The need for consistent nomenclature and assessment of growth phases in diauxic cultures of *Saccharomyces cerevisiae*

J. G. LEWIS,^{1*} C. J. NORTHCOTT,² R. P. LEARMONTH,¹ P. V. ATTFIELD² and K. WATSON¹

¹Department of Biochemistry, Microbiology and Nutrition, University of New England, Armidale, NSW 2351, Australia

²Yeast Physiology Research Group, Burns Philp Technology and Research Centre, PO Box 219, North Ryde, NSW 2113, Australia

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The need for consistent nomenclature and accurate assessment of late growth phases in diauxic yeast cultures is highlighted by the substantial variation of stress tolerance in *Saccharomyces cerevisiae* after the exhaustion of the initial fermentable carbon source. At present, a wide variety of assessment methods and confused terminology exists in the literature, leading to difficulties in the interpretation and comparison of published results. A method based on the depletion of ethanol accumulated during the respiro-fermentative growth phase is suggested as suitable for assessing subsequent growth phases and reporting results. Consistent application of nomenclature for growth phases is recommended to assist the interpretation of published experimental results. It is suggested that the phases of growth in diauxic batch culture should be referred to using the terms (1) initial lag phase, (2) respiro-fermentative phase, (3) diauxic lag phase, (4) respiratory phase, (5) stationary phase, and (6) death phase.

Introduction

The majority of work published on *Saccharomyces cerevisiae* has used cells grown in aerobic batch culture, where growth phases are pronounced and have a dramatic effect on cellular physiology. Many reports have dealt solely with cells during rapid growth on fermentable sugar, avoiding the complications of subsequent growth on ethanol and other by-products of fermentation. However, the diauxic nature of most strains of *S. cerevisiae* has led to difficulty in understanding and interpreting published work that has used cells in phases of growth following sugar exhaustion. There are two distinct areas of confusion in the reporting of such work: the methods used to assess growth phases, and the terminology used to describe them.

Methods used to assess the growth state of cultures have varied widely. 'Exponential' growth on fermentable sugars has been defined by viable count and optical density, as well as number of cell generations since inoculation (de Virgilio *et al.*, 1991) or before glucose exhaustion (Hottiger *et al.*, 1987), time after inoculation (Winkler *et al.*, 1991) and dry weight of cells (Warth, 1989). Growth phases after sugar exhaustion have also

been defined by viable count and optical density, as well as time after inoculation (de Virgilio *et al.*, 1991), exhaustion of glucose and decrease in percentage of budded cells (Boucherie, 1985), and exhaustion of ethanol (Blomberg *et al.*, 1988; François *et al.*, 1991). Unfortunately, many of these parameters do not clearly identify growth phases to workers unfamiliar with the specific cultural details of the yeast strain reported and make independent interpretation of published results or reproduction of comparable experiments extremely difficult. This problem is exacerbated by the inconsistent usage of terms describing growth phases. For example, the initial proliferation of yeast cells growing on fermentable sugars is commonly termed 'exponential' or 'logarithmic' growth, but also 'logarithmic fermentation' (Attfield, 1987) and 'respiro-fermentative' growth (e.g. Larsson & Gustafsson, 1987). While 'stationary phase' has had little variation in its nomenclature, the term has often been used incorrectly to describe diauxic lag and other phases after the exhaustion of glucose (Boucherie, 1985; Bataillé *et al.*, 1991), as well as true stationary phase after the exhaustion of all assimilable carbon substrates (Blomberg *et al.*, 1988; François *et al.*, 1991).

Work in the area of stress tolerance highlights the importance of accurately determining and reporting growth phase information. It has long been accepted

* Author for correspondence. Tel. 6167 732729; fax 6167 728235; email rlearmon@metz.une.edu.au

that, for most stresses, tolerance is linked to the growth rate and phase of yeast cells, and slow or non-growing cells have been reported as far more tolerant to heat (Watson, 1987), chemical and physical mutagens (Parry *et al.*, 1976) and freezing (Meyer *et al.*, 1975) than rapidly growing cells. However, while cells have often been referred to as 'stationary phase', cultural details are frequently too brief to allow objective assessment (e.g. Meyer *et al.*, 1975; Winkler *et al.*, 1991). Commonly, slow-growing cells in phases after the exhaustion of fermentable sugars are up to 1000 times more stress-tolerant than rapidly growing cells prior to sugar exhaustion. Consequently, it is often assumed that any variation in stress tolerance of cells in growth phases after sugar exhaustion is small, making accurate assessment of these growth phases unnecessary. However, in this paper we report considerable variation in stress tolerance during growth phases after the exhaustion of fermentable sugars, highlighting the need for their accurate assessment and terminology. We suggest a method for the routine assessment of these growth phases as well as nomenclature, based on already existing terms, to describe diauxic growth curves. A practice of reporting such information consistently, and in sufficient detail to permit full interpretation and comparison of results, is proposed.

Methods

Yeast strains and culture conditions. *Saccharomyces cerevisiae* strains DBY746 (ATCC 44773, a genetically defined laboratory strain), K7 (ATCC 26422, a saké strain), SG195 (ATCC 38554, a wild-type thermotolerant strain) and A9 (a wild-type baking strain), were maintained on YEP agar slopes containing 0.5% yeast extract, 0.5% bacteriological peptone, 1% glucose, 0.3% $(\text{NH}_4)_2\text{SO}_4$, 0.3% KH_2PO_4 and 1.5% agar (all w/v). Slopes were stored at 4 °C. Aliquots of 50 ml YEP broth in 250 ml Erlenmeyer flasks were inoculated from a slope and incubated overnight at 25 °C and 180 r.p.m. Such cultures were in respiratory growth phase (see Results and Discussion) when used to inoculate 500 ml YEP broth experimental cultures in 2-litre Erlenmeyer flasks stoppered with cotton wool bungs. These were incubated under the same conditions. Glucose was estimated using indicator strips (Diabur-Test 5000, Boehringer), which were not accurate above 0.5% (w/v), but proved consistent and sensitive at or below this level. Ethanol concentration was determined by alcohol dehydrogenase (ethanol test kit 332-UV, Sigma Diagnostics). The percentage of unbudded cells in the culture was monitored using light microscopy.

Stress resistance. Resistance of the cultures to freezing and thawing was tested as follows. A 10 ml portion of culture was centrifuged at 1500 g for 2 min at 25 °C, washed with 5 ml YEP without glucose, and resuspended in the original volume of YEP without glucose. Resuspension of all samples was necessary to avoid the influence of the changing composition of the culture medium on the cells during the stressing procedures. Sample volumes of 1 ml were distributed into 1.5 ml microfuge tubes, plunged into liquid nitrogen for 5 min and thawed in a 25 °C waterbath for 10 min. Resistance to heat stress was assessed by transferring a 4.1 ml sample from the resuspended culture to a 22 mm Pyrex test tube and heating at 60 °C for 30 s, rapidly raising

the temperature to 52 °C. The tube was then incubated in a 52 °C waterbath at 150 r.p.m. for 4.5 min, before being cooled in ice-water to 25 °C. Viable count of survivors after stressing was assessed by plating suitable dilutions in YEP without glucose on to YEP agar and incubating at 25 °C for 24–48 h. Stress tolerance was measured as the percentage of survivors after stress compared to an unstressed control. All results presented are representative of two to four experiments.

Results and Discussion

Stress tolerance

Fig. 1 presents heat and freeze–thaw tolerance of four yeast strains over an 80 h period. All showed typical sensitivity to stress during rapid growth on glucose, with a dramatic increase in tolerance upon exhaustion of the sugar. Stress tolerance changed markedly during subsequent culture phases, and strains A9 and DBY746 in particular demonstrated large changes in freeze–thaw tolerance. This variation may reflect metabolic activity such as the expression of heat-shock proteins (Bataillé *et al.*, 1991; Boucherie, 1985), accumulation of trehalose (Mackenzie *et al.*, 1988) and altered plasma membrane ATPase activity (Eraso *et al.*, 1987), all of which have been associated with stress tolerance (Van Laere, 1989; Panaretou & Piper, 1990). Observed variations in stress tolerance during different growth phases, together with reported changes in cellular physiology, highlight the necessity for accurate assessment of growth phases.

Growth phase assessment

Fig. 2 presents results of a typical aerobic batch growth curve for strain A9. Growth is initially the result of fermentation and assimilation of glucose, and subsequently the assimilation of the ethanol resulting from fermentation. Fig. 2(a) demonstrates the suitability of optical density, viable count, percentage budding, glucose concentration and ethanol concentration as markers of the cultures' exit from phase 1, and of entry into, and exit from, growth phases 2 and 3. Changes are rapid and distinct, allowing clear assessment of growth phase, provided that the growth curve is well characterized. In addition, data derived from microcalorimetry (Blomberg *et al.*, 1988) and flow cytometry (Scheper *et al.*, 1987) have been used to define these phases. However, the interpretation of exit from the phase of growth on ethanol (phase 4) and entry into stationary phase (phase 5) is more difficult, as markers such as optical density and viable count become too insensitive to be accurate. The rate of metabolic activity of the culture, inferred by microcalorimetry, is an extremely sensitive measure of entry into stationary phase (Blomberg *et al.*, 1988), but is not convenient for routine work. However, the rapid decrease in heat output of the culture associated with entry into stationary phase coincides with the exhaustion of

Fig. 1. Stress tolerance of (a) DBY746, (b) K7, (c) SG195 and (d) A9 in batch culture. ▼, Ethanol; ▽, glucose; ●, freeze-thaw stress survivors; ○, heat stress survivors.

ethanol in the culture, and thus ethanol concentration itself may act as a suitable marker. Although accumulated acetate may be present in the medium at the point of ethanol exhaustion, it is immediately and rapidly consumed and has little effect in extending respiratory growth (Blomberg *et al.*, 1988). Of four *S. cerevisiae* strains tested in our laboratory, three exhausted the accumulated ethanol within 35 h of the exhaustion of glucose and entered stationary phase (Fig. 1). The fourth strain, DBY746, assimilated ethanol very slowly, and had not reached stationary phase 70 h after the exhaustion of glucose. This demonstrates the unreliability of assuming that a culture will be in stationary phase 24 or even 48 h after inoculation. Death phase, the final phase in batch culture, refers to a significant and

continuous fall in population viability and so may only be defined in terms of viable count. Entry into death phase was not evident in these experiment (Figs 1 and 2) and would not normally be expected for several days (results not shown).

Growth phase terminology

Fig. 2 may be divided into five phases, several of which have counterparts in the traditional bacterial growth curve. Phases 1, 2 and 5 (Fig. 2) would traditionally be called lag, exponential and stationary phase, respectively. However, no consistent terms have been available to describe phases 3 and 4. We suggest below terms to describe these phases and revised terms for other parts of

Fig. 2. Growth of *S. cerevisiae* A9 in batch culture: (a) 0–15 h, (b) 0–75 h. ○, OD₆₄₀; ■, log viable cells ml⁻¹; ●, percentage of budding cells; ▼, ethanol; ▽, glucose. Data are from a representative experiment. Growth phases 1 to 5 are indicated by vertical dashed lines.

the growth curve in order to arrive at a consistent nomenclature. The terms reflect the physiological state of the cells, and most have already appeared in the literature.

Phases 1 and 3 (Fig. 2) may both be referred to as 'lag' phases, implying a period of retarded growth during metabolic adjustment to a new substrate. In order to differentiate between them we suggest the terms 'initial lag phase' and 'diauxic lag phase', both of which have previously been employed (e.g. Scheper *et al.*, 1987). While numerous terms have been used to describe phase 2 (Fig. 2), it is invariably characterized by high levels of fermentative activity and substantially repressed respiration. Terms such as 'exponential' and 'logarithmic' growth, although widely accepted and understood, describe mathematical abstractions of growth and give no indication of cellular physiology. In addition, growth on ethanol is also a period of exponential increase in cell number, although at a slower rate. We favour the term 'respiro-fermentative' growth (e.g. Larsson & Gustafsson, 1987), which reflects the physiological state of the cells, and emphasizes the predominantly fermentative nature of growth in phase 2. Phase 4 (Fig. 2) appears to have no widely used descriptive term and has often been erroneously described as 'stationary phase' on the basis that 'stationary phase' is any period after

the exhaustion of the initial fermentable carbon source. While stationary phase may immediately follow respiro-fermentative growth on sugar, for example in respiratory-deficient mutants, anaerobic cultures or cultures grown on non-fermentable substrates, this is not the case for most cultures, and phase 4 precedes the stationary phase. Phase 4 is a period of respiratory growth on ethanol and we favour the term 'respiratory growth phase', again reflecting cellular physiology. Existing terminology is adequate to describe phase 5 (Fig. 2), 'stationary phase', as there appears to be little confusion in the literature about what this term implies. The term is used exclusively to mean the complete cessation of growth due to the lack of essential nutrients, but maintenance of high cell viability. 'Death phase' was not evident in this experiment, but the term has been used exclusively to describe the decrease in population viability after extended starvation. We suggest that the terms 'stationary phase' and 'death phase' be retained without alteration.

In conclusion, although the importance of the growth phase in all aspects of cellular response is widely recognized, it is not always acknowledged in the way results are presented. This has led to reports which omit information needed to put results into their physiological context. Ideally, published results obtained from diauxic

batch culture should employ consistent nomenclature to describe growth phases. In addition, growth curves should be included in published results. For experiments involving only respiro-fermentative phase cells, optical density or viable count, as well as fermentable substrate concentration, should be included. For post-respiro-fermentative phase cultures, data should include growth curves and ethanol concentration in the culture. The consistent reporting of these parameters will allow definite identification of the growth phase of the culture and do much to make the interpretation of results easier for other workers.

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