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Acetophenone tolerance, chemical adaptation, and residual bioreductive capacity of non-fermenting baker's yeast (*Saccharomyces cerevisiae*) during sequential reactor cycles

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Bioreduction of acetophenone (ACP) to phenethyl alcohol (PEA) by baker's yeast (*Saccharomyces cerevisiae*), which is highly enantioselective, can be carried out entirely in a resting state using stored carbohydrate, suggesting that a high degree of chemical tolerance might be possible. However, viability and catalytic activity of precultured cells decline steeply within 24 h at initial ACP concentrations >0.2% (17 mM). Viability of cells at 0.4% ACP was 1/4 the viability at 0.2% ACP as determined by vital staining, and <1% based on colony-forming ability. This sensitivity was observed in suspensions with a cell content of nearly 30% (w/v). Longterm PEA production is strongly dependent on viability, indicating that the cumulative yield per batch of cells is maximized by maintaining a very low concentration of substrate (~0.2%). However, nonviable cells (CFU ml^{-1} <1% cells ml^{-1}) can achieve PEA yields up to 1/3 the maximum, an amount representing initial absorption of ACP without further uptake. Regarding population adaptability, when cells surviving the most selective (toxic) concentration of ACP (0.6%) were subcultured in an ACP-free medium and re-reacted, the 24-h percent viabilities (vital staining) and colony-forming frequencies exceeded those of non-selected cells. However, the surviving cells represented only a small fraction (~1%) of the recultured progeny. Even at ACP concentrations as low as 0.25% (w/v), surviving cells were unreliable in transmitting and maintaining ACP-tolerance. In addition, there was no evidence that the chemical yield of recultured ACP-tolerant cells (amount of PEA relative to initial amount of ACP) can consistently exceed the maximum yield of an equivalent density of previously unreacted (non-selected) cells. These results indicate that over a broad range of substrate concentrations, rapid replacement of cells may be more cost-effective than maintenance or reuse of viable cells.

Keywords: acetophenone; baker's yeast; bioreduction; chemical tolerance; phenethyl alcohol; *Saccharomyces cerevisiae*

Introduction

There has been much interest in use of whole cells of baker's yeast for enantioselective reduction of carbonyl compounds [1,2,4,5,7–9,17,20–22,25,27]. Whole cells provide a diversity of enzymes and activated cofactors at a lower expense than purified enzymes to which cofactors must be added [26]. Substrates such as aromatic ketones, which can be reduced in sugar-free solutions using cell-stored carbohydrates, offer the best opportunities to optimize efficiency of whole-cell reactors, for several reasons. The absence of sugar in the medium prevents unnecessary diversion of reducing power to ATP and biomass production, and inhibits buildup of fermentative waste products [2,4,13,14], helping to maximize duration, efficiency, and homogeneity of product recovery. Because cells do not need to be maintained in a physiological state supporting continual fermentation, a significant amount of bioreduction might be sustainable at toxic concentrations of substrate or reduced levels of viability. Substrate tolerance would also facilitate reuse of cells, depending on requirements for cell reactivation. This would be advantageous if substrate tolerance is achieved without a decrease in catalytic activity.

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In this paper, we investigate viability thresholds, catalytic activity, and reusability of baker's yeast reacted in sugar-free aqueous solutions containing different concentrations of acetophenone (ACP), solubilized with ethanol. The bulky phenyl group of acetophenone provides a challenging conversion problem [10] which may be solved by exploiting the diversity of enzymes in whole cells, and provides a model for evaluating the efficiency of microbial production of chiral secondary alcohols used as building blocks in chiral synthesis. The product of acetophenone reduction, phenethyl alcohol (PEA), is an important precursor in flavorings and drug synthesis. Baker's yeast is a favorable microbial agent because of simple growth requirements, population stability and uniformity, and inexpensive commercial availability of cells. Enantiomeric excess (S form) of phenethyl alcohol produced by reduction of acetophenone by baker's yeast has been as high as 90% [17] and 95% [1] for fermenting yeast, and 95% for non-fermenting yeast [28].

We found in preliminary experiments that consecutive additions of 2 ml ACP L^{-1} reactor solution spaced over several days resulted in more prolonged production and higher total yields of phenethyl alcohol than a single addition of 6 ml L^{-1}. This suggested that acetophenone becomes toxic or repressive when its immediate concentration exceeds 0.2%, and that chemical yield may depend on maintaining a certain level of cell viability. Here we evaluate the significance of maintaining cell viability, the correlation of cell

viability and bioreduction, and the maximum tolerance and adaptability of cell populations to increasing substrate concentrations. The questions our data will address are:

(1) Without prior reaction, what proportion of yeast cells remain viable during the period of significant PEA production at 0.2% ACP and higher concentrations? Viability is defined at two levels: number ml^{-1} of cells capable of replication, as indicated by colony formation, and percent of cells with residual metabolic activity, as indicated by vital staining.
(2) As a reaction proceeds, what are the quantitative relationships of chemical yield and duration of PEA production to cells ml^{-1} and percentage of viable cells? Over what range of values are relationships linear? What is the maximum proportion of total PEA yield that can be achieved by nonviable cells, ie, cells no longer capable of replication?
(3) Once a reaction has essentially gone to completion, what is the maximum yield of PEA that can be achieved by reacted cells transferred to fresh solutions of ACP without reculture?
(4) Do viability levels of recultured progeny of previously reacted cells exceed the viability levels of initial populations when reacted at equivalent or higher concentrations of ACP, suggesting physiological habituation or cell selection?
(5) Over a range of ACP concentrations, to what extent can the maximum PEA yield of recultured survivors equal or exceed that of an equivalent density of cells not previously exposed to ACP? This needs to be determined since ACP tolerance may be achieved by increasing the rate of conversion of ACP to PEA (assuming PEA is less toxic than ACP), by excluding ACP, or by converting ACP to products other than PEA.
(6) Can tolerance limits or chemical yield be raised by exposure of yeast populations to progressively higher concentrations of ACP? If so, do the selected cell lines maintain tolerance limits or bioreductive capacity when recultured and re-reacted with ACP? The latter questions were motivated by studies of population adaptation to added ethanol [6,11,15]. Our results are of general interest since previous studies have been concerned with chemical adaptability of rapidly fermenting populations in which membrane dynamics and cell turnover may be quite different from non-fermenting populations.

Methods

Preparation of yeast

Dried yeast (Sigma Type II *Saccharomyces cerevisiae*, Sigma Chemical Co, St Louis, MO, USA) was precultured in a nutrient medium containing 2% glucose, 2% peptone, and 1% yeast extract in distilled water. The medium was prepared by autoclaving 10 g of peptone and 5 g of yeast extract in 450 ml distilled water, and adding sterile glucose solution to yield 2% glucose. Dried yeast (10% w/v) was then added to the complete medium at room temperature. Suspensions in half-filled 250-ml flasks stoppered with a foam plug were shaken at 200 rpm at 30°C for 48 h. For pure cultures, suspensions were grown from isolated colonies from previously activated cultures, rather than directly from dried yeast.

Preparation of reaction suspensions

Cells were centrifuged in sterile tubes and cell pellets were washed with sterile water, and recentrifuged. Washed cells were added to solutions of ACP and ethanol in water. Solutions were prepared by mixing ACP and ethanol in a 1:2 ratio, and adding the mixture to water. This was necessary to solubilize the acetophenone which has a solubility in water of 0.7% [24]. Stock solutions of ACP and ethanol were not sterilized, but all solutions were prepared using sterile water and added to sterile vessels. Reactions were carried out at 30°C in 125-ml or 250-ml flasks shaken at 200 rpm. Flasks were rubber-stoppered to prevent loss of volatile ACP during shaking.

Approximately 10 g of wet yeast were added per 30 ml of reaction solution, an initial cell density of $\sim 5 \times 10^6$ ml^{-1}. In computing the amount of ACP needed to achieve a given concentration, it was assumed that the water content of the pelleted yeast paste was 55% by weight, a value determined in previous experiments.

Viability determinations

(a) Abundance of colony-forming units: Colony-forming units ml^{-1} were estimated by plating samples from serial 10-fold dilutions on Sabouraud dextrose agar. *Saccharomyces* colonies were evident by characteristic colony appearance and color. Cells from representative colonies were nevertheless gram-stained to verify the identity of the predominant colonies.

(b) Percent viable cells in suspension based on vital staining: In a preliminary experiment percent viability was determined using methylene blue [15]. Cells which remained colorless were scored as viable. In subsequent experiments, yeast were stained using the LIVE/DEAD FungoLight Viability Kit (Molecular Probes, Eugene, OR, USA). Comparisons of batches of cells stained by these methods showed that mean differences in percent viability were very similar, although absolute percentages were higher and more variable using methylene blue.

For samples containing $>10^6$ cells ml^{-1}, viable and nonviable cells were counted in 10 randomly-selected squares in the central grid of a hemocytometer. On average, approximately 200 cells were counted. For samples in which cell density was substantially depleted (0.4–0.6% ACP treatments after 24 h), cells were counted in all 25 squares of the central grid.

Measurement of acetophenone and phenethyl alcohol

HPLC (Dionex AI 4500) was used to measure concentrations of ACP and PEA. Well-mixed samples were removed from flasks with a 1-ml syringe and extracted with 3 ml of chloroform in a separatory funnel. Chloroform extracts were diluted 10:1 with methanol, the mobile phase solvent. The analytical column was ZORBAX ODS (Mac

Mod Analytical Co, PA, USA). Flow rate was 1 ml min^{-1}. Peaks were detected using a wavelength of 215 nm.

The efficiency of chloroform in extracting ACP and PEA from reactor solutions was verified experimentally. Peak areas of representative concentrations of ACP and PEA dissolved directly in chloroform were nearly identical to peak areas of the same concentrations of ACP and PEA well-mixed in water and extracted with chloroform. Differences in peak areas for ACP and PEA were both about 1%.

The efficiency of chloroform in extracting residual ACP from cells was determined in an experiment in which 6 ml of ACP was mixed into a suspension containing 1 L of water and 100 g yeast (dry weight), providing an initial theoretical concentration of ACP of 49.9 mmol. After 1 h of mixing (a period too short for significant PEA production), non-centrifuged and centrifuged (cell-free) samples were extracted with chloroform. The concentration of ACP in the extract of the centrifuged sample was 38.3 mmol. The concentration of ACP in the extract of the non-centrifuged sample was 49.1 mmol. This indicates that it is possible to extract almost all of the ACP trapped in cells.

Reproducibility of estimates of the ACP and PEA concentrations was 1.3–1.8% and 1.6–2.5%, respectively, based on analysis of multiple samples of concentrations of ACP, PEA, and yeast typical of experimental reactor flasks.

Results

(A) Effect of ACP on maintenance of cell viability

(1) ACP tolerance of previously unreacted yeast

Colony-forming units ml^{-1}: As shown in Table 1, ACP had very little effect on colony-forming units ml^{-1} after 1 h. The only discernible effect of substrate concentration was a slight drop in CFU ml^{-1} in solutions containing $\geqslant$0.5% ACP. After 24 h, however, colony production was sharply reduced at concentrations $>$0.3% ACP.

Vital staining: After 1 h, percent viability judged by fluorescence was more sensitive to ACP than when judged by colony counts (Table 1). Each measure of viability was carried out on the same sample of yeast. Small but mostly non-significant reductions in percent viability were evident at each 0.1% increment of ACP. After 24 h, much more significant reductions in viability were evident at each 0.1% increment of ACP. The steepest reductions were between 0.2% and 0.4% ACP, consistent with the steep colony-count reduction.

Based on vital staining no viable cells were observed in samples from flasks containing 0.6% ACP, as opposed to occasional colony-forming units. This may simply be a statistical effect of smaller sample size. Smaller samples of cell suspensions were used for hemocytometer counts of stained cells than volumes used for preparing dilutions for plating. On the other hand, in flasks containing 0.4–0.6% ACP, the reduction in colony-forming units after 24 h (over a thousand-fold) was much more severe than the reduction in percent viability based on vital staining, suggesting a significant residual population of 'living' but non-replicatable cells.

(2) ACP tolerance of recultured cells

Cells exposed to low concentrations of ACP: In a preliminary adaptation experiment, yeast which had been shaken in 0.2% ACP for 95 h were diluted and plated on Sabouraud dextrose agar. Four colonies were streaked for isolation. Material from isolated colonies from each plate was grown in separate flasks of preculture medium for 48 h. Pellets from the preculture were then transferred to the slightly higher concentration of 0.25% ACP and shaken for 94 h. Cell pellets from the four preculture flasks were combined into two reaction flasks. This provided approximately the same initial cell density as in reactor experiments. Percent viability by methylene blue staining [15] and PEA production were determined at 1, 24, 48, and 94 h. One group of cells experienced only 11% loss of viability throughout the 94-h period, whereas the second group experienced approximately a 50% loss in viability, mostly between 24 and 48 h. This indicates that even at relatively low ACP concentrations, ACP-tolerant cells may vary in adaptation to ACP.

Cells exposed to high concentrations of ACP: Cells from five colonies representing 24-h survivors of a 0.6% solution were grown separately in nutrient-rich preculture

Table 1 Effect of acetophenone (ACP) concentration on viability of previously unreacted, precultured yeast after 1 and 24 h

Initial conc. ACP		Colony-forming units ml^{-1} (mean ± SD)			% Viability based on staining (mean ± SD)		
% (v/v)	mM	1 h		24 h	1 h		24 h
0.0	0.0	$(5.78 \pm 0.16) \times 10^6$	$>$	$(5.01 \pm 0.17) \times 10^6$	96.7 ± 1.5	$>$	78.7 ± 5.0
0.1	8.3	## $\underline{(4.59 \pm 0.38) \times 10^6}$		$(4.82 \pm 0.15) \times 10^6$	# $\underline{91.3 \pm 4.0}$	$>$	# $\underline{67.0 \pm 6.0}$
0.2	16.7	## $(4.56 \pm 0.29) \times 10^6$	$>$	## $\underline{(3.83 \pm 0.25) \times 10^6}$	## 88.3 ± 3.5	$>$	## $\underline{58.3 \pm 5.0}$
0.3	25.0	# $(4.52 \pm 0.58) \times 10^6$		## $(3.93 \pm 0.22) \times 10^6$	## $\underline{82.7 \pm 2.5}$	$>$	## $\underline{36.3 \pm 6.7}$
0.4	33.3	$(5.08 \pm 0.81) \times 10^6$	$>$	## $\underline{(6.53 \pm 1.88) \times 10^3}$	## 79.0 ± 2.6	$>$	## $\underline{15.3 \pm 4.2}$
0.5	41.7	## $\underline{(3.80 \pm 0.12) \times 10^6}$	$>$	## $\underline{(0.63 \pm 0.23) \times 10^3}$	## 77.3 ± 2.1	$>$	## $\underline{5.7 \pm 1.5}$
0.6	49.9	## $(2.85 \pm 1.47) \times 10^6$	$>$	## $\underline{<10^2}$	## 78.0 ± 2.6	$>$	## $\underline{0.0}$

Viability estimated by colony counts and by hemocytometer counts of fluorescent-stained cells. Underlined values are significantly lower ($P < 0.05$) than the preceding increment of ACP. Values significantly lower than the ACP-free control are indicated by # ($P < 0.05$) or ## ($P < 0.01$); $>$ indicates a significant reduction in viability ($P < 0.05$) in the same flask between 1 and 24 h.

medium, then resuspended in 0.6% ACP for 24 h, with two replicated treatments per subculture. After 24 h, viability in the 0.6% ACP solution averaged 21%, compared with about 71% in the ACP-free controls (Table 2a). Regarding colony production, although one subculture achieved a 24-h CFU ml^{-1} which was 56–69% that of the controls, in four out of five subcultures, abundance of colony-forming units was 0.5–2.4% of the controls. These results indicate that, on average, only a small fraction of progeny of cells surviving initial exposure to a high concentration of ACP are able to maintain ACP tolerance, even after being recultured. Nevertheless, the ACP tolerance of recultured survivors greatly exceeds that of non-selected cells, which displayed an approximate 10^6 reduction in colony-forming units and a percent viability of zero.

In a second experiment, colonies from cells which survived the second 0.6% ACP solution described above were cultured for 48 h in glucose-peptone-yeast extract and resuspended in 0.8% and 0.9% ACP as well as 0.6% and 0%. The greater toxicity of the 0.8% and 0.9% solutions was immediately evident (Table 2b). After only 1 h, there was a substantial reduction in colony-forming units, which is proportional to the increase in concentration of ACP—ie, after 1 h, an average of 2.7×10^4 and 6×10^3 CFU ml^{-1} were indicated for the 0.8% and 0.9% solutions, respectively. In contrast, the 0.6% solution yielded 3.6×10^6 CFU ml^{-1}, only slightly less than the 0% control, 4.5×10^6, and similar to previous 1-h samples of 0.6% solutions. At 24 h, the 0.8% and 0.9% solutions yielded no colonies, and the 0.6% solution yielded $<10^2$ per ml. The 0% control yielded about the same CFU ml^{-1} as the 1-h control, ie, 4×10^6 CFU ml^{-1}. The near absence of colony-forming cells from the 0.6% solution was surprising since, in the previous experiment, recultured cells which were re-exposed to 0.6% ACP yielded at least 10^4 CFU ml^{-1} after 24 h, a modest degree of habituation. Thus, the degree of genetic or phenotypic habituation is quite variable.

(B) Bioreductive capacity of yeast in relation to initial concentration of ACP, population viability and previous exposure to ACP

Using populations of yeast which were not selected for ACP tolerance, we tested: (1) the effect of ACP concentration on PEA production independent of viability; and (2) whether cell populations reacted at different ACP concentrations retain their ability to produce PEA when recycled without reculture. This was done by measuring PEA production of cells shaken in 0.2%, 0.4%, and 0.6% ACP for 92–94 h, washing the cells, and then resuspending the cells and testing for PEA production in a fresh reaction solution containing a relatively non-toxic concentration of 0.2% ACP. We determined the effect of viability on PEA production using: (1) previously unreacted cells; and (2) recultured survivors of previous reactor cycles (progeny of ACP-tolerant cells). We also investigated whether the PEA yield of progeny of ACP-tolerant cells differs significantly from the PEA yield of previously unexposed cell lines in response to increasing concentrations of ACP.

Effect of ACP concentration and cell viability on PEA yield of initially-reacted cells

The 0.2% ACP treatment had a much higher yield of PEA and a longer period of PEA production than the 0.4% and 0.6% treatments (Figure 1). This suggested that PEA pro-

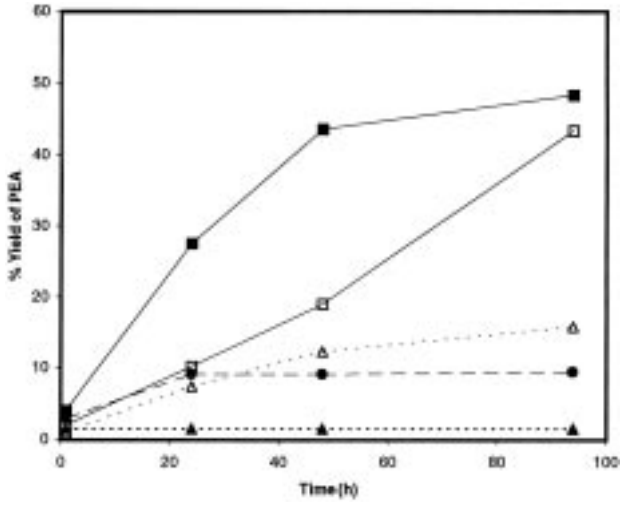

Figure 1 Cumulative field of PEA in relation to initial concentration of ACP. –■– Unreacted cells, 0.2% ACP; –●– unreacted cells, 0.4% ACP; – –▲– – unreacted cells, 0.6% ACP; –□– reacted cells (0.2% ACP), 0.25% ACP 89% viability; – –△– – reacted cells (0.2% ACP), 0.25% ACP 50% viability.

Table 2 Viability of recultured 24-h survivors of reaction solutions containing 0.6% ACP upon resuspension in 0.6–0.9% ACP

Current conc. ACP		Colony-forming units ml^{-1} (mean ± SD)			% Viability based on staining (mean ± SD)		
% (v/v)	mM	1 h		24 h	1 h		24 h
(a). Recultured populations of cells recovered from original reaction solution (0.6%-treatments of Table 1)							
0.0	0.0	$(5.64 \pm 0.23) \times 10^6$	>	$(5.00 \pm 0.14) \times 10^6$	73.5 ± 2.1		71.0 ± 1.4
0.6	49.9	## $(3.87 \pm 0.41) \times 10^6$	>	## $(4.90 \pm 4.02) \times 10^4$	## 52.3 ± 5.6	>	## 21.9 ± 4.8
(b). Cells recultured from survivors of second 0.6%-treatment							
0.0	0.0	$(4.51 \pm 0.43) \times 10^6$		$(4.62 \pm 0.27) \times 10^6$	nd		nd
0.6	49.9	# $(3.64 \pm 0.40) \times 10^6$	>	## $(0.4 \pm 0.1) \times 10^2$	nd		nd
0.8	66.7	## $(2.69 \pm 0.77) \times 10^4$	>	## 0	nd		nd
0.9	75.0	## $(0.6 \pm 0.2) \times 10^4$	>	## 0	nd		nd

Symbols same as Table 1; nd = no data.

duction depends on preventing toxicity rather than on simply the initial uptake and retention of ACP by cells, dead or alive.

The relation of viability to chemical yield is shown in Figure 2. In staining determinations carried out at 24 h and 96 h there was about a 1:1 relationship between percent viability and percent yield of PEA. At the maximum 24-h yield of PEA (27.5%) the CFU ml^{-1} as a percent of the control (66.3%) was similar to percent viability based on staining (58.5%). However, at an intermediate PEA yield of 9%, CFU ml^{-1} was negligible. This could indicate that yields up to 9% are produced prior to loss of replication ability, or that production of PEA is continuous between 1 and 24 h but is only responsive to enzymatic activity of cells regardless of replicative ability.

PEA yield of re-reacted cells

When cells previously exposed to 0.2%, 0.4%, or 0.6% ACP were re-reacted in a fresh 0.2% solution, there was essentially no PEA production.

PEA yield of recultured ACP-tolerant cells

Cells surviving 0.2% ACP for 95 h were recultured. They were then added in equal amounts to 0.25% ACP and reacted for 92–94 h. The PEA yield, which was highly variable, appeared to depend on the degree to which percent viability, an indicator of current ACP-tolerance, was maintained, ie, a population more tolerant of ACP (89% viability after 92–94 h) had a higher yield of PEA (43%) than a population less tolerant of ACP (50% viability, and a PEA yield of 16%). Differences in PEA yield were evident after 22.5 h (Figure 1, 'Reacted cells (0.2% ACP); 0.25% ACP'). Less than a two-fold difference in percent viability resulted in almost a three-fold difference in PEA yield. In each case, however, the reaction rate of recultured cells was slower than the reaction rate of naive cells exposed to 0.2% ACP (Figure 1).

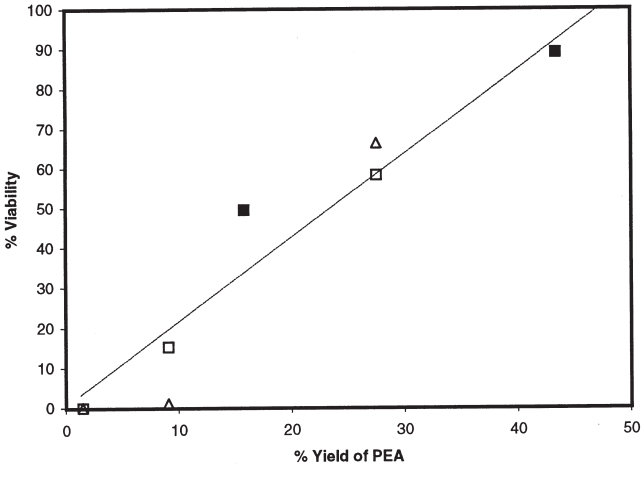

Figure 2 Yield of PEA *vs* viability irrespective of ACP concentration. ■ 96 h % stained; □ 24 h % stained; △ 24 h % CFU.

Discussion

Adaptability and chemical yield of previously unreacted cells

Limits of substrate tolerance: Our viability data verified a preliminary finding of a steep decline in viability after 24-h exposure to ⩾0.3% ACP, and 96-h exposure to ⩾0.2% ACP. The hypothesis that this was due to substrate- rather than product-toxicity was supported by a separate experiment in which precultured cells shaken for 48 h in an ACP-free solution containing 0.4% PEA and 1.2% ethanol had nearly 100% viability, with CFU ml^{-1} proportional to total cell concentration. Cells exposed to 0.4% ACP + 1.2% ethanol over the same period were non-viable, as indicated by negative vital staining and absence of colonies.

The baker's yeast toxicity threshold of 0.2–0.3% ACP we observed was similar to that observed after 10 h of exposure to 0.2–0.3% benzaldehyde in a solution containing 5% sucrose and having a pH of 4.5 [16]. This suggests that aromatic carbonyl substrates of similar molecular weight can have similar toxicity thresholds in solutions differing in concentration of co-substrates and pH. At some point, however, co-substrate composition and pH may enhance substrate toxicity. For instance, in a solution containing 11.8% glucose and having a pH of 4.0, growth of baker's yeast was inhibited by 0.1% ACP, and uptake of glucose was inhibited by 0.05% ACP [29]. Modeling of solution effects is currently hindered by a lack of standardization. For instance, in [16] flasks contained <1% (w/v) wet yeast *vs ca* 30% wet yeast in our study. In [29], the initial yeast concentration was not specified.

Differentiation of viability responses: After 24 h at toxic concentrations of 0.4–0.6% ACP, percent viability based on vital staining declined more gradually than colony-forming units, indicating that a large fraction of cells incapable of replication can maintain metabolic functions. Thus it is important to specify the type of viability response being measured in studies of cell longevity, or in studies correlating longterm catalytic activity with population status. This has also been indicated in studies of ethanol tolerance [12,23]. However, chemical toxicity may slow down cell division without preventing it [11], thus the magnitude of difference in viability estimated by colony counts *vs* percent viability may depend on the length of time plates are incubated.

At 0.0–0.3% ACP, CFU ml^{-1} relative to the 1-h or 24-h control was consistently *greater* than percent viability based on vital staining. This was unexpected, since replicative ability should be more restrictive than survival. For instance, in a study of longterm effect of added ethanol on yeast [12], colony-forming ability declined more rapidly than viability judged by methylene-blue, regardless of ethanol concentration (0.0–5.0%) (see also [6]). We found that the fluorescent viability stain, which is based on uptake of glucose, is a less liberal indicator of viability than is reduction of methylene blue. We are aware of only one study of yeast [19] in which percent viability based on staining was less stable than colony-forming ability. How-

ever, in this case fermenting yeast were densely packed in beads, possibly disrupting the cell membranes.

Dependence of chemical yield on yeast viability: Our data suggest that sustained PEA production depends significantly on viability, defined by vital staining or replicative ability. A small increase in ACP concentration above 0.3% resulted in a steep decline in PEA yield and viability. Any chemical advantage of increased substrate concentration is offset by cell inactivation.

A correlation of sustained PEA production with continual cell activity is also supported by studies in progress showing that preculturing significantly enhances longterm yield of PEA at ACP concentrations of 0.2–0.3% but not 0.6%. Precultured cells, which presumably contain more stored carbohydrate than non-cultured cells, enhance PEA yield only at ACP concentrations permitting sustained cell viability.

The cell-viability requirement implies that, for batches of unprotected cells, maximum longterm yield of PEA should require feeding so that ACP is maintained at maximum non-toxic levels, ~0.2%. Maintenance of low substrate concentration may have other benefits, such as maximizing S-enantiomeric excess by restricting substrate access to competing oxidoreductases [cf 2].

Maintenance of viability may also be needed to maximize biotransformation of other carbonyl compounds. For instance, nonviable cells (unable to form colonies) removed from benzaldehyde solutions after 5 h were deficient in initiating both benzyl alcohol and L-PAC production when added to fresh reactor solutions [16].

Chemical yield of non-viable cells: Although the yield of PEA is small in batch reactions in which viability is lost, some PEA may be produced in cells with declining metabolic activity, or in cells which are dead. In the experiment in which yeast were reacted at 0.6% ACP, cell viability and carbon dioxide production had declined to near zero at 25–47 h yet PEA continued to accumulate until the final sample was taken at 170 h. However, the moles of PEA produced did not exceed the moles of ACP present in cells after initial saturation. Thus, once cells have died, there is no substantial replacement of ACP in the cell phase.

Longevity and reuse of the biocatalyst: Cells which had continuously reduced ~0.2% ACP, which is non-toxic within a period of 48 h, were unreactive when transferred without preculture to fresh non-toxic solutions of ACP. Thus the biocatalyst cannot be reactivated without regeneration in an ACP-free medium, and catalyst longevity depends on the time that the initial precultured population can support reduction of ACP.

The fact that cells originally exposed to 0.2% ACP were nonproductive is interesting, since the population maintains a significant percentage of viable cells. As shown in Table 1, viability after 24 h was high. Although viability was not measured at 96 h, it is assumed that a significant proportion of cells was alive at 96 h given the continual production of PEA and the high viability at 24 h compared with higher concentrations of ACP. If reacted cells lose the capacity to regenerate reducing power, or lose the use of enzymes favoring reduction of acetophenone, then reacted cells should not be recycled or regenerated, even after being exposed to only 0.2% ACP.

Adaptability and chemical yield of recultured cells

Development of chemical tolerance: Previous adaptation studies have been concerned with rapidly fermenting cells rather than populations of non-growing, non-fermenting cells. For instance, yeast surviving 7% ethanol have a significantly higher viability over a broad range of ethanol concentrations than non-adapted yeast [11]. In another study [6], yeast viability (CFU ml^{-1}) was higher in populations exposed to a toxic concentration of ethanol for 3 h than for 1 h, suggesting that populations became enriched for ethanol-tolerant cells (see also [3]).

ACP tolerance of progeny of cells surviving 0.6% ACP exceeded that of non-selected cells, but the degree of adaptation is insignificant and transient. Only ~1% of the progeny of cells surviving 0.6% ACP maintained ACP tolerance, even after reculture. This suggests a low level of genetic or phenotypic stability since all progeny of the original surviving colonies should be identical genetically. Even at concentrations of 0.25% ACP, surviving cells were quite variable in transmitting and maintaining ACP tolerance. The low frequency of chemical adaptation to ACP contrasts with that observed in response to ethanol [3,6,11], but is comparable to benzaldehyde [18].

Chemical yield of tolerant cells: ACP tolerance could be achieved by greater exclusion of ACP, evidenced by a lower conversion rate, or by a more rapid or more extensive conversion of ACP, preferably coordinated with more efficient PEA production. In our experiments, the maximum rate of PEA production of populations propagated from surviving reacted cells did not exceed that of populations derived from previously unreacted (non-selected) cells, indicating that ACP tolerance is not necessarily correlated with more efficient conversion of substrate. However, cells were tested only at non-inhibitory concentrations of 0.2–0.25% ACP. Mahmoud *et al* [18] found that progeny of cells surviving 0.6% benzaldehyde showed a higher rate of L-PAC production in response to continuous addition of very low doses of benzaldehyde, *ca* 0.1%, in an air-bubble column, but the degree of enhancement was not significant statistically. Cells surviving 0.6% benzaldehyde did not exhibit higher rates of L-PAC production when incubated in a growth medium without benzaldehyde and then added in four doses over 4 h in a medium containing 0.6% benzaldehyde. Thus, over a broad range of substrates and substrate concentrations, it is possible that rapid replacement of cells would be more cost-effective than maintenance or reuse of viable cells, with the break-even point depending on the vigor of initial reaction rates, rates of deactivation of cells, and costs incurred by more frequent preparation of dried yeast.

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