at a constant acceleration rate (a) that enables a fast adaptation of the yeast metabolism to the varying growth rates and keeps the culture under a quasi-stationary steady state conditions in relation to the specific growth rate (μ). This cultivation procedure enables a fast screening of cell physiology under a wide range of growth rates, providing reliable data in shorter time than the classical chemostat technique (*9,10*).

In a previous work the enological yeast *Sacch. uvarum* was isolated from white wine musts of Alentejo, Portugal (*11*). This strain is a Crabtree-positive yeast, therefore it produces ethanol whenever sugars are present in excess, even under excess of oxygen. In this work we have studied the *Sacch. uvarum* physiology grown on grape must based medium under chemostat, A-stat and fed-batch fermentation conditions, aiming to determine the μ_{crit} and the maximal biomass yield ($Y_{X/S}$) for oxidative and respiro-fermentative metabolism. Particularly, the A-stat technique was found to provide fast and reliable information about yeast physiology.

Material and Methods

Microorganism

A yeast strain designated *Saccharomyces uvarum* CCMI 885 was isolated from Alentejo grape musts (*11*). By present taxonomy it should be regarded as *Saccharomyces cerevisiae* var. *uvarum* The strain was maintained in YMA slants, and it is deposited at the Culture Collection of Industrial Microorganism (CCMI) of INETI, Lisboa, Portugal (*12*).

Cultivation media

Sacch. uvarum cultivations were carried out in diluted grape must, supplemented with minerals, vitamins and nitrogen/phosphate source. The grape must media were supplemented with (per liter): citric acid, 10 mg; $FeCl_3$, 0.10 mg; H_3BO_3, 0.25 mg; $CuSO_4$, 0.02 mg; KI, 0.05 mg; $MnSO_4 \cdot H_2O$, 0.22 mg; Na_2MoO_4, 0.10 mg; $ZnSO_4$, 0.38 mg; NaCl, 50 mg; $MgSO_4$, 510 mg; $CaCl_2 \cdot H_2O$, 50 mg; biotin, 0.01 mg; nicotinic acid, 0.25 mg; pyridoxine, 1.0 mg; inositol, 1.0 mg; pantothenate, 1.0 mg; thiamine, 1.0 mg; $K_2HPO_4 \cdot 3H_2O$, 3,27 g; $(NH_4)H_2PO_4$, 14,5 g. For continuous cultivation, the feed composition was, slightly fermented grape must previously diluted to a final concentration of C-substrates of 20.6 g/L (7.7 g/L glucose + 10.4 g/L fructose + 2.5 g/L ethanol). Fed-batch fermentation was performed in the same basal medium, but the feed grape must containing 111 g/L of total C-substrates (43.7 g/L glucose + 60.1 g/L fructose + 7.3 g/L ethanol) concentration.

Chemostat cultivation

Continuous cultivations were carried out in a 3 L NBS Bioflo III bioreactor (New Brunswick, New Jersey, USA), using 2 L of working volume. Yeast was grown at 30 °C, with an aeration of 2 L/min and a constant pH=4.5, automatically controlled by the addition of 4 M NaOH. The stirring speed was set to 500 rpm and increased whenever dissolved oxygen was below 20 %. After an initial 8 h batch fermentation, dilution rate (D) was set to 0.05 h^{-1} and then slowly increased to 0.10 h^{-1} to avoid cell cycle synchronisation (*9*). At D=0.10 h^{-1}, a C-limited steady state was achieved within 5 retention times. A second steady state was then established at D=0.14 h^{-1} and samples from these two steady-state cultures were harvested directly to a vacuum filtration system.

A-stat procedure

At the second steady state, established at D_0=0.14 h^{-1}, a smooth increase in dilution rate was set (controlled by the AFS software from NBS, v. 3.42) with a constant acceleration rate (a) of 0.011 h^{-2} so that D changed with time as follows:

$$D = (D_0 + at) \qquad /1/$$

For calculation of specific growth rate in the A-stat procedure we used the continuous culture growth rate expression obtained from biomass balance. As dilution rate (D) varies linearly during A-stat cultivation procedure and volume (V) remains constant, it was assumed that μ could be calculated from biomass experimental data by the following approximation of the biomass balance equation

$$\mu = \frac{1}{X_m}\frac{\Delta X}{\Delta t}D_m \qquad /2/$$

where: X_m = mean biomass concentration between two consecutive data points; ΔX = biomass concentration difference between two consecutive data points; Δt=time interval between two consecutive data points; D_m=mean dilution rate value between two consecutive data points.

Samples from this culture were taken at regular 1 hour intervals and harvested in a similar way as in chemostat cultivation.

Wash-out technique

The experimental procedure was used as described in (*13*). An initial steady state was attained at D=0.29 h^{-1} and, then, D was suddenly shifted to 0.45 h^{-1}. The decrease of biomass concentration (as dry weight) was monitored during 2.5 hours.

Fed-Batch fermentation

Fed-batch culture was carried out in the same bioreactor and cultivation conditions that were used before for the continuous cultures (t=30 °C; pH=4.5; 500 rpm stirring speed; 2 L/min of air flow). Initial batch fermentation was performed in 1.2 L of growth medium

and the feed addition began after 16.2 h of batch fermentation. During fed-batch fermentation the C-feed was computer-controlled and varied exponentially according to equation /1/. The mathematical equations /3/, /4/ and /5/ represent a fed-batch system, with growth limited by carbon uptake, where specific growth rate (μ) is constant and residual carbon substrates virtually null in the culture media.

$$Q(t) = (\mu\ X_0\ V_0\ e^{\mu t}/Y_{X/S}\ S_0) \qquad /3/$$

$$V(t) = V_0\ (1\text{-}K+K\ e^{\mu t}) \qquad /4/$$

$$X(t) = X_0\ e^{\mu t}/(1\text{-}K+K\ e^{\mu t}) \qquad /5/$$

The feeding lasted 8 hours.

Analytical methods

Culture samples were used for determination of biomass concentration through filtration (4.5 μm membrane filters) followed by drying during 3 hours at 100 °C and weighing. Glucose, fructose and ethanol concentrations in the culture media were analysed by HPLC using a SugarPak™ column (Waters, Mildford, USA). Bioreactor off-gases were dried by passing through a vessel containing silica, O_2 and CO_2 concentrations were measured by means of a paramagnetic and an infrared analyser (1440 gas analysers, Servomex, Holland), respectively.

Results and Discussion

In the present work, the physiology of *Saccharomyces uvarum* yeast was studied under chemostat, A-stat and fed-batch cultivation conditions. The growth medium used in the different cultivations of *Sacch. uvarum* strain was grape must. In view of the fact that this strain is to be applied in vinifications, the production of biomass in the same medium provides a better adaptation of the yeast metabolism to the final substrate (*8*). Besides, it is known that, in some circumstances, baker's yeast producers add ethanol to the sugar-based cultivation medium to improve efficiency and economics (*14*). Ethanol results in higher growth yields than molasses sugars (fructose and glucose) because it is more reduced (*10*). For this reason, slightly fermented grape must was used in this work, meaning that some ethanol (2.5 g/L) was already present in the initial cultivation media.

A-stat cultivation

In order to carefully study the yeast behaviour in a wide range of growth rates, the dilution rate (D) in the accelerostat technique (A-stat) varied between an initial value of 0.14 h^{-1} and a final value of 0.41 h^{-1}, with a constant acceleration rate of 0.011 h^{-2}. Fig. 1A shows the biomass and ethanol concentration profiles, as well as carbon dioxide produced during the A-stat cultivation. For dilution rates of up to 0.21 h^{-1} biomass concentration in the culture was almost constant and no ethanol was accumulated, indicating that the yeast was growing under purely oxidative metabolism. During this cultivation period the A-stat culture exhibited behaviour similar to the classical chemostat with a perfect agreement between calculated specific growth rate and dilution rate (Fig. 1B). Above this dilution rate value (0.21 h^{-1}), A-stat culture showed a sharp decrease in biomass concentration and ethanol began to accumulate in the culture medium, meaning that the yeast growth turned to respiro-fermentative metabolism and the critical specific growth rate was attained. The critical specific growth rate value (0.21 h^{-1}) exhibited by this yeast under A-stat cultivation is close to the value (0.25 h^{-1}) reported by Paalme *et al.* (*9*) for baker's yeast grown in a similar medium by the same technique. Those changes in the culture caused a disturbance in the system that provoked a shift between the calculated specific growth rate and the dilution rate. This means that under this specific growth condition the rate of acceleration of D (a=0.011 h^{-2}) is higher than the stabilisation time of the yeast metabolism. Nevertheless, the expected decrease of biomass yield ($Y_{X/S}$) during the respiro-fermentative metabolism was clearly observed (Fig. 1B). During the oxidative growth period, biomass yield exhibited values in the range of 0.61–0.63 g cell dry weight/g total carbon-substrate (glucose+fructose+ethanol) decreasing from 0.61 g g^{-1} for D=0.21 h^{-1} to 0.34 g g^{-1} for D=0.41 h^{-1} during the respiro-fermentative growth. These values are similar to those found by Paalme *et al.* (*10*) for baker's yeast grown on a mixture of glucose/ethanol (200 mM/200 mM) using the same cultivation technique (A-stat).

Chemostat cultures

The chemostat cultures were performed in order to determine the metabolic rates and stoichiometric parameters under perfect steady-state conditions. The obtained data were compared with the values obtained under A-stat cultivation. Table 1 shows the specific con-

Table 1. Metabolic rates and stoichiometric parameters calculated from data obtained under three chemostats and A-stat culture at three dilution rates

	D	q_{glc}	q_{fru}	q_s	q_{CO2}	$Y_{X/S}$
	(h^{-1})		(mmol-C $g^{-1}h^{-1}$)		(mmol $g^{-1}h^{-1}$)	(g cel g^{-1}subs)
Chemostat	0.10	2.5	3.0	5,5	3.2	0.52
	0.14	2.8	3.7	6.5	2.8	0.64
	0.29	7.6	10.3	17.9	6.6	0.53
A-stat	0.16	3.1	4.2	7.3	2.9	0.63
	0.21	4.3	5.8	10.1	3.7	0.61
	0.29	7.4	10.0	17.4	5.7	0.53

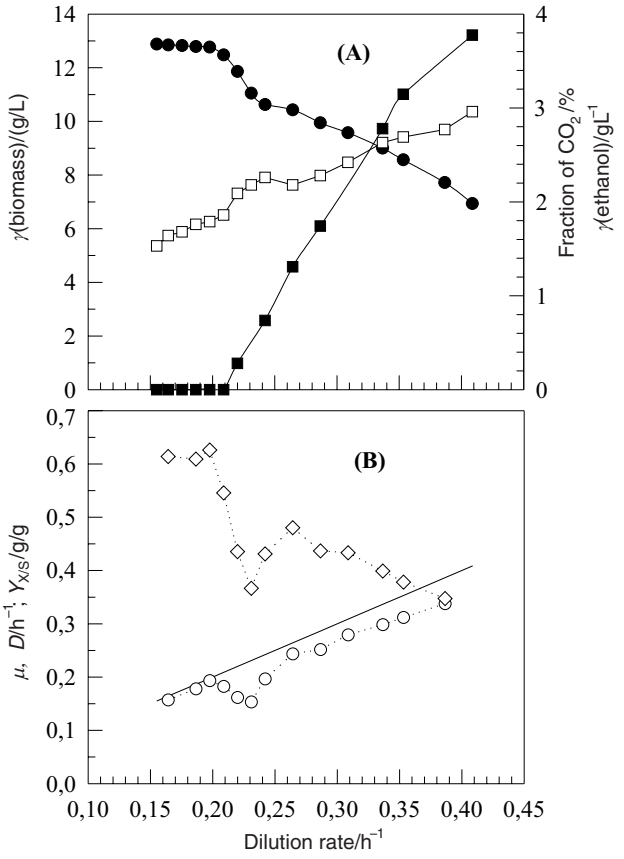

Fig. 1. A-stat cultivation of *Sacch. uvarum* between D=0.14 h^{-1} and D=0.41 h^{-1}
A) ● biomass dry weight; ■ ethanol; □ carbon dioxide
B) ○ specific growth rate; — dilution rate; □ biomass yield

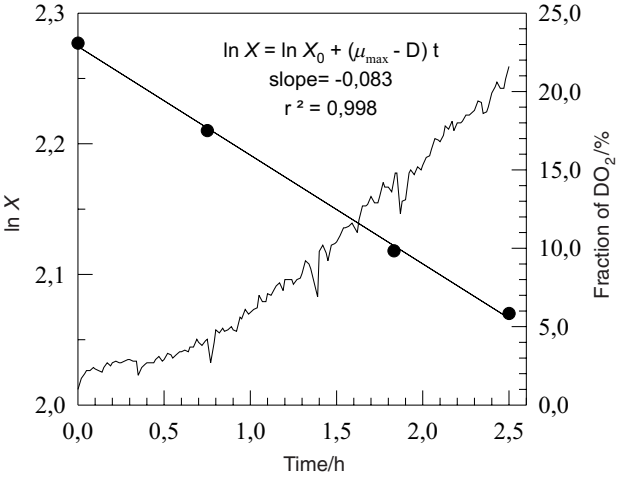

Fig. 2. Biomass wash-out rate for *Sacch. uvarum* grown in a chemostat at D=0.45 h^{-1}; According to Herbert *et al.* (*18*) the wash-out rate is equal to (μ_m–D) ●, biomass dry weight — dissolved oxigen profile, expressed as percent saturation

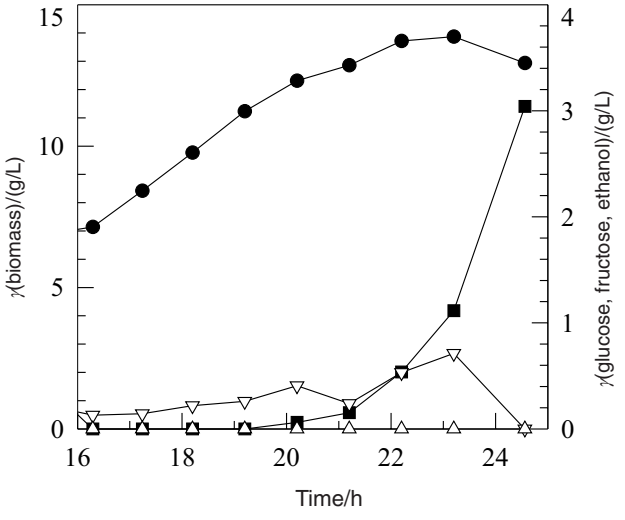

Fig. 3. Fed-bach fermentation of *Sacch. uvarum* grown on grape must; ● biomass dry weight; Δ residual glucose; ∇ residual fructose; ■ ethanol

sumption rates for glucose, fructose and total sugars (q_{glc}, q_{fru}, q_S), the specific carbon dioxide production rate and the biomass yield in respect to carbon compounds ($Y_{X/S}$) exhibited during chemostat cultivation and A-stat cultivation at three different dilution rates. The same tendencies were observed, when the yeast metabolism turned from pure oxidative to respiro-fermentative metabolism, under different cultivation techniques. The key parameter for the shift from oxidative to respiro-fermentative growth is the rate of glycolysis in Crabtree-positive yeasts (*15*). Enfors *et al.* (*15*) found a critical specific sugar consumption rate (q_S) of 0.36 g g^{-1} h^{-1} for baker's yeast grown on molasses, which contains approximately the same proportions of glucose and fructose. Woehrer and Roehr (*16*) reported for a baker's yeast a critical specific glucose consumption rate of 0.35 g g^{-1} h^{-1}, with a corresponding critical specific growth rate of 0.18 h^{-1}. These values compare well with the specific total sugar consumption rate (0.30 g g^{-1} h^{-1}) at the critical specific growth rate of 0.21 h^{-1} obtained under A-stat cultivation for *Sacch. uvarum* (Table 1).

The maximum specific growth rate (0.37 h^{-1}) estimated from the wash-out method (Fig. 2) was smaller than the μ_{max} obtained under A-stat cultivation conditions (0.41 h^{-1}), which also had been described before (*9,10*). This behaviour can be explained by the fact that in the accelerostat procedure the change of dilution rate is smooth (a=0.011 h^{-1}) compared with the wash-out method where the change in dilution rate is abrupt.

Fed-Batch fermentation

During the fed-batch fermentation (Fig. 3) a feed rate that imposed to the culture a constant specific growth rate of 0.19 h^{-1} was used. This value is slightly lower than the μ_{crit} (0.21 h^{-1}) found in the A-stat technique and it was chosen with the purpose of avoiding unstable growth conditions during the feed stage. When the yeast metabolism shifts from purely oxidative to respiro-fermentative growth a highly unstable growth behaviour is observed, as reported by Lei and Jörgensen (*17*). From the results plotted in Fig. 3 it is clear that, during the first 4 h of feeding, the yeast metabolism was purely oxidative, since no ethanol was accumulated in

the culture media. During this period the calculated specific growth rate was 0.19 h^{-1} and biomass increased from 7.1 g/L to 12.3 g/L. After 4-hours of feeding, ethanol was formed and C-sources, namely fructose, accumulated in the culture medium. The effective specific growth rate (μ_{eff}) on this period of fermentation decreased to values below the specific growth rate imposed by the feed rate (0.19 h^{-1}). This means that, at this time, the culture media was already limited by other growth factors, most probably, oxygen availability or toxic metabolites accumulation. From these observations we can conclude that the critical specific growth rate (μ_{crit}) obtained under the A-stat cultivation can be used as a valid value for fed-batch cultivation.

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SYMBOLS:

$Q\ (t)$	feed flow at time t (L h^{-1})
$V\ (t)$	work volume at time t (L)
$X\ (t)$	biomass concentration at time t (g L^{-1})
K	$X_0/Y_{X/S}\ S_0$
μ	specific growth rate (h^{-1})
S_0	total carbon feed substrates concentration (g L^{-1})
V_0	initial work volume (L)
X_0	initial biomass concentration (g L^{-1})
$Y_{X/S}$	biomass yield, in respect to total carbon substrates conversion (g g^{-1})
μ_{crit}	critical specific growth rate (h^{-1})
D	dilution rate (h^{-1})
D_0	initial dilution rate (h^{-1})
a	dilution acceleration rate (h^{-2})
q_{glc}	specific glucose consumption rate (mmol-C g^{-1} h^{-1})
q_{fru}	specific fructose consumption rate (mmol-C g^{-1} h^{-1})
q_S	specific total sugars consumption rate (mmol-C g^{-1} h^{-1})
q_{CO_2}	specific carbon dioxide production rate (mmol g^{-1} h^{-1})

Studij fiziologije *Saccharomyces uvarum* CCMI 885 u uvjetima šaržnog uzgoja s prihranjivanjem, te u kemostatu i akcelerostatu

Sažetak

Fiziološke studije vinskog kvasca *Saccharomyces uvarum* CCMI 885 (*Saccharomyces cerevisiae* var. *uvarum*) provedene su tijekom različitih uvjeta uzgoja, tj. uzgoja u kemostatu, u šaržnom postupku s prihranjivanjem i u akcelerostatu. Kontinuirani uzgoj proveden je u moštu grožđa koji sadrži smjesu heksoza (glukoza/fruktoza) i etanol s ukupnom koncentracijom izvora ugljika od 20,6 g/L. Kako bi se proučilo ponašanje kvasca pri različitim brzinama rasta, brzina razrjeđenja (D) pri akcelerostatskom postupku (A-stat) mijenjala se od početne vrijednosti 0,14 h^{-1} do konačne vrijednosti od 0,41 h^{-1}, uz konstantnu brzinu

akceleracije od 0,011 h^{-2}. Tijekom postupka opažen je prijelaz od čisto oksidativnog do respiracijsko-fermentativnog metabolizma, a kritična specifična brzina rasta (m_{crit}) iznosila je 0,21 h^{-1}. Kulture ustaljenog stanja u kemostatu proučavane su pri trima različitim brzinama razrjeđenja: 0,10, 0,14 i 0,29 h^{-1}. Metaboličke promjene i prinosi kvasca bili su slični onima postignutim tijekom akcelerostatskog postupka (A-stat). Maksimalna specifična brzina rasta tog soja, dobivena postupkom ispiranja, iznosila je 0,37 h^{-1}, što je niže nego u A-statu (0,41 h^{-1}). Šaržni uzgoj s prihranjivanjem proveden je na istoj podlozi, tj. u moštu grožđa s brzinom prihranjivanja koja je održavala specifičnu brzinu rasta od 0,19 h^{-1}, pri čemu je kultura kvasca imala samo oksidativni rast. Stoga je uzgoj u A-statu uspješan postupak koji omogućuje brze i pouzdane podatke o fiziologiji kvasca.