Effects of Growth Media pH and Reaction Water Activity on the Conversion of Acetophenone to (S)-1-Phenylethanol by Saccharomyces cerevisiae Immobilized on Celite 635 and in Calcium Alginate

Nicholas P. Coleman
University of Kentucky

Czarena Crofcheck
University of Kentucky, crofcheck@uky.edu

Sue E. Nokes
University of Kentucky, sue.nokes@uky.edu

Barbara L. Knutson
University of Kentucky, bknutson@engr.uky.edu

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Effects of Growth Media pH and Reaction Water Activity on the Conversion of Acetophenone to (S)-1-Phenylethanol by *Saccharomyces cerevisiae* Immobilized on Celite 635 and in Calcium Alginate

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ABSTRACT: Biologically catalyzed reactions often produce enantiomers of the product; however, only one configuration is desired. Reaction conditions are known to affect enantiomer ratios and reaction kinetics, but little is known regarding the effect of processing conditions on whole-cell biocatalysis. Saccharomyces cerevisiae cells were grown in batch on glucose at pH = 4, 5, and 7, and then immobilized on Celite beads or in calcium alginate beads and used as the biocatalyst for the conversion of acetophenone in hexane to (S)-1-phenylethanol at water activities of 0.37, 0.61, and 0.80. S. cerevisiae was used as a model microorganism for the whole-cell catalyzed reaction. The initial reaction rate (IRR) and the final (S)-1-phenylethanol concentration were quantified for each treatment. The highest IRR value (94.9 μmol/h) and the highest final concentration of (S)-1-phenylethanol (17.8 mM) were observed with cells grown at pH 5 or 7, with the main effect of growth medium pH highly statistically significant. The main effect of water activity and the interactions of the two were not statistically significant (α = 0.05). The cells immobilized in calcium alginate beads favored a water activity of 0.61, resulting in an IRR of 916.2 μmol/h/g dw, averaged over pH. The highest final concentration of (S)-1-phenylethanol (4.8 mM) was achieved with cells grown at pH 5 or 7. Calcium alginate beads gave the highest initial reaction rate with a growth pH of 7 and a water activity of 0.61. However, pH of 5 and water activity of 0.61 resulted in the highest final concentration of (S)-1-phenylethanol.

Keywords. Acetophenone, Calcium alginate, Celite, Immobilization, Water activity, Whole cells.

Chiral organic molecules produced chemically typically have high racemic yields. Therefore, biocatalytic reactions of pro-chiral compounds to produce more enantiomerically pure mixtures has been explored through the use of purified enzymes (Lam et al., 1986; Shin and Kim, 1999). However, the cost of purification of enzymes can be prohibitive, which has led to the investigation of using whole cells of Saccharomyces cerevisiae to catalyze reactions (Griffin et al., 1998; Leon et al., 1998).

Whole cells offer several advantages over purified enzymes as biocatalysts (Chakraborty et al., 2005). Whole cells do not require an expensive purification step, they are capable of multi-step reaction, and the requirement for providing cofactors exogenously is less critical than with enzymes (Gervais et al., 2000). According to Chun and Agathos (1989), the use of immobilized whole cells was “the only possible approach for the continuous production of complex compounds through multistep reactions requiring cofactors” (Chun and Agathos, 1989).

ORGANIC SOLVENTS AND THE MODEL REACTION

Recent advances in the bioreduction of ketones to chiral alcohols by yeast have been recognized as having significant potential for large-scale production (Cheng and Ma, 1996). Conversion of acetophenone to phenylethanol by Saccharomyces cerevisiae UVAY101 has been well-studied (Buque et al., 2002; Griffin et al., 2001; Griffin et al., 1998). The reaction is known to require NADH and alcohol dehydrogenase (ADH), which are both present in the cell. However, NADH needs regeneration through the use of a reducing agent, as shown in figure 1. The small levels of NADH in the cell require the addition of 2-hexanol as a sacrificial substrate to recycle NAD⁺ to NADH. In previous work with immobilized yeast, 2-hexanol was selected as the co-substrate because it yielded the highest reaction rates of the various alcohols that were tested (Griffin, 2000).

Organic solvents are used in whole-cell catalysis because the solubility of the reactants and products improves relative to the solubility of these products in an aqueous environment (Halling, 2000; Ghanem and Aboul-Enein, 2004; Vidinha et al., 2004). In addition, the desired product will partition from the aqueous phase into the organic phase, effectively removing cellular products from the aqueous phase, thereby limiting end-product inhibition (Halling, 1994).
**EFFECT OF GROWTH pH**

The phenomenon of “pH memory,” or the idea that enzymes acting in low-water media are affected by the pH in their previous aqueous environments, has been widely studied (Halling, 2000). The pH of the aqueous solution containing harvested baker’s yeast prior to calcium alginate immobilization was also considered by Griffin et al. (1998) as a factor that influenced the reduction of acetophenone in hexane. It was discovered that an increase of the pH of the cell suspension solution prior to immobilization from 3 to 9 (as maintained by various buffers) decreased the initial reaction rate in hexane. However, a pH of 10 resulted in an initial reaction rate nearly as high as that at a pH of 3, with another drastic drop in rate at a more basic pH.

**WATER ACTIVITY**

It is generally accepted that some amount of water is necessary for an enzymatic reactions to occur, as water provides the enzyme with a certain amount of flexibility so that the active site is accessible to the substrate. Omar and Robb (1995) stated that even though the amount may be quite small, water plays a significant role in biocatalysis in organic media. However, if excessive water is present in organic solvents, the enzyme becomes destabilized because of local denaturation of the active site (Broos et al., 1995). The control of water activity ($a_w$) of reactions occurring in organic solvents allows the investigator to separate the effects of other parameters being studied from a change in water activity throughout the progression of the reaction. Water activity control also allows for the study of the effects of various water activities and enzyme hydration on the reaction itself (Halling, 1992; Zacharis et al., 1997). One widely accepted method for controlling water activity in organic solvents is the use of salt hydrate pairs (Kvittingen et al., 1992). These salt hydrate pairs act as a “water buffer,” exchanging water molecules to maintain a water balance much like a pH buffer will exchange hydrogen ions.

Enzymes in low-water media have been shown to differ in behavior from fully hydrated enzymes. Some, in fact, lose all ability to catalyze reactions without sufficient water. Celite has been shown to maintain water activity over a range of water concentrations within organic media (De Martin et al., 1999). This proved effective at maintaining catalytic functionality of enzymes in low-water conditions and may do the same for whole cells.

**IMMobilization**

Immobilization is an accepted method for protecting cells from the molecular toxicity of organic solvents. The two main immobilization techniques investigated here were: (1) the immobilization of whole cells on Celite beads, and (2) the immobilization in calcium alginate beads.

Celite is a diatomaceous earth that is compatible with both aqueous and organic media. Its industrial uses include the filtration of drugs, pharmaceuticals, beverages, acids, and petrochemicals (B. Hurst, personal communication, Santa Barbara, Cal., 2 July 2001). The use of Celite in enzyme immobilization offered promising results in aqueous phosphate buffer (Huang et al., 1997) and in hexane (Lee and Akoh, 1998).

Calcium alginate is one of the more commonly used polymeric beads for immobilizations (Park and Chang, 2000; Gervais et al., 2003; Milagre et al., 2006). When a sodium alginate mixture comes in contact with a divalent cation such as calcium, the alginate forms a tight matrix and solidifies quickly into a solid particle. Cells immobilized in alginate beads have been shown to catalyze reactions in organic solvents. Specifically, Griffin et al. (1998) have shown that baker’s yeast can be used to reduce acetophenone to phenylethanol in hexane when entrapped in alginate beads.

**OBJECTIVES**

The objective of this study was to examine the effects of processing conditions on the biocatalytic activity of immobilized S. cerevisiae. The processing conditions examined were growth medium pH and subsequent reaction water activity. The immobilization methods used were entrapment in calcium alginate beads and adsorption onto Celite beads. The reaction studied was the conversion of acetophenone to phenylethanol by S. cerevisiae UVAY101 with the addition of 2-hexanol as a sacrificial substrate to recycle NAD+ to NADH.

**MATERIALS AND METHODS**

**MATERIALS**

*Saccharomyces cerevisiae* UVAY101 was generously provided by Dr. Daniel Carta, University of Virginia, as frozen samples in micro-centrifuge tubes. Sodium alginate (medium viscosity), calcium chloride, and all growth media chemicals were purchased from Sigma Chemical Company (St. Louis, Mo.). Acetophenone, hexane and 2-hexanol were purchased from Aldrich Chemical Company (Milwaukee, Wisc.). Celite R-635 was kindly donated by Advanced Minerals Corporation (Santa Barbara, Cal.).

**YEAST PROPAGATION**

Propagation of the yeast samples for library storage was accomplished as follows. The fluid from one thawed sample
tube was placed in a 250 mL seed flask, which had been autoclaved at 121°C and 15 psi for 15 min. The seed flask also contained 50 mL of media consisting of 30 g/L lactate, 30 g/L glycerol, 20 g/L peptone, and 10 g/L yeast extract. This was then incubated at 30°C on an orbital shaker tray at 175 rpm for 24 h. From this flask, 4 mL were used to inoculate subsequent flasks (as needed) containing identical media and incubated at identical conditions for 48 h. From these flasks, 0.75 mL of broth was mixed with 0.75 mL of a 40% glycerol solution (that would act as a cryogenic preservation agent) in sterile micro-centrifuge tubes. Tubes were stored at -46°C for future use.

**Yeast Cultivation**

Growth of cells for use as biocatalysts occurred in a two-stage process. A medium containing 30 g/L lactate, 30 g/L glycerol, 20 g/L peptone, and 10 g/L yeast extract was prepared and adjusted to a pH of 5. Then, 50 mL of medium was added to a 250 mL Erlenmeyer flask and autoclaved at 121°C and 15 psi for 15 min. One frozen yeast sample was thawed and used to inoculate this seed flask. The seed flask was then incubated at 30°C and 175 rpm for 6 h. The fermentation flasks (three for calcium alginate immobilization and nine for Celite immobilization) consisting of 60 g/L lactate, 20 g/L peptone, and 10 g/L yeast extract were prepared and adjusted to pH values of 4, 5, and 7. These flasks were autoclaved and inoculated with 4 mL of medium from the seed flask. These flasks were incubated at 30°C and 175 rpm for 12 h. Cells were harvested by centrifugation at 2300g for 25 min and the supernatant decanted to yield a cell pellet.

**Immobilization**

Celite beads were pretreated by washing three times with deionized water and heated in a furnace at 600°C overnight to remove volatile solids. Beads were then autoclaved for 15 min at 121°C and 15 psi. The harvested cell pellet was resuspended in 50 mL of 0.02 M Tris-HCl buffer (pH 7). Five grams of Celite beads were added to the cell pellet solution and shaken at 175 rpm for 2 h at 25°C to allow cells to adhere to the Celite. The supernatant was then decanted, and the beads were washed with deionized water to remove any free cells. The Celite-immobilized cells were then dried for 2 h at ambient conditions on a paper towel.

In order to immobilize cells in calcium alginate beads, the cell pellet was resuspended in 25 mL of 20 mM Tris-HCl buffer solution (pH 7). This cell solution was then added to 75 mL of 0.75% sodium alginate. Sodium alginate solution (pH 7) was prepared and adjusted to a pH of 5. Then, 50 mL of medium was added to a 250 mL Erlenmeyer flask and autoclaved at 121°C and 15 psi for 15 min. One frozen yeast sample was thawed and used to inoculate this seed flask. The seed flask was then incubated at 30°C and 175 rpm for 24 h. From this flask, 4 mL were used to inoculate subsequent flasks (as needed) containing identical media and incubated at identical conditions for 48 h. From these flasks, 0.75 mL of broth was mixed with 0.75 mL of a 40% glycerol solution (that would act as a cryogenic preservation agent) in sterile micro-centrifuge tubes. Tubes were stored at -46°C for future use.

**Water Budget Calculations**

After immobilization, for each treatment combination, 2.5 g of dried calcium alginate beads or 5 g of dried Celite beads were placed in a bottle containing 50 mL of 0.1 M acetophenone and 0.1 M 2-hexanol in hexane. Salt hydrate pairs, which are widely used to control water activity in organic solvents (Halling, 1992), were then added to these bottles to maintain the appropriate water activity (0.80, 0.61, or 0.37). In order to calculate the appropriate theoretical amount of each salt hydrate to add to each bottle, a water budget was calculated for each reaction condition (Kvittingen et al. 1992). Water sorption isotherms were generated by air-drying beads at ambient conditions to various levels and measuring both moisture content (wet basis) and water activity with an AquAlab Series 3 water activity meter (Decagon Devices, Pullman, Wash.).

Considering that hexane becomes saturated with water when briefly exposed to ambient air, it was assumed that the hexane was saturated with water when it was originally added to the reaction mixture. That is, the original water activity was 1.0. The desired water activity level was directly related to the water mole fraction with respect to hexane and the appropriate salt hydrate pair, as shown in table 1.

It was assumed that water in the reaction medium is only bound to the hexane and not to the acetophenone or 2-hexanol because: (1) the volume of both reactants in the hexane is extremely small compared to the hexane volume, and (2) this amount of water is two orders of magnitude less than the amount of water stored in the cells, so it is relatively insignificant in this calculation. For each treatment, 150% of the theoretically calculated salt hydrate was added along with 50% of that theoretical weight of the alternate pair. This was done as a factor of safety to make sure that there was enough of each form to maintain the appropriate water activity.

**Reaction**

The bottles were placed in an incubator at 25°C and 175 rpm for the duration of the reaction. Preliminary data showed that the reaction proceeded at a constant rate (the initial reaction rate) for the first 4 to 6 h, and then the product concentration leveled off within about 15 to 18 h. Accordingly, 1.6 mL samples were taken at 0, 2, and 24 h for analysis. The samples were taken by electronic pipette and placed in 2 mL crimp-top vials with silicate seals to contain the hexane. Samples were immediately analyzed by high-performance liquid chromatography (HPLC).

**Analysis**

All injection sample sizes were 10 µL with a 100 µL sample loop. A calibration curve for (S)-1-phenylethanol was constructed with known concentrations. Samples were analyzed for both the desired product, (S)-phenylethanol, and its undesired enantiomer, (R)-phenylethanol, with HPLC with UV-VIS detection (Varian ProStar 320) and a chiral column (Chiralcel OB, 0.46 cm inside diameter by 25 cm length). The mobile phase was a hexane/water mixture (90/10), and the

<table>
<thead>
<tr>
<th>Water Activity</th>
<th>Water Mole Fraction</th>
<th>Salt Hydrate Pair</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>0.00006</td>
<td>--</td>
</tr>
<tr>
<td>0.80</td>
<td>0.00005</td>
<td>Na2SO4 · 10/0</td>
</tr>
<tr>
<td>0.61</td>
<td>0.00004</td>
<td>Na2HPO4 · 7/2</td>
</tr>
<tr>
<td>0.37</td>
<td>0.00025</td>
<td>Na2S2O3 · 5/2</td>
</tr>
</tbody>
</table>

Table 1. Water mole fraction required to obtain various water activities in hexane (adapted from Voutsas et al., 2001) and the corresponding salt hydrate pair, valid at 25°C (adapted from Halling, 1992).
flow rate was 0.35 mL/min. Detection time for each sample lasted 1 h, and the peak for (S)-1-phenylethanol occurred at 19.2 min. There was no (R)-phenylethanol production detected in any sample throughout the experiment. A similar result was reported by Griffin et al. (2001) using immobilized yeast in hexane. They measured an extremely high enantioselectivity for the reaction studied, and hypothesized that, for the conditions of their experiment, only one ADH enzyme remained active, while others present in the cell were inactivated. This result is consistent with the results obtained with an isolated alcohol dehydrogenase. Product concentrations, as determined by HPLC, were plotted over time. The initial reaction rate was calculated as the change in the (S)-1-phenylethanol level over time, within the first 2 h. The final product concentration was measured at 24 h, where the product versus time plot had leveled off. These two parameters (initial reaction rate and final product concentration) were used as the response variables to measure treatment effects.

**EXPERIMENTAL DESIGN**

The calcium alginate immobilization experiment was analyzed as a split plot design where the whole plots were growth pH (4, 5, or 7) and the split plots were reaction water activity (0.80, 0.61, or 0.37). Experimental units (growing *S. cerevisiae* cells) were randomly assigned to each whole plot (growth media flask at each pH). Once immobilized, these cells were randomly assigned within each split plot (reaction vessel at various water activities). Because of the nature of immobilization, where all of the cells put into the calcium alginate are ultimately immobilized, the split plot design was selected. Three complete replications of the experiment were performed.

The Celite immobilization experimental design consisted of a $3 \times 3$ factorial treatment structure in a completely randomized design. Experimental units (cells in each growth media flask) were randomly assigned to each treatment. The pH treatments were growth media with pH of 4, 5, or 7. Water activity treatments were reaction media with $a_w$ of 0.80, 0.61, or 0.37. With this immobilization technique, the amount of cells added during the immobilization step. As a result, a full factorial was a better experimental design, where the effectiveness of cell immobilization will also be a factor in the final performance of the immobilized cells. Three complete replications of the experiment were performed.

**RESULTS AND DISCUSSION**

**CELL LOADING**

Cell loading of calcium alginate beads was determined under the assumption that all cells harvested from fermentation flasks were completely immobilized in beads. Results are shown in table 2.

### Table 2. Cell loading for calcium alginate beads with immobilized cells grown at various pH (mg dry cell weight/g beads).

<table>
<thead>
<tr>
<th>Growth pH</th>
<th>Cell Loading (mg dcw/g bead)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>4.1</td>
<td>0.15</td>
</tr>
<tr>
<td>5</td>
<td>4.9</td>
<td>0.58</td>
</tr>
<tr>
<td>7</td>
<td>5.5</td>
<td>0.49</td>
</tr>
</tbody>
</table>

**WATER BUDGET CALCULATIONS**

Figures 2 and 3 show the sorption isotherm describing the relationship between moisture content and water activity. After drying at ambient conditions for 2 h on a paper towel, most of the free water on the outside of the beads had evaporated, and the water activity of the beads was consistently measured at about 0.99 for the calcium alginate beads and 0.95 for the Celite beads.

**INITIAL REACTION RATE**

Table 3 shows the main effects of the treatment combinations on the IRR of (S)-1-phenylethanol by *S. cerevisiae* immobilized in calcium alginate beads. Reaction water activity was the only treatment to show a significant effect, with $a_w = 0.61$ yielding the highest IRR. This water activity showed an IRR that was 99% higher than $a_w = 0.80$ and 829% higher than $a_w = 0.37$. Although this number seems high, it should be noted that there was no initial reaction rate detected at $a_w = 0.37$ with cells grown at pH 4. An analysis of variance showed that the interaction of growth medium pH and water activity was not significant ($\alpha = 0.05$).

Similar, but more limited studies with the same yeast strain and the same reaction were carried out by Gervais (2002). The study involved yeast grown at a pH of 5,
immobilized in calcium alginate with no control of water activity in the hexane, and resulted in an IRR value of 700 (μmol/h/g dcw), which falls within the range of values observed in this study.

An analysis of variance for the effects of growth medium pH, reaction water activity, and their interaction on the initial reaction rate (IRR) of (S)-1-phenylethanol production showed that the effect of growth medium pH on IRR was highly significant (α = 0.01) and the effect of reaction water activity was also significant (α = 0.05). The interaction of the two, however, was not significant. Table 4 shows the treatment combination means as well as the main effect means on IRR of (S)-1-phenylethanol production by \textit{S. cerevisiae}. The reactions that occurred at a water activity of 0.80 and 0.61 exhibited a 28% higher IRR than those at a water activity of 0.37. Similarly, cells grown at a pH of 5 and 7 showed an 80% greater IRR than those grown at pH of 4.

**Final (S)-1-Phenylethanol Concentration**

Results from analysis of variance showed that the growth medium pH had a significant effect on total product formation at the 0.05 significance level. Reaction water activity was shown to be highly significant (α = 0.01), but the interaction of the two was not significant. The main treatment effects are shown in table 5. The cells grown at a higher pH (5 or 7) were not significantly different from each other, but produced more (S)-1-phenylethanol (>37%) than cells grown at pH of 4. All reaction water activities produced significantly different total product amounts. Those at \(aw = 0.61\) produced 76% more (S)-1-phenylethanol than those at \(aw = 0.80\) and 1919% more than those at \(aw = 0.37\).

An analysis of variance for effects of growth medium pH, reaction water activity, and the interactions of the two on final (S)-1-phenylethanol concentration showed that the effects of both the growth medium pH and reaction water activity were highly significant, but the interaction of the two was not significant. The individual treatment means as well as the main effect means are shown in table 6. The same trends that occurred with IRR can be seen in total product formation. The cells grown at pH 5 or 7 produced more (S)-1-phenylethanol (>46%) than those grown at a pH of 4. In addition, those cells that catalyzed the reaction at a water activity of 0.80 or 0.61 produced 39% more total product than those that catalyzed the reaction at \(aw = 0.37\).

**Growth Media pH**

All cells have an optimal pH range in which they operate most efficiently, mostly because the amino acids that make up all proteins within a cell require a certain level of protonation to function. As the proton levels increase or decrease, the cell becomes unable to carry out basic processes and dies because the pH is not within the appropriate range. There is an optimum pH value within this range at which the cell operates most efficiently. If the hydronium ion concentration deviates from this optimum level, the proteins will change shape and begin to lose efficacy, causing a reduction in the reaction rates of all cellular enzymes.

It has been shown that lyophilized enzymes in organic solvent tend to show a “pH memory” of the aqueous environment prior to freeze-drying. However, this is not likely the cause for variations in the response variables with changes in growth medium pH. First of all, lyophilized enzymes are unable to change their protonation state because there is no medium through which protons can be transferred, whereas whole cells are able to regulate internal cellular pH to a certain extent. In addition, the whole cells are not dried, so they still have a certain amount of free water surrounding them that would provide a medium through which protons could be transferred. And prior to immobilization, all cells (regardless of growth medium pH) are resuspended in the same buffer solution for the immobilization process. The only variation is in the pH of the medium in which the cells are grown. This leads to the conclusion that the growth media conditions affect the catalytic activity of the whole cells.

Alcohol dehydrogenase (ADH) is the enzyme that catalyzes the reduction of acetophenone to phenylethanol within...
the cell. The optimal pH for ADH as reported by Sigma Aldrich is 8.2, supporting the trend that cells grown at the higher pH values (5 and 7) were more effective at the bioconversion. Changes in pH can affect the structure of the alcohol dehydrogenase active site, inhibiting its catalytic activity. However, ADH is not the only enzyme involved in the reaction. Because ADH operates within the matrix of the mitochondria, the transport of reactants into the cell and products out of the cell requires crossing three membranes: the cellular membrane and the outer and inner mitochondrial membranes. Because this transport is facilitated by membrane proteins across each membrane, any loss of protein function could cause a reduction in reaction rate (Lodish et al., 2000).

This is likely the reason that the growth medium pH affected the biocatalytic activity of the yeast. Cells grown in the range of pH 5 and 7 were under conditions that were optimal for production of all enzymes involved in the bioconversion. Cells grown at pH 4 were in an environment in which the high concentration of hydronium ions caused the enzymes to lose functionality and, therefore, lose their ability to catalyze the reaction as efficiently.

**WATER ACTIVITY**

Because a certain amount of water is required for enzymes to function, reaction conditions with extremely low water activity may inhibit the catalytic activity of the yeast. Increasing the water activity (or water content) of the biocatalyst’s surroundings has been shown to increase the activity of the catalyst (Omar and Robb, 1995; Zacharis et al., 1997; Zaks and Klíbanov, 1988). As stated earlier, enzymatic conversions in organic solvents are sensitive to high amounts of water, likely due to protein aggregation and inactivation (Chowdary and Prapulla, 2002). Presumably, the same inactivation would not be a problem with whole cells, as the hydrophilic outer cellular membrane layer would prevent aggregation. However, Jayasinghe et al. (1994) found that in certain solvents (namely toluene and carbon tetrachloride) an increase in water content past the optimal value leads to a decrease in bioconversion catalyzed by yeast cells. Although hexane was not part of their study, it is possible that the same holds true with hexane.

One possibility deals with the solubility of the reactants and products. If water levels become too high, it is possible that the solubility advantages achieved with bioconversions in organic solvents could become nullified, with the molecules of interest being less soluble in water. This possibility was supported by the results from the experiment with calcium alginate immobilized yeast cells. However, Celite in organic solvent has the unique ability to maintain a constant water activity over a range of water concentrations (De Martin et al., 1999). Controlling the water activity may have resulted in the entrapment of water in the pores of the Celite or because of ionic interaction between the Celite and water. In the study by De Martin et al. (1999), the Celite pore size and the organic solvent were different; however, the same ability to control water activity may have affected the conditions in the hexane in this study. This property may interact with the salt hydrate pairs’ ability to control water activity and therefore maintain a water activity different from the planned \( a_{w} \). This theory was unable to be tested because the hexane interfered with the sensor in the AquaLab water activity meter, rendering it ineffective.

Another possibility is that the high levels of salts used to maintain water activity may affect the integrity of the calcium alginate beads. If the beads rupture in the hexane, then the cells would be unprotected from the organic solvent and may become inactivated.

**IMMOBILIZATION**

It is difficult to compare the initial reaction rate results from the two experiments with each other because the rates for calcium alginate immobilized cells are normalized with respect to the dry cell mass within the immobilization matrix, and the rates from cells immobilized on Celite are not. This was because the amount of cells immobilized on Celite was difficult to quantify. In the calcium alginate immobilization process, it is reasonable to assume that all cells within the cell pellet became entrapped in the beads, and therefore the amount of cells in the beads can be determined. However, the Celite immobilization process is not as clear, in that there are still some cells within the suspension solution after the immobilization process. The presence of cells in solution was confirmed by plating out samples of the suspension solution before and after the immobilization process. There was no detectable difference between plate counts before and after immobilization, so the number of cells immobilized could not be determined. Cells were obviously immobilized because the reaction progressed as expected, but quantifying the immobilized cell mass was beyond the scope of this study. If the initial reaction rates are compared without being normalized with respect to cell mass, then the maximum initial reaction rate for Celite-immobilized cells was 8.5 times higher than calcium alginate immobilized cells (94.9 \( \mu \text{mol/h} \) compared to 11.2 \( \mu \text{mol/h} \)).

The maximum final (S)-1-phenylethanol concentration in the Celite experiment was 3.7 times higher than in the calcium alginate experiment (17.8 mM compared to 4.76 mM). It is possible that more cells were present in the Celite conversion bottles, causing the reaction to proceed further, so that if the final concentrations were normalized for cell mass they would be more equivalent. Another possibility deals with diffusion limitations. The Celite immobilization process is an adhesion process, and all the cells are on the surface of the immobilization matrix, causing very little diffusion limitations. Because there are cells trapped within the calcium alginate beads, it is possible that there are diffusion limitations present, which could slow the reaction catalyzed by calcium alginate immobilized cells.

**CONCLUSIONS**

In the calcium alginate immobilization experiment, it was found that the growth medium pH only had a significant effect on the final (S)-1-phenylethanol concentration, with cells grown at a pH of 5 and 7 being the most effective biocatalysts. The treatment that produced the highest initial reaction rate (916.2 \( \mu \text{mol/h/g dcw} \)) was a reaction water activity of 0.61. The same treatment combination yielded the highest final product concentration (4.76 mM).

In the Celite experiment, the growth medium pH had significant effects on both the initial reaction rate and the final product concentration. Cells grown at a pH of 5 or 7 yielded the highest initial reaction rate and final product concentration. The reaction water activity also had significant effects
on both the initial reaction rate and final concentrations, with water activities of 0.61 and 0.80 producing more product at a higher rate than a water activity of 0.37. The highest observed initial reaction rate (94.91 μmol/h) occurred with cells grown at a pH of 5 or 7 and a reaction water activity of 0.61 or 0.80. The highest final (S)-1-phenylethanol concentration (17.8 mM) occurred with the treatment combination of growth media pH 5 or 7 and reaction water activity of 0.61 or 0.80.

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