THE EFFECT OF INOCULUM PREPARATION ON THE FULLY AERATED GROWTH OF *SACCHAROMYCES CEREVISIAE* WITH A GLUCOSE SUBSTRATE

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(Received November 21, 1986)

The effect of inoculum preparation on the fully-aerated batch growth of *Saccharomyces cerevisiae* on glucose is described. The time allowed for inoculum adaptation and the conditions under which adaptation occurred, were observed to have a significant effect on the major metabolic variables: growth rate, specific sugar uptake rate, specific ethanol production rate, specific oxygen uptake rate, specific carbon dioxide production rate, the biomass yield, and the ethanol yield. Adaptation was allowed to occur under conditions of limited aeration (in shake flasks) and under defined aerobic conditions ($\text{DOT} > 75\%$ saturation). With increasing time, differences in the extent of fermentation and respiration were found. Adaptation in shake flasks resulted in a significant increase in the fermentation capacity of the yeast, while adaptation under strongly aerobic conditions resulted in an increase in the respiratory capacity. These results suggest that inoculum preparation is a significant variable which can be manipulated to obtain varying growth characteristics under fully aerobic batch growth conditions.

The growth characteristics of *Saccharomyces cerevisiae* in batch culture are influenced by the conditions to which the yeast is subjected, before it is used as a starter culture. Factors reported to have an effect are; the carbon source (1–6), the sugar concentration (4, 7), the physiological age of the culture (7, 8), the extent of aeration (7, 9, 10), and the inoculum level (11). Respiratory adaptation first described in 1950 (12) has been widely reported in the literature. Broadly, respiratory adaptation can be defined as the changes in enzymes and co-factors, induced by oxygen, which accelerate oxygen uptake. The increase in oxygen consumption or respiratory capacity have been ascribed to an adaptive synthesis of cytoch-

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romes (12, 13), levels of enzymes of the TCA cycle (1-4, 14, 15), and changes in the number and type of mitochondria (16, 17). Most experiments reporting respiratory adaptation describe short term changes (<24 hr) in organisms subjected to a shift from an anaerobic to aerobic environment (1-4, 7, 17, 18). However, studies of adaptation of yeast over longer time intervals have also been reported (7, 10, 14, 19). Given the extent of adaptation obtained with varying conditions, many fundamental studies on yeast metabolism do not state the method of inoculum preparation in sufficient detail to allow neither the effect of any given culture history to be quantified nor any quantitative comparison between various works.

The importance of respiratory adaptation and inoculum preparation in metabolic control studies in continuous culture has recently been shown with respect to the "Crabtree Effect" (14, 19-21). Differences in the preparation of the inoculum will result in differing metabolic parameters and varying levels of fermentation and respiration. Such variations may significantly affect the conclusions drawn about the control of fermentation and respiration. Industrially, little attention is paid to the development of the optimum inoculum preparation. As a result, significant variations in both metabolic rates (specific growth rate, specific sugar uptake rate, specific ethanol production rate) and yields (biomass yield, ethanol yield) occur. Optimizing these rates and yields for any particular industrial application can result in a reduction in the capital and operating costs of the plant.

This paper describes the batch growth of the yeast *S. cerevisiae* on glucose following various methods of inoculum preparation.

MATERIALS AND METHODS

Organism. *Saccharomyces cerevisiae* 248 UNSW 703100 (20, 22, 23). This yeast was maintained on slopes, the medium containing; malt extract (Oxoid Ltd., London) 5 g/l, glucose 4 g/l, agar 15 g/l, vegemite (a local brand of yeast extract, Kraft Foods Ltd., Melbourne, Australia) 4 g/l. The culture was stored at 4°C.

Viability. A viability of almost 100% was verified using the methylene blue method (24, 25).

Growth medium. A fully defined medium was used in all experiments. It had the following composition (g/l): glucose, 10; (NH₄)₂SO₄, KH₂PO₄, 3.4; Na₂HPO₄ 0.41, citric acid, 0.21; MgCl₂·6H₂O, 0.014; CaCl₂, 3.5 × 10⁻³; MnCl₂·4H₂O, 2.5 × 10⁻³; CoCl₂, 0.6 × 10⁻³; H₃BO₃, 0.2 × 10⁻³; CuCl₂·2H₂O, 0.4 × 10⁻³; ZnCl₂, 2 × 10⁻⁵, biotin, 0.08 × 10⁻³; *p*-amino benzoic acid, 8 × 10⁻³; riboflavin-5-phosphate, 8 × 10⁻³; pyridoxine hydrochloride, 0.016; thiamine, 0.016; and inositol, 0.08. All chemicals were analytical grade.

Growth conditions. All batch cultures were grown under strictly defined conditions of aeration, temperature, and pH. The dissolved oxygen level was maintained greater than 75% saturation (monitored by a Titron 502 oxygen electrode with a Titron 240 VAC 50-60 Hz polarizing unit). The temperature was controlled to 30°C by a West Gardian controller (Type RTQAT3RR/M) with a

single platinum resistance thermometer. The pH of the fermenter was 5.0, maintained by a Leeds and Northrup pH controller (model 7073-11) and preamplifier module 316524, with a Phillips electrode (type no. CHA 11). The fermentation apparatus used, described previously (26) facilitated accurate measurement of gas exchange.

Inoculum preparation. The inoculum was prepared by transfers in either shake flasks or fermenters used for batch work (described in "growth conditions"). Two methods of shake-flask preparation were used:

1. Two hundred fifty-ml flasks containing 100 ml of liquid media, incubated in a Paton Reciprocating shaking water bath (model RW1812) at 100 oscillations per minute (opm), a stroke length of 21 mm, and at 30°C.

2. One-liter flasks containing 100 ml of liquid media incubated at 300 oscillations per minute.

All transfers were made before the sugar concentration of the medium fell to approximately 2 g/l. Adaptation was allowed to occur for defined periods (10–150 generations) after which a final fermenter was inoculated. A generation time was defined as the time required for the cell density to double.

Inoculation procedure. An actively growing inoculum, taken from the first exponential growth phase was used in all experiments. A small sample of culture (prepared as indicated above) was quickly transferred to a fermenter equilibrated to the correct experimental conditions of temperature, pH and aeration. The initial concentration of the yeast in the fermenter was approximately 0.002 g/l. Therefore approximately 10 hr elapsed before accurate measurements could be made of dry weight, sugar and ethanol concentrations, and gas exchange.

This method of inoculation was used to overcome problems of long lag times and non-linear exponential growth phases.

Gas analysis. The percentage of oxygen in the exit gas from the fermenter was measured by a Taylor Servomex O₂ analyser (model 137), and the carbon dioxide by an MSA Lira infrared analyser (model 303). To eliminate inaccuracies due to the presence of water vapor, the exit gas is chilled to 1–3°C, by a Hartmann and Braun Gas Cooler (Model CGEK) prior to analysis.

Sugar/ethanol. These were analyzed using high performance liquid chromatography (Waters Associates Pty Ltd.), using a Dextropak column with a Differential Refractive Index Detector (model R401). The solvent, water, was pumped at 1.0 ml/min (model 600A pump).

Calculation of major metabolic parameters. The specific growth rate was calculated from the slope of the semilogarithmic plot of x versus t and the maximum specific growth rate (μ) was obtained by linear regression. The standard deviation for these calculations was found to be 0.01 hr⁻¹.

The cell yield on sugar $Y_S = \Delta X / -\Delta S$ was defined as the amount of cells produced per gram of sugar consumed, where X = dry cell weight concentration (g/l) and S = residual sugar concentration in fermenter (g/l). The ethanol yield Y_E was defined as

$$Y_E = \frac{\Delta E}{\Delta X}$$

where E = ethanol concentration (mmol/l).

Overall yields rather than incremental yields were calculated. It was found that the accuracy of sugar and dry weight analysis was insufficiently high to enable consistent incremental yields to be calculated. The standard deviation of cell yield and ethanol yield measurements was approximately 2%.

The specific sugar uptake rate (Q_s) was calculated from

$$Q_s = -\frac{1}{X} \frac{dS}{dt} = \frac{\mu}{Y_s}$$

Similarly specific ethanol production rate (Q_E) was defined as:

$$Q_E = \frac{1}{X} \frac{dE}{dt} = Y_E \cdot \mu$$

The standard deviations of the specific sugar uptake rate and the specific ethanol production rate were found to be approximately 4%.

The rates of oxygen uptake (Q_{O_2}) and carbon dioxide evolution (Q_{CO_2}) were measured using previously described methods (26).

Both inlet and outlet gas flow rates must be measured in conjunction with outlet gas concentrations before these rates can be calculated. In this paper the outlet-gas flow rate was not measured, but calculated from a simple nitrogen balance (26). The standard deviations of the specific oxygen uptake rate and specific carbon dioxide production rate were approximately 13%.

RESULTS AND DISCUSSION

Tables 1, 2, and 3 summarize the major metabolic variables of aerobic growth of *S. cerevisiae* 248 UNSW 703100 in batch culture with glucose as the limiting substrate.

From the results obtained, it was found that two variables have an effect on the major metabolic parameters: the time allowed for adaptation and the conditions under which adaptation occurred. The use of 250-ml shake flasks, agitated at 100 rpm has been shown to result in a much lower than optimum oxygen transfer to the medium (27). Preparation of the inoculum in these flasks resulted in an increase in the specific growth rate (from 0.33 to 0.40 hr⁻¹) when the adaptation time was increased from approximately 15 to 130 generations (see Table 1).

Symbols: $Q_{CO_{2[ave]}}$, specific carbon dioxide production rate (ml g-cell⁻¹ hr⁻¹); Q_E , specific alcohol production rate (mmol g-cell⁻¹ hr⁻¹); $Q_{O_{2[ave]}}$, specific oxygen uptake rate (ml g-cell⁻¹ hr⁻¹); Q_s , specific glucose uptake rate (mmol g-cell⁻¹ hr⁻¹); t , time (hr); Y_E , ethanol yield (mmol g-cell⁻¹); Y_s , biomass yield from sugar (g-cell g-sugar⁻¹); ΔE , ethanol increment (mmol/l); ΔS , sugar decrement (g/l); ΔX , cell mass increment (g/l); μ , specific growth rate (hr⁻¹).

Table 1. Experimental data from batch growth of *S. cerevisiae* 248 UNSW 703100 with glucose as the limiting substrate. Adaptation of inoculum in 250-ml shake flasks.

Adaptation no. of generations	μ	Y_S	Y_E	Q_S	Q_E
15	0.33	0.13	54	14	18
130	0.40	0.12	57	18	22
130+10*	0.42	0.12	58	19	24

10*: 10 generations in fermenter with defined conditions.

Fig. 1. Specific oxygen uptake rate and specific carbon dioxide production rate after adaptation for 15 generations in 250-ml shake flasks.

▼, QO_2 ; ▽, QCO_2 ; ----, cell concentration (x).

The QO_2 and QCO_2 observed in batch growth after the inoculum was prepared for 15 generations, were significantly different from that obtained after preparation for 130 generations. Balanced growth was not achieved after 15 generations since the QO_2 and the QCO_2 did not reach steady values during the duration of the batch. The QO_2 from 130 to 51 ml g-cell⁻¹ hr⁻¹ and the QCO_2 increase is statistically significant. It was found that, provided sufficient time was given for adaptation (130 generations), these specific rates may reach steady values within the duration of the batch (20) (see Fig. 2). This fact throws doubt on any kinetic data for the growth of *S. cerevisiae* which does not exhibit steady values for the specific exchange rates. Previous batch works reported with *S. cerevisiae* do not state in sufficient detail the method of inoculum preparation and hence most previous data are inadequate for an assessment of the control of fermentation and respiration

Fig. 2. Specific oxygen uptake rate and specific carbon dioxide production rate after adaptation for 130 generations in 250-ml shake flasks.

▼, QO_2 ; ▽, QCO_2 ; ----, cell concentration (x).

Table 2. Experimental data from batch growth of *S. cerevisiae* 248 UNSW 703100 with glucose as the limiting substrate. Adaptation of inoculum in 1.0-liter shake flasks.

Adaptation no. of generations	μ	$QO_{2[ave]}$	$QCO_{2[ave]}$	Y_S	Y_E	Q_S	Q_E
20	0.45	73	631	0.14	58	18	26
150	0.45	97	512	0.14	52	18	23

except for transient conditions.

An increase in agitation speed (to 300 rpm) and a decrease in the ratio of the volume of the medium to the volume of the flask (that is, 100 ml in 1-liter flask) will increase the rate of oxygen transfer, although the extent of the increase has not been quantified. Balanced growth, as measured by constant QO_2 and QCO_2 production rates was achieved after 48 hr. The average specific oxygen uptake rate and the average specific carbon dioxide production rate of these experiments are shown in Table 2.

In fermenters where the measurement of oxygen tension is possible and air supply or stirrer speed can be altered, aeration can be adequately defined. The use of such a fermenter, with controlled conditions of pH, temperature, and aeration (DOT > 75%), for inoculum preparation resulted in no adaptation occurring after 20 generations (Table 3). Balanced growth occurred after 20 generations as measured by approximately constant specific oxygen uptake rate and specific

Table 3. Experimental data from batch growth of *S. cerevisiae* 248 UNSW 703100 with glucose as the limiting substrate. Adaptation of inoculum in fermenters with defined conditions of temperature, pH, and aeration.

Adaptation no. of generations	μ	$QO_{2[\text{ave}]}$	$QCO_{2[\text{ave}]}$	Y_S	Y_E	Q_S	Q_E
10	0.41	117	634	0.14	50	16	20
20	0.45	78	613	0.16	56	16	25
30	0.45	90	473	0.14	57	18	26
50	0.44	99	542	0.15	51	17	23
75	0.45	85	525	0.14	54	18	24
100	0.45	97	538	0.14	46	18	21
150	0.45	105	494	0.15	41	17	19

carbon dioxide production rate.

By comparing Tables 1, 2, and 3, it can be seen that the conditions under which inoculum preparation occurs has a statistically significant effect on the magnitude of the major metabolic parameters. The specific growth rate can be varied from 0.33 to 0.45 hr⁻¹, the cell mass yield from 0.12 to 0.16 g-cell g-sugar⁻¹ and the specific sugar uptake rate from 14 to 18 mmol g-cell⁻¹ hr⁻¹. The yield of ethanol can vary from 41 to 58 mmol g-cell⁻¹ and the specific ethanol production rate from 18 to 26 mmol-ethanol g-cell⁻¹. The method of inoculum preparation will also determine whether or not balanced growth is obtained.

The effect of long-term adaptation under conditions of poor aeration on subsequent adaptation to conditions of adequate aeration was tested by preparing an inoculum in 250-ml shake flasks for approximately 130 generations, then in a fermenter with defined conditions for a further 10 generations (Table 1). The growth observed compared to growth after 130 generations in 250-ml flasks, did not vary significantly. Adapting the yeast in conditions of optimum pH, temperature and high aeration (from a culture maintained on a slope) required 20 generations before optimum growth was attained. Allowing the culture to adapt first in poorer conditions of aeration did not lower this time.

Fundamental studies of yeast metabolism often involve an estimation of the extent of fermentation and respiration. The "Crabtree Effect" (28-30) and the "Pasteur Effect" (31-33) are two such examples. The "Crabtree Effect" is defined as the repression of respiration under fully aerobic, non-limiting conditions. The "Pasteur Effect" is defined as the action of oxygen in diminishing carbohydrate uptake. In both cases data on specific oxygen uptake rates, specific ethanol production rates and sugar uptake rates, are used to estimate the extent of any repression of respiration or any reduction in sugar uptake rate. The common method of inoculum preparation (in shake flasks) may lead to errors in the interpretation of results. It is clear from this work that repression of respiration or reduction of sugar uptake may be due to differences in the length of adaptation or in the extent of aeration when the inoculum was prepared, not the result of other

imposed conditions, for example, high sugar concentrations. Batch growth of yeast is carried out in two major industries (bakers yeast and ethanol). Since the work described in this paper was performed under fully aerobic conditions, it has direct relevance to the bakers yeast industry. Whilst the direct application of this work to the ethanol industry may not be relevant, it is clear that rates of fermentation are significantly affected by the method of inoculum preparation. The pretreatment of resting cells with oxygen has previously been shown to stimulate fermentation (34). Hence this indicates that the performance of the yeast in ethanol production may also be enhanced by careful selection of inoculum preparation conditions. The effect of adaptation under conditions of very low aeration remains to be examined.

In the production of bakers yeast, the biomass yield, the ethanol yield, and the subsequent growth of the yeast on sugar/ethanol are the parameters optimized to reduce capital and production costs. Traditionally fermentation conditions have been varied to optimize production. From the results discussed in this paper the preparation of the inoculum is a significant variable which can be manipulated to help obtain optimum operating conditions.

CONCLUSIONS

The characteristics of fully aerated batch growth of *S. cerevisiae* with glucose as the limiting substrate is dependent upon the time allowed for adaptation in the preparation of an inoculum and the conditions under which adaptation occurs.

Inoculum preparation in shake flasks with poor agitation resulted in an increase in the fermentation capacity of the yeast when time of adaptation increased from 15 to 130 generations. Adaptation in conditions of aeration where DOT > 75% of saturation, resulted in an increase in the extent of respiration. Traditional control concepts based on the measurement of the extent of either fermentation or respiration are open to question. The conditions of inoculum preparation are rarely stated in sufficient detail and it is unlikely that the extent of the inoculum effects (as described in this work) on the major metabolic variables has been considered.

Support for this research was provided by Mauri Foods, a Division of Burns Philp Pty Ltd., North Ryde, NSW, Australia.

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