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Continuous Production of Acetaldehyde by Immobilized Yeast with *In Situ* Product Trapping

Comparison of Alcohol Dehydrogenase and Alcohol Oxidase Routes

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ABSTRACT

Two alternative enzymatic routes to continuous biological acetaldehyde production from ethanol by immobilized yeast were compared: ADH of *Candida utilis* and AOX of *Candida boidinii*. The ADH route, performed by immobilized cells in the presence of tris buffer continuously trapping the acetaldehyde formed, was found superior to the AOX route. It exhibited significantly higher operational stability in both batchwise or continuous operation in fluidized bed reactor. The ADH system exhibited a stable acetaldehyde production level for 5 d, followed by gradual decrease in activity, which could be readily fully regenerated *in situ* by flushing with growth medium. Efficient continuous product trapping by tris buffer was essential to maintain continuous operation.

Index Entries: Acetaldehyde; alcohol dehydrogenase; alcohol oxidase; *Candida utilis*; immobilized yeast; product recovery.

INTRODUCTION

Acetaldehyde is a widespread natural flavor in many fruits (oranges, strawberries), food products (yogurt, bread, cooked meat), and beverages (beer, wine [*1,2*]). Because of its low boiling point (21°C), acetaldehyde is

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rapidly lost during food processing, e.g., preparation of juice concentrate (*1*). However, its impact as a flavor may be readily restored via the addition of pure acetaldehyde as added flavor to food products. In recent years, there has been a tendency to switch from using chemically derived flavors, colorants, and other food additives to naturally derived "bioflavors" obtained via extraction from plants, prepared through enzymatic or microbial transformations of natural precursors or made via biosynthesis (*2–4*). Acetaldehyde may be prepared from ethanol via two main enzymatic routes (2): alcohol dehydrogenase (ADH) catalyzing:

$$CH_3CH_2OH + NAD^+ \xrightarrow{ADH} CH_3CHO + NADH + H^+$$

and alcohol oxidase (AOX) catalyzing:

$$CH_3CH_2OH + O_2 \xrightarrow{AOX} CH_3CHO + H_2O_2$$

These enzymatic routes may be applied either as purified enzymes or as intracellular enzymic activity operating from within dead or viable cells, which seems preferable since enzyme isolation and purification are saved. The biological production of acetaldehyde from ethanol has to overcome several intrinsic problems: Because of its low boiling point, acetaldehyde is rapidly lost from the system. Furthermore, acetaldehyde is an inhibitor and chemically reactive (*5,6*). The substrate ethanol is also inhibitory (*7*), and the byproduct, hydrogen peroxide, formed by the alcohol oxidase route is highly toxic (*8*). In order to overcome these difficulties, trapping of the acetaldehyde formed via reaction with tris buffer (*6*), hydrogen peroxide degradation by catalase (*9*) and restricted low ethanol input (*10*) were suggested.

The use of immobilized viable cells in an appropriate bioreactor configuration may be very effective in integrating *in situ* product recovery and maintenance of a low level of inhibitory product concentration, as recently shown by several labs, including the authors' (*11–16*). Mostly, *in situ* product removal or trapping was essential to maintain high operational stability with higher yields.

Acetaldehyde production by whole cells was reported mainly for *Candida utilis* (alcohol dehydrogenase route [*10*]), *Candida boidinii*, and *Pichia pastoris* (alcohol oxidase route [*5,6,17*]). Attempts to use immobilized cells for acetaldehyde production were reported only for the latter (*18,19*). Immobilization of *Pichia pastoris* cells in calcium alginate gel resulted in lower reaction rates, but the immobilized cells exhibited higher thermal stability than freely suspended cell control. Repeated batchwise conversions resulted in gradual loss of activity (50% within three cycles [*18*]). The most probable explanation for the decay in alcohol oxidase activity is the effect of accumulating byproduct, hydrogen peroxide, which is not fully degraded by intracellular catalase (*8*). This problem may become crucial

for prolonged continuous ethanol oxidations, making this enzymatic route less attractive than the alternative alcohol dehydrogenase route.

In this presentation, a comparative study on factors affecting the operational stability of two immobilized yeast systems representing the alcohol dehydrogenase route (*Candida utilis*) and the alcohol oxidase route (*Candida boidinii*) is described. Both systems, representing genera of food-compatible yeast (*20*), were coupled with acetaldehyde trapping by means of tris buffer in an aerated fluidized-bed reactor to determine which intracellular enzymic route is preferable for prolonged continuous operation.

MATERIALS AND METHODS

Cells

Candida utilis (NRRL Y-900) was obtained as a freeze-dried culture from Northern Regional Research Center, Peoria, Ill. *Candida boidinii* (CBS 5777) was obtained on agar slant from Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

Polymers

Sodium alginate (cat. no. 30105) was purchased from BDH, Poole, UK. Polyacrylamide hydrazide of mol wt 100,000 or 180,000, and acylhydrazide content of 0.8 meq/g, was prepared after (*21*).

Chemicals

Ethanol (cat. no. 983), acetaldehyde (cat. no. 800004), calcium chloride (cat. no. 2389), and glyoxal (cat. no. 804192) were all purchased from Merck, Darmstadt, FRG. Tris buffer (cat. no. T-1503) was purchased from Sigma, St. Louis, MO. 2,4 Dinitro phenyl hydrazine (DNPH, cat. no. 42210) was purchased from Fluka, Buchs, Switzerland. All other chemicals employed were of analytical grade.

Cultivation of Cells

Candida boidinii cells were first grown on YM culture medium (prepared after [*19*]) in 2% agar plates. Cell colony was transferred into 50 mL of the above-mentioned YM liquid medium in a 250 mL Erlenmeyer flask and shaken at 30°C (180 rpm) for 10–12 h. Five-milliliter aliquots were then transferred into 50 mL of the same medium and shaken for an additional period of 12 h. The cells were then allowed to grow on 5% (v/v) ethanol as sole carbon source for 2 h (*22,23*). Cells were harvested by centrifugation (800 rpm, 5 min, yielding 0.9 g [WW] cell pellet), washed with saline, centrifuged again, and stored at 4°C.

Candida utilis cells were grown, harvested, and stored essentially as described above for *C. boidinii*, employing the recommended YM medium composition as provided with these cells by the NRRL collection.

Cell Immobilization

Calcium alginate gel: an aqueous 3% (w/v) solution prepared as previously described (24). Cells were added (1 g [ww]/15 mL of 3% [w/v] sodium alginate solution), and the suspension homogenized by magnetic stirring for 15 min. The cell suspension was then added dropwise into a 1% (w/v) $CaCl_2$ solution employing the injector described in ref. 24 at a flow rate of 2.5 mL/min via a conduit of internal diameter of 1.05 mm. Compressed air was applied through the outer conduit at a flow rate of 8 L/min to generate droplets 2.0 mm in diameter. Following curing of 30 min at room temperature, the beads were separated and washed three times with 0.25*M* tris buffer (pH 8).

PAAH beads: Cells were immobilized in PAAH beads, bearing the same cell input as alginate beads, essentially as previously described (24,25). The outer alginate envelope was removed from these beads by incubation under 0.1*M* phosphate buffer (pH 8) at 30°C for 30 min. The PAAH beads were then washed three times with 0.25*M* tris buffer (pH 8).

Enzyme Assays

Alcohol dehydrogenase activity of *C. utilis* and alcohol oxidase activity of *C. boidinii* were assayed by applying 1 g (ww) of cells—either freely suspended or immobilized—into 30 mL (final vol) of 6% (v/v) ethanol in 0.25*M* tris buffer (pH 8) in a 250-mL Erlenmeyer flask and incubating on a gyratory shaker at 180 rpm at 30°C. In the case of immobilized cells, the beads retaining the cells were equilibrated three times with cold (4°C) substrate solution to ensure the same final substrate concentration and reaction mixture volume as with freely suspended cells. Samples (1 mL) were removed periodically (up to 6 h), and suspended cells or particulate material were removed by centrifugation (2 min Eppendorf minicentrifuge 5414S) and the supernatant analyzed for ethanol and acetaldehyde content.

Continuous Acetaldehyde Production by Immobilized Yeast

Five grams (ww) of cells immobilized in 70 mL of beads were loaded into the fluidized-bed reactor (working vol: 150 mL) described in Fig. 1, previously employed by us for continuous steroid dehydrogenation by

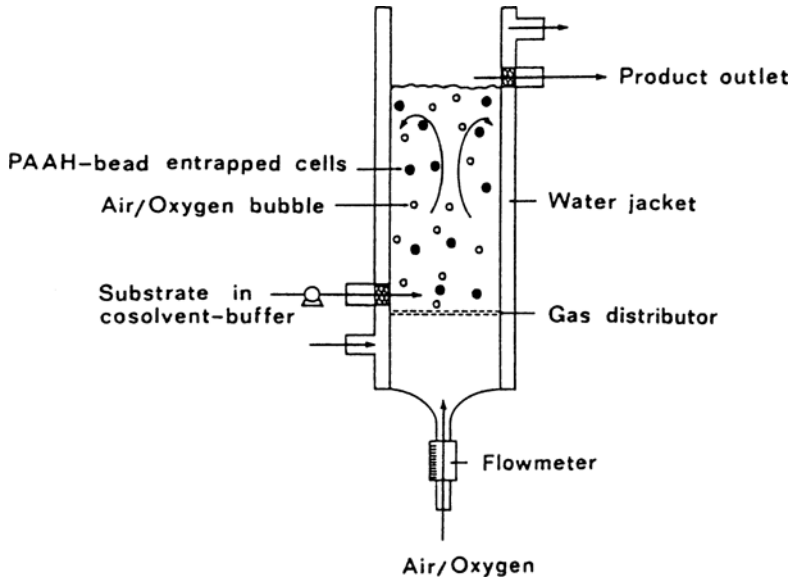

Fig. 1. Aerated fluidized-bed reactor (working vol: 150 mL).

immobilized *A. simplex* cells (*12*). Ethanol solution (6% [v/v] unless otherwise stated) in 0.25*M* tris buffer (pH 8) was pumped in at a flow rate of 0.3 mL/min and aeration was provided via a sintered glass gas dispenser located at the reactor bottom (Fig. 1) at a flow rate of 20 mL/min. The temperature was maintained at 30°C by means of a water jacket. Samples were removed from the top of the reactor periodically. Free cells or suspended particulate material was removed by centrifugation, and the supernatant analyzed for ethanol and acetaldehyde content.

Analysis

Ethanol concentration was determined by gas chromatography as previously described (*24*). Acetaldehyde concentration was determined spectrophotometrically by a modification worked out in this study to the "classical" determination of aldehydes and ketones by precipitation following Schiff's base formation with 2.4 dinitrophenyl hydrazine (DNPH [*26*]). A sample (0.5 mL) of acetaldehyde solution in tris buffer is added into 4.5 mL of 3 mg/mL DNPH solution in 2*N* HCl. The mixture was incubated for 24 h at 4°C for precipitation of DNPH-acetaldehyde conjugate. One milliliter of the supernatant was then removed, centrifuged (3 min on Eppendorf minicentrifuge 5414S), diluted ×5 with 2*N* HCl, and OD_{405} measured. Acetaldehyde concentration—within the range 0–150 m*M*—was calculated from an appropriate calibration curve.

RESULTS AND DISCUSSION

Optimization of Working System Parameters

Spectrophotometric Determination of Acetaldehyde in Tris Buffer

Acetaldehyde, produced under the expected reaction conditions in the presence of tris buffer, at physiological or slightly alkaline pH values, is immediately trapped as Schiff's base with tris. This process is reversible, and the acetaldehyde may be released by drastically lowering the pH. Because of its low boiling point, most of the acetaldehyde thus liberated will evaporate at temperatures exceeding room temperature. This effect was previously employed for the determination of acetaldehyde in such mixtures by trapping the gas phase following reaction medium acidification and analyzing its acetaldehyde content by gas chromatography (*17,19*). Because routine handling of samples via this procedure seemed to be tedious, an alternative simple colorimetric assay was developed for this study. The method is based on reacting a sample containing acetaldehyde–tris conjugate with excess of very acidic ice-cold solution of 2,4 dinitrophenylhydrazine (DNPH). Under these conditions, the acetaldehyde is gradually liberated from its conjugate with tris, trapped by Schiff's base formation with the hydrazine derivative, and the insoluble adduct precipitates. Since DNPH has strong absorbance at 405 nm, the decrease in OD_{405} is proportional to the acetaldehyde content, which precipitated with equimolar amount of this reagent. Optimization of this procedure resulted in the assay conditions described in the Materials and Methods section. Under these conditions, the linear calibration curve for the 0–150 m*M* acetaldehyde concentration range was readily obtained.

Cell Growth, Induction, Harvest, and Storage

Study of growth curves under the conditions described in the Materials and Methods section showed that both cells entered the stationary phase after 12 h. The biomass obtained for *C. utilis* was, however, about twice as much as that obtained for *C. boidinii* (calculated OD_{660} 28 vs 14, respectively [data not shown]). Following growth, the cells were induced by incubation in the presence of ethanol for 2 h (after Hill et al. [23]). Following cell harvest and washing, storage at +4°C vs freezing and storing at −18°C were compared. In both cell systems, it was clearly shown that freezing and thawing of the cells resulted in a sharp decrease in activity: the ADH activity of frozen and thawed cells of *C. utilis*, and as the AOX activity of frozen and thawed cells of *C. boidinii* were less than a quarter of that attained by the same cells stored at +4°C. It was observed, however, that some decrease in activity on prolonged storage at +4°C

also occurred: ADH residual activity of *C. utilis* was 95% following 3 d and 85% following 23 d of storage, whereas AOX residual activity of *C. boidinii* was significantly lower: 80% and 40% respectively.

Effect of Tris Buffer Concentration and pH

The approach of effectively trapping the acetaldehyde formed by the tris buffer may be favored by employing high tris concentrations. The use of 0.5*M* tris was reported by Duff and Murray (*5,6,18*). This concentration, however, was found to be inhibitory for acetaldehyde production by both enzymic systems studied by us: AOX activity of *C. boidinii* was even lower in the presence of 0.5*M* tris (pH 8) than in its absence (10 m*M* acetaldehyde produced within 5 h vs 30 m*M* produced in the presence of 50 m*M* phosphate [pH 8]). Optimization of tris input for acetaldehyde formation indicated that 0.25*M* tris (pH 8) allowed for maximal levels of acetaldehyde by both systems (data not shown). This concentration was routinely employed in all the following experiments. Screening different pH values with 0.25*M* tris buffer indicated that pH 8 was optimal for both systems studied (data not shown).

Effect of Ethanol Concentration

Armstrong et al. (*10*) reported that minimal 3.5 g/L ethanol concentration is required for acetaldehyde production by *C. utilis*. This finding was confirmed by us. Some increase (up to 20%) in acetaldehyde production was observed by elevating ethanol input up to 8% (v/v). AOX activity of *C. boidinii* was not affected by ethanol, and similar acetaldehyde levels were recorded for concentration range of ethanol input of 2–10% (v/v) (data not shown).

Effectiveness of Acetaldehyde Trapping by Tris Buffer in Aerated Reactor

The effectiveness of tris for trapping acetaldehyde was tested first—in the absence of cells—in the reactor described in Fig. 1. The reactor was loaded with 150 mL of 0.25*M* tris buffer (pH 8) containing 100 m*M* acetaldehyde and 6% (v/v) ethanol. The solution was aerated for 24 h at 30°C. Samples were removed periodically for acetaldehyde and ethanol analysis. Acetaldehyde concentration was fully stable within the first 8 h, whereas ethanol concentration decreased slightly to 95% of input concentration. Following 24-h aeration, acetaldehyde concentration decreased to 90% and ethanol decreased to 70% of their respective input concentrations. These results indicate that employing flow rate leading to residence time of up to 8 h will minimize acetaldehyde losses (assuming concentrations up to 0.1*M*). This set of conditions was routinely employed in the following continuous-operation experiments.

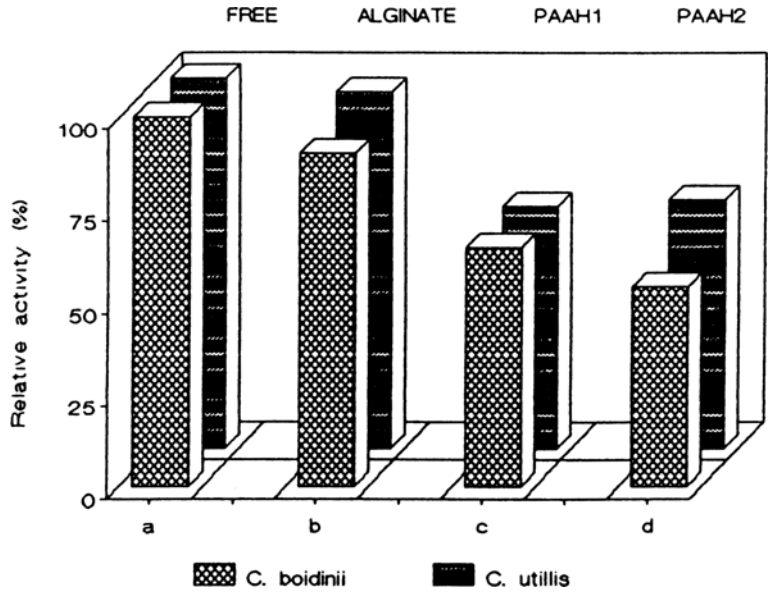

Fig. 2. Effect of immobilization in 3% (w/v) alginate, 5% (w/v) PAAH (mol wt 100,000, PAAH 1), and 3% (w/v) PAAH (mol wt 180,000; PAAH 2) on ADH activity (*C. utilis*) or AOX activity (*C. boidinii*) of immobilized yeast.

ADH-Based vs AOX-Based Immobilized Yeast Systems for Acetaldehyde Production

Effect of Immobilization

Both systems could be readily immobilized in 3% (v/v) calcium-alginate gel with nearly full retention of input activity (Fig. 2). Immobilization in PAAH beads led to about 25% decrease of input activity, a result that may be interpreted as some acetaldehyde binding to acylhydrazide groups present on this polymeric matrix, thus artificially lowering the recorded result. There was no significant difference in immobilization effect on the ADH- or AOX-based systems. On the basis of these observations, Ca-alginate beads entrapped cells were routinely employed in subsequent studies.

Repeated Batchwise Application of Free and Immobilized Cells

Freely suspended and immobilized cells (1 g each) were repeatedly employed for acetaldehyde production under the conditions described under Enzyme Assays in the Materials and Methods section. Immobilized

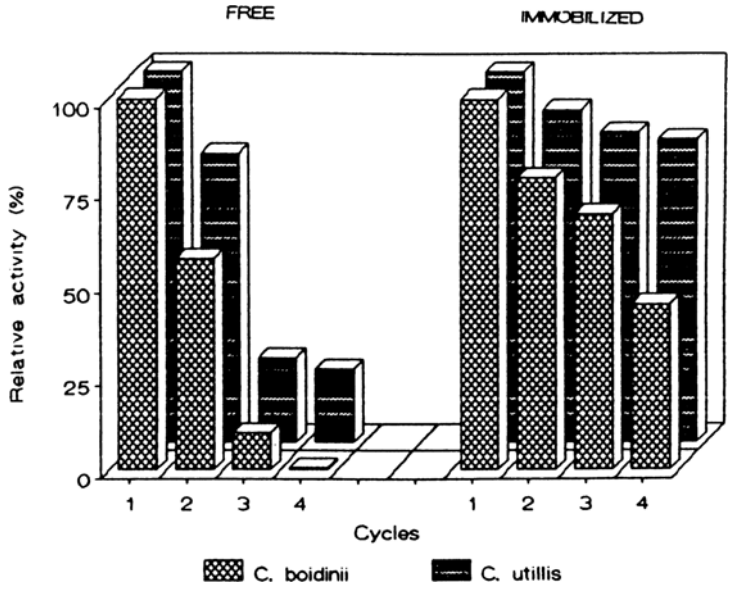

Fig. 3. Residual activity following repeated batchwise acetaldehyde production by freely suspended or Ca-alginate-entrapped ADH activity of *C. utilis* and AOX activity of *C. boidinii*. Activities were assayed as described in Materials and Methods for 5 h, washed three times with 0.25*M* tris (pH 8), and stored at +4°C overnight between repeated experiments.

cells were significantly more stable than the corresponding free cells (Fig. 3). The AOX-based system was significantly less stable—both as free and immobilized cells—than the corresponding ADH-based system, although a decrease in activity was observed in all experiments. This finding is in accord with a similar decrease in activity of alcohol-oxidase containing immobilized *P. pastoris* cells (*18*).

Continuous Acetaldehyde Production by Immobilized C. utilis *and* C. boidinii

The results of a comparative study of continuous acetaldehyde production by the two cell systems are presented in Fig. 4. Although both systems attained similar initial production levels after 20 h of continuous operation, the activity of AOX of *C. boidinii* was unstable, gradually and continuously declining to about half of the initial activity within 85 h. The ADH activity of *C. utilis* exhibited a stable level of acetaldehyde production for up to 130 h, followed by a gradual decrease to half residual activity at 160 h of continuous operation. This observation indicates that the ADH system seems to be more stable and, thus, preferable to the AOX-based system, under these conditions, for continuous acetaldehyde production.

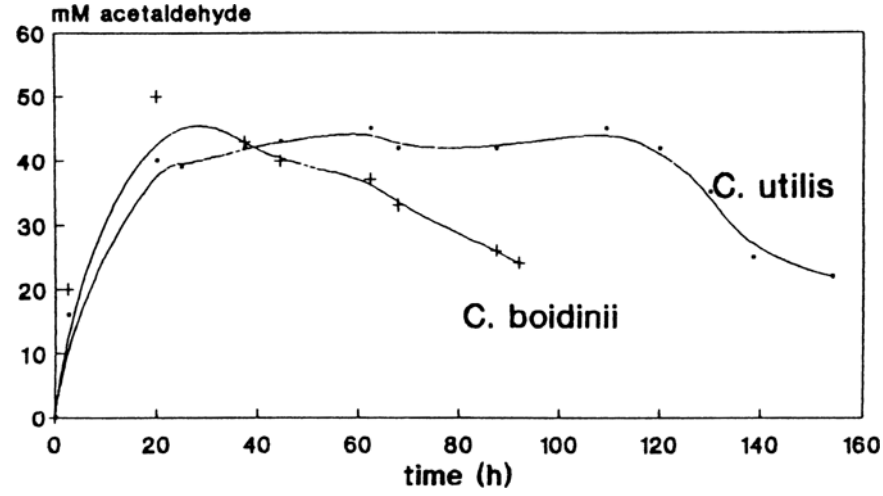

Fig. 4. Comparison of operational stability of continuous acetalhyde production from 6% (v/v) ethanol in 0.25*M* tris buffer (pH 8) by intracellular ADH (*C. utilis*) and AOX (*C. boidinii*) of Ca-alginate-entrapped yeast in fluidized-bed reactor (for details, *see* Materials and Methods).

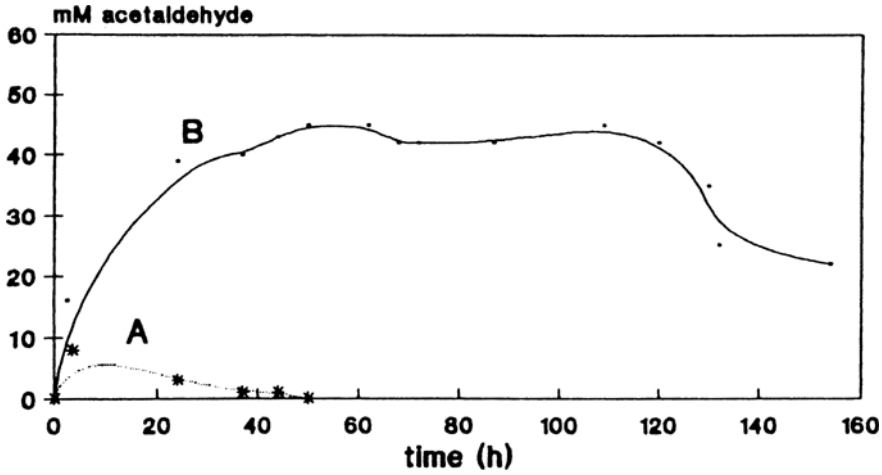

Fig. 5. Buffer effect on continuous acetaldehyde production by ADH of Ca-alginate-entrapped *C. utilis*: A: HEPES buffer (50 mm, pH 8); B: tris buffer (0.25*M*, pH 8).

Demonstration that Acetaldehyde Trapping Is Essential for Continuous Acetaldehyde Production

The presence of tris in the reaction medium allowing for continuous acetaldehyde production by immobilized *C. utilis* is essential for maintaining a stable production level: When HEPES buffer was employed instead of tris, and thus, acetaldehyde trapping could not occur, an initial

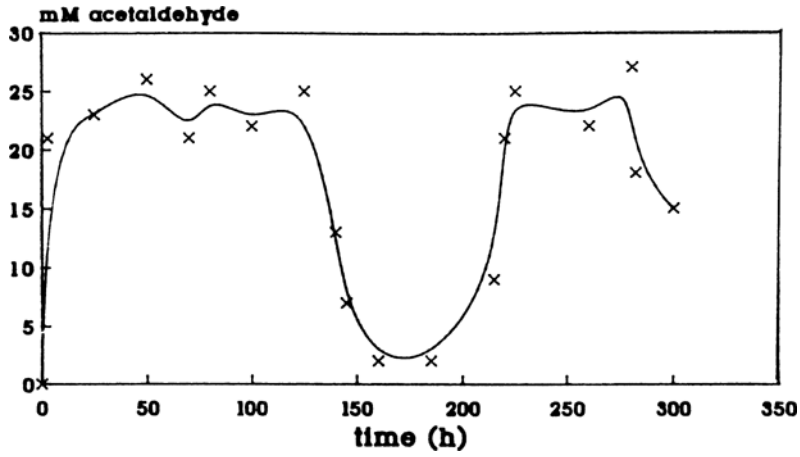

Fig. 6. Continuous production of acetaldehyde by PAAH(2)-entrapped *C. utilis* from 4% (v/v) ethanol in 0.25*M* tris buffer (pH 8). Growth medium (YM) introduction was carried out after 145 h, and incubation continued for 24 h, followed by renewed substrate flow.

acetaldehyde production level of only 10% of that attained by the corresponding tris-containing system was observed (Fig. 5). Furthermore, this low level decayed quickly, in <40 h (curve A vs B, Fig. 5), indicating that acetaldehyde trapping is essential not only for product recovery, but also for protecting the cells, even at low production levels.

Extension of Continuous Acetaldehyde Production by Immobilized C. utilis

The decline in the continuous acetaldehyde production level by immobilized *C. utilis*, observed after 130 h of continuous operation, raises the question of whether this time scale is the limit for this process. Furthermore, following continuous operation for 130 h, the Ca-alginate beads swelled, increasing from 2.0 to 2.5 mm in diameter, and softened. The following changes were therefore introduced into the system aiming toward improving its operational stability: (1) gel matrix was changed to PAAH beads, previously shown to be stable for several weeks of continuous operation in aerated fluidized-bed reactors (*12*); (2) ethanol input was lowered to the minimum required (4% [v/v]) to minimize its possible interference. The result was a similar profile of stable acetaldehyde production for 130 h, although acetaldehyde concentration level was lower than in the previous experiments (Fig. 6 vs Fig. 4). Following a decline in acetaldehyde level to 20% of maximal activity, the medium in the reactor was substituted by YM medium and aerated batchwise for 24 h to allow for regeneration of the envisaged activity. On resuming substrate feeding, acetaldehyde production level could be fully restored and maintained for a second production period (Fig. 6). This observation indicates that

the ADH activity of immobilized *C. utilis* may be readily regenerated, thus allowing alternate production and regeneration phases.

CONCLUSION

The results of the comparative study of acetaldehyde production with continuous product trapping by ADH-based and AOX-based immobilized yeast clearly indicate that the ADH route is preferable. The ADH activity of *C. utilis* was superior to the activity of *C. boidinii* for most variables investigated: easier cell growth, better storage stability, significantly higher operational stability as immobilized cells for both repeated batchwise and continuous operations. Furthermore, this activity could be readily restored via *in situ* regeneration. The presence of tris buffer and effective acetaldehyde trapping was shown to be critical not only in preventing product loss by evaporation, but also in protecting the producing cells from its toxic effects. This finding, coupled with the fact that *C. utilis* is considered safe, food-grade cells (*12*) make this yeast the preferable candidate for subsequent continuous process development for "bio"-acetaldehyde production.

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