Extraordinarily Rapid Appearance of a β -Fructofuranosidase (Exo-inulase)-hyperproducing Mutant in Continuous Culture of *Kluyveromyces fragilis*

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A wild-type strain of the yeast *Kluyveromyces fragilis* was grown in continuous culture on inulin-, fructose- and glucose-limited media. In the presence of organic nitrogen, all three carbon sources supported an extremely rapid changeover to a homogeneous mutant cell population that exhibited hyperproduction of β -fructofuranosidase (exo-inulase). The non-specific role of the carbon substrate in the takeover suggested co-regulation of inulase synthesis and early glycolytic pathway enzymes such as hexokinase, resulting in gratuitous hyperproduction of inulase. The rate of mutant appearance increased linearly with a decrease in dilution rate as well as carbon/nitrogen ratio, implying that the responsible forces were related to the residual concentration of the limiting carbon substrate. Under optimum conditions, mutant cells were detected within only 40 h (2.9 generations) from the start of continuous culture, at least 6.5 times faster than predicted. The maximum rate of displacement was as high as 54% per generation, or more than 12 times greater than predicted from the differential growth rate between the wild-type and mutant strain. These extraordinarily rapid takeovers point to a very fast and ongoing genetic change triggered by growth under severe stress.

INTRODUCTION

Continuous culture represents a powerful technique for the selection of desirable microbial strains from mixed cultures or of mutants from monocultures (Harder *et al.*, 1977; Dykhuizen & Hartl, 1983). Mutant selections can be directed by manipulating environmental conditions, in particular carbon substrate composition and concentration. Nutrient-limited continuous cultures have produced fitter strains with either higher levels of substrate-capturing or growth-limiting enzymes, or alternatively, enzymes with structural changes resulting in improved catalytic properties, such as greater substrate affinity and shifts in pH and temperature optima. Multiple forms of mutations participate in conferring the genetic adaptation. Thus, the *Escherichia coli* mutant cells that dominated a lactose-limited continuous culture within 30 generations contained 20% of cellular protein as β -galactosidase due to a transition from inducible to constitutive control supplemented by tandem gene duplication (Horiuchi *et al.*, 1962). Growth of a strain of *Saccharomyces cerevisiae* over 1000 generations in glycerophosphate-limited culture yielded mutants with increased levels of a structurally modified acid phosphatase; genetic analysis showed that gene duplication through transposition of the phosphatase gene to a different chromosome and a mutation in a repressor gene had occurred (Francis & Hansche, 1972; Hansche *et al.*, 1978; Lange & Hansche, 1980).

Using continuous culture to avoid catabolite repression, high titres of the non-specific β -D-fructofuranosidase (exo-inulase; EC 3.2.1.26) were produced by a strain of *Kluyveromyces fragilis* on the inducing substrates inulin (a fructan of 35 residues), sucrose and fructose

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(GrootWassink & Hewitt, 1983). However, the population seemed unstable, resulting in hysteresis effects on fructose and appearance of a mutant cell population on fructose/galactose mixtures. A representative mutant clone showed stable production of enhanced levels of inulase (up to ~7.5% of dry cell wt) on a much wider range of carbon substrates in batch and continuous culture (GrootWassink & Hewitt, 1983; Lam & GrootWassink, 1985).

In this paper we examine the role of some cultivation conditions in the appearance of the inulase-hyperproducing mutants in continuous cultures of *K. fragilis*.

METHODS

Yeast strains. The homothallic diploid *Kluyveromyces fragilis* (*Kluyveromyces marxianus* var. *marxianus*; Kreger-van Rij, 1984) ATCC 12424 (wild-type) and its mutant ATCC 52466 (inulase hyperproducer; previously PRL Y53A, GrootWassink & Hewitt, 1983) were used. Stock cultures were grown on slants containing 5 g yeast extract l⁻¹, 20 g glucose l⁻¹ and 20 g Bacto-agar l⁻¹ (10 ml medium), and stored at 4 °C under glycerol. Water-suspended cells from freshly grown agar slant cultures were used as inoculum for liquid media at a ratio of one slant in 1 litre.

Fermenter cultures. Cultures of approximately 2.5 l working volume were grown continuously at 30 °C in a 7.5 l bench-top fermenter (Microferm, New Brunswick). Medium was pumped into the fermenter from a medium sterilizer (Nutrient Vessel, New Brunswick). Spent culture and air left the fermenter through a fixed overflow tube. Aeration was 1–2 l min⁻¹ and agitation 300–500 r.p.m., ensuring a dissolved oxygen tension above 20% of saturation (GrootWassink & Hewitt, 1983). The pH was maintained at 4.5 by automatic addition of HCl or NaOH (4 M). Continuous cultivation at a fixed dilution rate began after initial batch growth for approximately 20 h. Samples were taken aseptically throughout the course of the fermentation by collecting 20–30 ml of spent culture in containers cooled on ice.

The media used in the continuous cultures contained yeast extract (Difco), Yeast Nitrogen Base without amino acids (Difco) and vitamin-free Casamino acids (Difco) as sources of nitrogen, minerals and vitamins. Inulin, fructose and glucose (Sigma) were used as carbon sources. Concentrations of the various components are detailed in the Figure legends.

Analyses. Biomass levels were monitored by microfiltration (0.45 µm, Millipore) of culture samples, washing the resultant yeast mat twice with 5–10 ml deionized water and determining its dry wt after freeze-drying.

Total inulase (cell-associated plus soluble) was assayed by incubating diluted whole culture samples (0.1 ml) with 4% (w/v) sucrose (final concentration) in 0.1 M-sodium acetate buffer, pH 5.0 (0.9 ml) at 50 °C. After 15 min, the reaction was quenched by 5-fold dilution with 0.4 M-Tris/HCl, pH 7.2. The amount of glucose liberated during incubation was determined with the Statzyme glucose kit (Worthington) using a Clinical Chemistry Analyzer (System 203S, Gilford). Inulase activities are expressed as U mg⁻¹, defined as µmol sucrose hydrolysed min⁻¹ (mg dry cell wt)⁻¹. Sucrose was used as the assay substrate since it offered greater convenience and accuracy than inulin (GrootWassink & Hewitt, 1983).

Wild-type and inulase-hyperproducing mutant cells were differentiated and enumerated by plating on glycerol agar. Visual detection was effected by exposure of the colonies to a mixture of sucrose and a chromogenic reagent for glucose. Distinctive red zones developed readily around mutant colonies. The method was described in detail by Tsang & GrootWassink (1985).

RESULTS

Mutant appearance on different carbon sources

It is expected that during growth on inulin a culture will seek to obtain the maximum benefit from the carbon and energy source by maximizing inulase activity. To reach this state, however, the normal physiological adaptation expected during the initial growth phase in continuous cultures of wild-type *K. fragilis* was quickly superseded by a genetic change in the cell population (Fig. 1). The inulase content closely followed this change, stabilizing at 68 U mg⁻¹ after 34 generations when the original inducible wild-type cells had been completely displaced by mutant cells with a hyperproducing phenotype. Cloning and subculturing of many single cell isolates revealed homogeneity and genetic stability of the new population under carbon and nitrogen limitation. The appearance of mutant cells was apparently not due to a growth yield advantage as the biomass level remained constant throughout the entire cultivation period.

To verify the role of inulase activity in directing the selection, a culture was grown on fructose, the predominant degradation product of inulin. On this substrate inulase formation is induced

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Appearance of mutant cells in inulin-limited continuous culture of wild-type *K. fragilis*. The culture was grown on 0.5% yeast extract and 1% inulin at a dilution rate of 0.05 h⁻¹. ●, Inulase content; ○, Percentage of mutant cells; ■, Dry wt (upper panel), dry wt (lower panel).

Fig. 2. Appearance of mutant cells in fructose-limited continuous culture. The culture was grown on 0.5% yeast extract and 1% fructose at a dilution rate of 0.05 h⁻¹. Symbols, see Fig. 1.

Fig. 3. Appearance of mutant cells in glucose-limited continuous culture. The culture was grown on 0.5% yeast extract and 1% glucose at a dilution rate of 0.05 h⁻¹. Symbols, see Fig. 1.

Fig. 4. Stability of wild-type *K. fragilis* on a defined medium. The culture was grown on 0.4% Yeast Nitrogen Base and 1% fructose at a dilution rate of 0.05 h^{-1} . Symbols, see Fig. 1.

(GrootWassink & Hewitt, 1983), but the apparent redundancy of the enzyme should obviate any need for hyperproduction and therefore genetic adaptation, in contrast to the situation on inulin. However, the results were quite incompatible with this notion since the appearance of mutant cells was much more rapid and pronounced on fructose than on inulin (Fig. 2). Complete displacement of the wild-type cells was effected within 15 generations from the start of continuous culture, with the mutant cell population again yielding 68 U mg^{-1} inulase. The takeover was so rapid it only took about 7 generations from the time the first mutant cells were detected to completion.

Glucose also does not require inulase for its utilization, but nevertheless supports the formation of a low level ($\sim 5 \text{ U mg}^{-1}$) of enzyme in continuous culture (GrootWassink & Hewitt, 1983). In previous experiments at dilution rates above 0.085 h^{-1} glucose did not evoke any irregular inulase profiles, implying culture stability. In this study, extended cultivation at 0.05 h^{-1} , however, gave a different result (Fig. 3). An apparent steady state was established between 4 and 10.5 volume exchanges, after which an abrupt change in culture composition caused a dramatic increase in the inulase level. The final concentration of 40 U mg^{-1} was only 60% of those observed above on inulin and fructose. This difference was not due to the appearance of a less productive cell population on glucose since testing of a representative number of mutant cell clones isolated from the various carbon sources did not reveal any variability in production characteristics. The lower enzyme yield was probably due to the weak inducing capacity of glucose in continuous culture. The significance of the slight drop in biomass level associated with the population changeover is not clear. The trend suggests that the mutant cell population had a lower specific growth yield, perhaps due to the tremendous waste of energy associated with overproducing inulase (an estimated extra 4.3% of dry cell wt) and some other enzymes. It shows that the takeover was definitely not based on improved efficiency in carbon use.

Mutant appearance on defined media

To determine the importance of the nitrogen source in the appearance of mutant cells, a culture was grown on a defined medium containing ammonium sulphate (Yeast Nitrogen Base) plus fructose. The defined medium was designed to contain excess nitrogen, incorporating 25% more than present in the above carbon-limited media with yeast extract. No mutant cells could be detected for at least 28 generations (Fig. 4). Intermediate levels of inulase ($\sim 20 \text{ U mg}^{-1}$) were produced. Supplementation of the same medium with Casamino acids (0.4%) restored the rate

Fig. 5

Fig. 5. Appearance of mutant cells in continuous culture at 0.025 h^{-1} . The culture was grown on 0.5% yeast extract and 1% fructose. Symbols, see Fig. 1.

Fig. 6

Fig. 6. Appearance of mutant cells in continuous culture at 0.1 h^{-1} . The culture was grown on 0.5% yeast extract and 1% fructose. Symbols, see Fig. 1.

of appearance to values similar to those observed before with yeast extract (data not shown). Inulase levels also returned to 68 U mg^{-1} . Thus, it appears that the accumulation of mutant cells was dependent on the availability of an organic nitrogen source to exert maximum pressure on the carbon supply.

Effect of dilution rate on mutant appearance

A decrease in dilution rate from 0.1 h^{-1} to 0.025 h^{-1} increased the maximum rate of mutant cell appearance from 12 to 54% per generation, and concomitantly reduced the mutant detection lag from 14 to 5.5 generations (Fig. 5, reference Fig. 2, and Fig. 6). Since the residual substrate concentration is lower at low dilution rate, the results suggested that the selection pressure was determined by the residual carbon concentration, a measure of the restriction in the supply.

Effect of carbon/nitrogen ratio on mutant appearance

Although Monod kinetics imply that the residual concentration of a growth-limiting carbon substrate is independent of the concentration of the nitrogen source in the feed medium, the use of a complex medium might cause a deviation. Secondly, a complex nitrogen source like yeast extract which also provides the minerals, vitamins and a small amount of carbon might, depending on its concentration, change the overall physiology and growth characteristics of the organism. Therefore, appearance of mutant cells was compared at various fructose/yeast extract (F/Y) ratios, in lieu of carbon/nitrogen ratios, by keeping the fructose concentration constant and changing that of the yeast extract. Decreasing the F/Y ratio from 4 to 1 resulted in a reduction of the mutant detection lag from 11 to 2.9 generations and in a maximum rate of

Fig. 7

Fig. 8

Fig. 7. Appearance of mutant cells in continuous culture at a low carbon/nitrogen ratio. The culture was grown on 1% fructose and 1% yeast extract (F/Y ratio = 1) at 0.05 h⁻¹. Symbols, see Fig. 1.

Fig. 8. Appearance of mutant cells in continuous culture at a high carbon/nitrogen ratio. The culture was grown on 1% fructose and 0.25% yeast extract (F/Y ratio = 4) at 0.05 h⁻¹. Symbols, see Fig. 1.

appearance of 45% per generation (Fig. 7, reference Fig. 2, and Fig. 8). It was surprising that the appearance was still very strong at an F/Y ratio of 4, as the lower biomass and the lower inulase yield indicated a shift in the medium from carbon to nitrogen limitation. This suggested that physical restriction in the carbon supply is not an exclusive requirement for mutant appearance, at least not in the case of fructose. Perhaps the culture can create, in a qualitative sense, its own carbon limitation by diverting readily metabolizable fructose to slowly metabolizable byproducts such as glycerol (GrootWassink & Hewitt, 1983). The overall results showed that the pressure towards mutant accumulation was definitely affected by the concentration of a complex nitrogen source, even with carbon limitation.

Effect of cell density on mutant appearance

The accumulation of microbial metabolites in culture liquids can also cause deviations from Monod kinetics because of a change in the specific growth yield. Consequently, the course and outcome of mixed culture and mutant appearance can be greatly affected. Assuming that metabolite concentrations are related to cell densities, time-courses of mutant appearance were compared at various concentrations of the feed medium, F/Y ratio of 2. Increasing the medium concentration from 0.5 to 2% fructose gave a roughly proportional increase in the cell density, i.e. a biomass yield from 2.8 to 10.8 mg ml⁻¹ corresponding to 4 to 14 × 10⁸ c.f.u. ml⁻¹ (Fig. 9, reference Fig. 2, and Fig. 10). This increase was responsible for a reduction in the mutant detection lag from 10 to 4 generations, and a slight increase in the maximum rate of appearance from 31 to 39% per generation. The results showed that higher cell densities might not have any effect on the specific growth yield but nevertheless stimulate accumulation of the mutant.

Fig. 9

Fig. 10

Fig. 9. Appearance of mutant cells in continuous culture at low cell density. The culture was grown on 0.25% yeast extract and 0.5% fructose at 0.05 h⁻¹. Symbols, see Fig. 1.

Fig. 10. Appearance of mutant cells in continuous culture at high cell density. The culture was grown on 1% yeast extract and 2% fructose at 0.05 h⁻¹. Symbols, see Fig. 1.

DISCUSSION

The clonal isolates exhibiting the inulase-hyperproducing phenotype have been termed mutants since they were found to be completely stable during batch culture on a range of carbon substrates, including inulin, sucrose, fructose, glucose, galactose, lactose, glycerol, lactate and ethanol. Except for carbon-limited growth on lactose (Tsang & GrootWassink, 1988), they were also stable in continuous culture. A representative inulase-hyperproducing mutant strain, *K. fragilis* ATCC 52466, has been used in several regulation and production studies (GrootWassink & Hewitt, 1983; Hewitt & GrootWassink, 1984; Lam & GrootWassink, 1985).

Contrary to expectations, the appearance of inulase-hyperproducing mutant cells in carbon-limited continuous culture of *K. fragilis* was elicited not only by the substrate inulin but also by fructose and glucose. Thus, it appears that the genetic adaptation constituted a general response to a lack of carbon, in particular to those carbon sources that support inulase formation in the wild-type. Other carbon sources such as lactose, galactose, ethanol and lactate that do not support inulase formation in the wild-type in continuous culture, also have never elicited appearance of the mutant. These carbon sources, however, support excellent and stable production of inulase in continuous culture if inoculated with a hyperproducing mutant strain (GrootWassink & Hewitt, 1983). These combined observations suggest that the appearance of the inulase-hyperproducing cells was brought about indirectly, probably through a regulatory mechanism that couples inulase formation to that of transport proteins and/or early glycolytic pathway enzymes of key importance in fructose and glucose catabolism. Hexokinase might be a good candidate enzyme since its levels were consistently higher (up to ~8-fold) in an inulase-hyperproducing mutant compared to the wild-type, when tested in batch culture on various fermentable and non-fermentable carbon sources (unpublished results). This phenomenon of co-regulation occurs widely, most clearly with enzymes of distinct metabolic pathways encoded by operons. Others, however, are unlinked and unrelated. For example in *E. coli*, three unknown periplasmic proteins are co-regulated with alkaline phosphatase, together constituting over 10%

of total cell protein in derepressed wild-type cells (Morris *et al.*, 1974). In yeast, synthesis of melibiase (α -galactosidase) and galactose catabolic enzymes is co-induced (Kew & Douglas, 1976) through the sharing of regulatory elements, termed a regulon (Johnston & Hopper, 1982). Similarly, lactose or galactose induces the expression of the lactose-galactose regulon (Wray *et al.*, 1987). High levels of invertase and maltase are produced simultaneously in regulatory mutants of yeast resistant to carbon catabolite repression (Zimmermann & Scheel, 1977).

The cell populations selected on the various substrates were identical with respect to the inulase-hyperproducing phenotype. In fact, cell clones showed no difference from the mutant *K. fragilis* ATCC 52466, previously isolated in this laboratory from a continuous culture on a mixture of fructose and galactose (GrootWassink & Hewitt, 1983). Thus, the same forces leading to their appearance must have prevailed under all continuous culture conditions.

Since the displacement appeared to result in a homogeneous mutant population, it may be assumed that only two competing strains were dominating the cultures at any one time, simplifying an attempt to interpret the rates of mutant appearance based on selection alone. It may be further assumed that the accumulation of the hyperproducing mutant cells proceeded according to the relationship $R_t = R_0 e^{st}$ where R_t is the ratio of the number of mutant to wild-type cells after selection at time t , R_0 is the same ratio at the beginning of selection, and s is the selection rate representing the differential growth rate. This model appears to be appropriate for the present situation as, in our experience, during this and other studies there have been no indications of interaction between the two strains other than the competition for the same limiting substrate. Also no preferential washing-out from the fermenter due to clumping or wall growth of one of the strains has been observed, a prerequisite for the validity of the model (Dykhuizen & Hartl, 1983). The selection rate, s , is obtained by plotting $\ln R_t$ at various time intervals during the course of selection as a function of time, and determining the slope. Transformation of the experimental data for selections at various dilution rates, carbon/nitrogen ratios and cell densities, however, did not yield straight lines but biphasic curves with the inflection at around $\ln R_t = 0$, i.e. when the two cell populations were equal in number (Figs 11*a*, 12*a* and 13*a*). Since the second part of the curves comprised more data points, their slopes were used to calculate the selection rate. Plots of the rates against fermentation parameters showed a negative linear relationship with dilution rate (Fig. 11*b*) and the carbon/nitrogen ratio (Fig. 12*b*). No clear effect of cell density was discernible (Fig. 13*b*).

In the case of dilution rate, its effect is more pronounced if the selection rate is expressed per generation [calculated as $(\ln 2)/D$], i.e. around $1.8 \text{ generation}^{-1}$ at $D = 0.025 \text{ h}^{-1}$ and $0.14 \text{ generation}^{-1}$ at $D = 0.1 \text{ h}^{-1}$. The finding contrasts strongly with the view sometimes held that the selection rate is independent of generation time (Dykhuizen & Hartl, 1983). However, if it is reasoned that the competitiveness between the two strains lies in their capacity to assimilate the growth-limiting substrate, the dilution rate (generation time) should have a major effect as it sets the residual concentration of the substrate in continuous culture. If organic nitrogen is used, the effect of the carbon/nitrogen ratio may be interpreted along the same lines: the greater the relative nitrogen concentration the greater the demand for carbon thus decreasing the residual concentration, which in turn results in an increased selection rate. According to the Monod model, residual substrate concentration is not affected by cell density, and therefore no effect on the selection rate would be expected.

The selection rate relevant to any particular state of continuous culture is a measure of the difference between the specific growth rates of the two strains. Theoretically, this difference can be calculated using the Monod equation. For this, the kinetic parameters $\mu_{\max}$ (maximum specific growth rate) and K_s (saturation constant) were estimated from batch cultures grown in yeast extract/fructose. The $\mu_{\max}$ of the wild-type and the inulase-hyperproducing mutant were very similar at $\sim 0.43 \text{ h}^{-1}$, while the K_s was 0.016 g l^{-1} for the wild-type and 0.011 g l^{-1} for the mutant (unpublished data). At $D = \mu = 0.05 \text{ h}^{-1}$, the corresponding residual substrate concentration for the wild-type culture at steady state would be 0.0021 g l^{-1} , which translates into a specific growth rate of 0.069 h^{-1} for the mutant. Therefore, at very early stages of selection when the wild-type culture would not as yet have been affected in any practical way, the mutant would grow 1.38 times as fast as the wild-type, exhibiting a selection rate of 0.019 h^{-1} . If the

Fig. 11

Fig. 12

Fig. 13

Fig. 11. Effect of dilution rate on (a) the course of appearance of mutant cells and (b) the rate of mutant appearance. Dilution rates (a) were 0.025 h⁻¹ (●), 0.05 h⁻¹ (■) and 0.1 h⁻¹ (▲). Data from Figs 2, 5 and 6.

Fig. 12. Effect of carbon/nitrogen ratio on (a) the course of appearance of mutant cells and (b) the rate of appearance. F/Y ratios (a) were 4 (●), 2 (■) and 1 (▲). Data from Figs 2, 7 and 8.

Fig. 13. Effect of cell density on (a) the course of appearance of mutant cells and (b) the rate of appearance. Cell densities (a) were 4 × 10⁸ (●), 8 × 10⁸ (■) and 14 × 10⁸ (▲) c.f.u. ml⁻¹. Data from Figs 2, 9 and 10.

selection with the F/Y ratio of 1 and the dilution rate of 0.05 h⁻¹ (Fig. 12) is singled out for verification, large differences come to light between the experimental and calculated selection rate. The experimental rate was roughly twelve times greater during the first phase and over three times greater during the second phase. Furthermore, if it is assumed that the mutant appeared, although undetectably, immediately after the initiation of continuous culture, it can be calculated from the above selection equation using $s = 0.019 \text{ h}^{-1}$ that it would take 268 h for the mutant to accumulate from a frequency of 10^{-4} to the detectable level of 1.6×10^{-2} , while in fact it only took 40 h (2.9 generations) (Fig. 12). The initial frequency of mutants was probably much lower than 10^{-4} but this figure represents the upper limit since not a single mutant clone was detected within a control population of 10000 wild-type clones scored over the course of this study. If indeed the initial frequency was much closer to that classically cited for spontaneous chromosome mutations, such as 10^{-6} , the mutant detection lag would theoretically increase to around 500 h.

The overall results point to an extraordinarily fast displacement of the wild-type cell populations in the continuous cultures, a phenomenon that appears to require the participation of processes other than mere selection due to growth rate differentials between the competing strains. We propose the presence of mutation mechanisms that are activated under extremely stressful conditions such as growth at a fructose concentration of only 0.0021 g l⁻¹ (see above). They appear capable of increasing the mutation frequency to levels that have a major impact on

the rate of strain displacement. In fact, we have observed displacement rates of over 50% per generation, suggesting each new genome produced was of the mutant type. A genetic switch similar to the mating type interconversion in homothallic *S. cerevisiae* with transposition of mating type cassettes at up to 50% per cell per generation (Herskowitz & Oshima, 1981) might be responsible. High-frequency genetic changes did not seem to be plasmid related as we have been unable to detect these elements in *K. fragilis* using agarose gel electrophoresis.

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