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# Regioselective biocatalytic hydrolysis of (*E*,*Z*)-2-methyl-2-butenenitrile for production of (*E*)-2-methyl-2-butenoic acid

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**Abstract**—*Acidovorax facilis* 72W nitrilase catalyzed the regioselective hydrolysis of (*E*,*Z*)-2-methyl-2-butenenitrile, producing only (*E*)-2-methyl-2-butenoic acid with no detectable conversion of (*Z*)-2-methyl-2-butenenitrile. (*E*)-2-Methyl-2-butenoic acid, produced in aqueous solution as the ammonium salt, was readily separated from (*Z*)-2-methyl-2-butenenitrile, and isolated in high yield and purity. The combination of nitrile hydratase and amidase activities of several *Comamonas testosteroni* strains were also highly regioselective for the production of (*E*)-2-methyl-2-butenoic acid from (*E*,*Z*)-2-methyl-2-butenenitrile.


## 1. Introduction

(*E*)-2-Methyl-2-butenoic acid (**1**), commonly known as tiglic acid, and (*Z*)-2-methyl-2-butenoic acid (**2**), commonly known as angelic acid, are useful starting materials for the preparation of flavor and fragrances, and in the preparation of pharmaceutical intermediates. **1**[1–4] and **2**[1,4,5] have each been prepared by a variety of chemical methods, including oxidation of the corresponding (*Z*)-alcohol, dehydration/hydrolysis of 2-hydroxy-2-methylbutyronitrile, carboxylation of 2-butenyl-2-lithium, and carbonylation of 2-chloro-2-butene or 2-bromo-2-butene. 2-Methyl-3-butenenitrile (**3**), a commercially-available by-product of adiponitrile manufacture,[6] can be readily isomerized to a mixture of (*E*)-2-methyl-2-butenenitrile (**4**) and (*Z*)-2-methyl-2-butenenitrile (**5**) using basic alumina,[7] but separation of this mixture by distillation prior to hydrolysis of the individual nitriles is difficult due to the similar chemical properties of the geometric isomers. Chemical processes for nitrile hydrolysis are not known to result in the regioselective hydrolysis of mixtures of geometric isomers; for example, reaction of (*E*,*Z*)-2-methyl-2-butenenitrile (**6**) with sulfuric acid at elevated temperatures followed by distillation produced a mixture of 19.5% **1** and 80.5% **2**.[8]

Enzyme-catalyzed hydrolysis of nitriles to the corresponding carboxylic acids is often preferred to chemical methods because the reactions are often run at ambient temperature, do not require the use of strongly acidic or basic reaction conditions, and produce the desired product with high selectivity at high conversion.[9] Regioselectivity for hydrolysis of the (*E*)-isomer of several (*E*,*Z*)-3-substituted-acrylonitriles has been demonstrated using the recombinant nitrilase AtNIT1 from *Arabidopsis thaliana*.[10] Nitrilase substrate specificity for α,β-unsaturated nitriles can be fairly limited; *Bacillus pallidus* Dac521 nitrilase showed no activity for hydrolysis of (*Z*)-2-pentenenitrile (**7**), but was capable of hydrolyzing acrylonitrile, methacrylonitrile, or crotononitrile.[11] The combined nitrile hydratase and amidase activities of *Rhodococcus* sp. AJ270 has been used for the enantioselective biotransformation of racemic β-substituted-α-methylenepropionitriles, where the amidase was shown to discriminate between the two amide hydration products produced by the nitrile hydratase.[12] The present report describes the use of *Acidovorax facilis* 72W nitrilase (Scheme 1), or the combination of nitrile hydratase and amidase activities of several strains of *Comamonas testosteroni*, for the facile preparation of **1** from **6**.

## 2. Results and discussion

### 2.1. Regioselective hydrolysis of 6 by *Acidovorax facilis* 72W nitrilase

The reaction of **4**, **5** and **6** with *A. facilis* 72W nitrilase was first performed using intact cells of *E. coli* SS1001,[13] a recombinant catalyst which expresses *A. facilis* 72W nitrilase. **4** was completely hydrolyzed to **1**, whereas under identical reaction conditions there was no detectable

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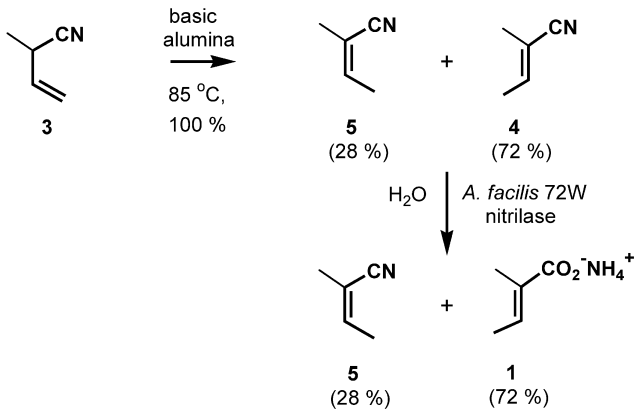


**Scheme 1.** Conversion of **3** to **1** and **5** by racemization of **3** to **6**, and subsequent regioselective hydrolysis of **4** using *A. facilis* 72 W nitrilase.

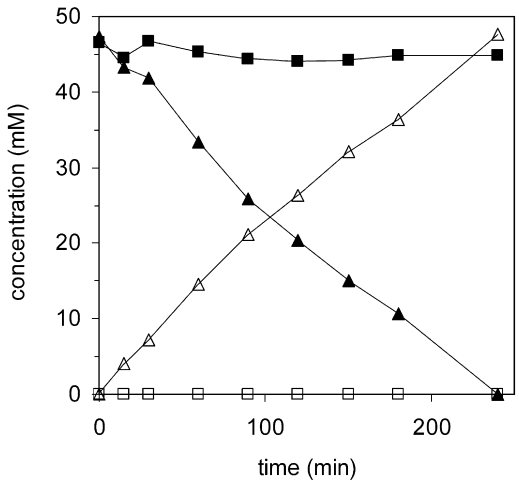


**Figure 1.** Time course for reaction of **6** ([**4**]=47 mM, [**5**]=47 mM) with *E. coli* SS1001 (9.5 mg dcw/mL) at 25 °C; **4** (▲), **5** (■), **1** (△), **2** (□).

hydrolysis of **5** (Table 1), even at extended reaction times. *E. coli* SS1001 also hydrolyzed only the (*E*)-isomer of a 1:1 mixture of **6** prepared from the two individual geometric isomers (Fig. 1), and a similar result was obtained using a 72:28 mixture of **6** prepared by isomerization of **3**. Several additional nitriles were tested as substrates for the *A. facilis* 72W nitrilase in order to determine if the presence or absence of an α-alkyl substituent, or the geometric isomerism of α,β-unsaturated nitriles, were a contributing factor for *A. facilis* 72W nitrilase substrate specificity. There was no regioselectivity observed for hydrolysis of a mixture of (*E*, *Z*)-2-pentenenitrile (**8**), and both isomers were converted to the corresponding carboxylic acids (*E*)-2-pentenoic acid (**9**) and (*Z*)-2-pentenoic acid (**10**) at similar rates. **3** was completely converted to 2-methyl-3-butenoic acid (**11**) at a significantly faster rate than for **4**.

*A. facilis* 72 W nitrilase was previously shown to regioselectively hydrolyze 2-methylglutaronitrile (**12**) or 2-methyleneglutaronitrile (**13**), with little or no hydrolysis of the cyano group adjacent to the 2-methyl- or 2-methylene substituent of these two dinitrile substrates.[14] Hydrolysis of **12** produced 4-cyanopentanoic acid (**14**, >98% yield) and 2-methylglutaric acid (**15**, <2% yield) with no detectable production of 2-methyl-4-cyanobutanoic acid at complete conversion of **12**, whereas **13** produced 4-cyano-4-pentenoic acid (**16**) as the only hydrolysis product, with no detectable hydrolysis of the cyano group vicinal to the α-methylene substituent at extended reaction times. This nitrilase has now been found to completely hydrolyze **3**, and to show no regioselectivity for hydrolysis of **8** (*E*/*Z*=22:78); in contrast, the AtNit1 nitrilase from *Arabidopsis thaliana* was completely (*E*)-regioselective for the hydrolysis of (*E*,*Z*)-2-butenenitrile.[10] The presence or absence of a methyl or methylene substituent at the α-position relative to a cyano group, and the geometric isomerism of α,β-unsaturation are each not independently predictive of the substrate specificity of *A. facilis* 72W nitrilase. The lack of significant hydrolysis of the more sterically-hindered cyano group of **12** or **13** was apparently related to the presence of a second, less-hindered cyano group, and not the presence of the α-methyl or α-methylene substituent.

## 2.2. Immobilized-cell nitrilase reactions

*E. coli* SS1001 cells were immobilized in both alginate[15] and carrageenan beads,[16] and *A. facilis* 72 W cells were immobilized in alginate beads,[15] and the resulting immobilized cell catalysts examined for the hydrolysis of **4** in **6** (*E*/*Z*=72:28) (Table 2). At concentrations of **6** greater than ca. 1.1 wt% (0.14 M) at 25 °C, the aqueous phase of the reaction mixture was saturated with **6**, and a second organic

**Table 1**. Regioselectivity of *E. coli* SS1001 and *A. facilis* 72W nitrilase

| Substrate | Conc. (mM) | Catalyst | mg dcw/mL[a] | Temp. (°C) | Time (h) | Product or recovered substrate (% yield)[b] |
|---|---|---|---|---|---|---|
| **4** | 99 | *E. coli* SS1001 | 9.5 | 35 | 2.5 | **1** (98) |
| **5** | 98 | *E. coli* SS1001 | 9.5 | 35 | 3 | **5** (100) |
| **6** (*E*/*Z*=1:1) | 95 | *E. coli* SS1001 | 9.5 | 25 | 4 | **1** (99), **5** (100) |
| **6** (*E*/*Z*=1:1) | 199 | *E. coli* SS1001 | 9.5 | 35 | 6 | **1** (99), **5** (100) |
| **6** (*E*/*Z*=72:28) | 101 | *E. coli* SS1001 | 9.5 | 25 | 6 | **1** (96), **5** (100) |
| **6** (*E*/*Z*=72:28) | 104 | *A. facilis* 72W[c] | 12 | 25 | 16 | **1** (100), **5** (100) |
| **7** | 99 | *E. coli* SS1001 | 9.5 | 35 | 2 | **10** (100) |
| **8** (*E*/*Z*=22:78) | 101 | *E. coli* SS1001 | 9.5 | 35 | 4 | **9** (100), **10** (100) |
| **3** | 101 | *E. coli* SS1001 | 9.5 | 35 | 1 | **11** (100) |
| **12** | 100 | *E. coli* SS1001 | 10 | 25 | 1 | **14** (98.7), **15** (1.3) |
| **13** | 100 | *A. facilis* 72W[c] | 13 | 27 | 2 | **16** (100) |

[a] Dry cell weight (dcw) *E. coli* SS1001 or *A. facilis* 72W unimmobilized cells.
[b] For reactions with **6** and **8**, yields were based on initial concentration of corresponding isomer.
[c] A suspension of *A. facilis* 72W cells in 0.35 M phosphate buffer (pH 7.0) was heated at 50 °C for 0.5 h prior to use to inactivate undesirable nitrile hydratase/amidase activity.[14]

**Table 2**. Regioselective hydrolysis of **6** using immobilized nitrilase catalysts

| Substrate | Conc. (M) | Catalyst[a,b] | Temp. (°C) | Time (h) | Product or recovered substrate (% yield)[c] |
|---|---|---|---|---|---|
| **6** (*E*/*Z*=72:28) | 2.00 | *E. coli* SS1001/alginate beads | 35 | 22 | **1** (100), **5** (100) |
| **6** (*E*/*Z*=72:28) | 0.40 | *E. coli* SS1001/carrageenan beads | 35 | 18 | **1** (96), **5** (100) |
| **6** (*E*/*Z*=72:28) | 0.40 | *A. facilis* 72W/alginate beads | 35 | 18 | **1** (100), **5** (100) |

[a] 7.5% dry cell weight (dcw) *E. coli* SS1001 or *A. facilis* 72W immobilized in 2.75 wt% alginate or 3.0 wt% carrageenan.
[b] 20 wt% immobilized cell catalyst beads in reaction mixture.
[c] Yields based on initial concentration of corresponding isomer.

**Table 3**. Hydrolysis of **4**, **5**, and **6** at 25 °C using *C. testosteroni* microbial cell catalysts

| Substrate | Conc. (M) | *C. testosteroni* catalyst | mg dcw/mL[a] | Time (h) | Product or recovered substrate (% yield)[b] |
|---|---|---|---|---|---|
| **4** | 0.10 | 5-MGAM-4D | 8 | 0.25 | **1** (100) |
| **5** | 0.10 | 5-MGAM-4D | 8 | 0.25 | **5** (73), **18** (27), **2** (0) |
| **4** | 0.10 | S5C | 10 | 2.5 | **1** (99) |
| **5** | 0.10 | S5C | 10 | 2.5 | **5** (75), **18** (23), **2** (0) |
| **4** | 0.10 | S2B-1 | 12 | 1.5 | **1** (99) |
| **5** | 0.10 | S2B-1 | 2.4 | 1.5 | **5** (92), **18** (8), **2** (0), |
| **6** (*E*/*Z*=72:28) | 0.10 | S2B-1 | 1.2 | 47 | **1** (100), **5** (18), **18** (75), **2** (0) |

[a] Dry cell weight (dcw) *C. testosteroni* unimmobilized cells.
[b] For hydrolysis of **6**, yields were based on initial concentration of corresponding isomer.

phase consisting of undissolved **6** was present; rapid stirring of this mixture produced an emulsion of **6** in the aqueous phase containing the suspended catalyst beads, and complete conversion of the **4** was obtained. Reactions employing the immobilized-cell nitrilase catalysts were run in the absence of added buffer. As the reaction proceeded, the reaction mixture was self-buffering at a pH of ca 7.2 due to the formation of the ammonium salt of **1**, and the reaction was run to complete conversion of **4** with no pH control. At the end of a reaction, the catalyst was recycled by decanting the product mixture from the immobilized-cell catalyst and adding a fresh charge of water and **6** (*E*/*Z*=72:28); no significant loss of activity of the catalyst over 2–3 catalyst recycles was observed.

The nitrilase activity of the immobilized cell biocatalysts was stable at concentrations of **6** as high as 2.0 M (as a two-phase reaction) and to similar concentrations of **1** over the course of a single reaction, as well as over the course of a series of reactions which employed catalyst recycle. The nitrilase utilizes the sulfhydryl group of a cysteine in the active site of the enzyme for its catalytic activity,[13] and α,β-unsaturated nitriles, amides and carboxylic acids can be highly reactive towards sulfhydryl groups, irreversibly inactivating the enzyme.[17] The lack of significant inactivation of enzyme activity by the starting material or product is an indication of the robustness of *A. facilis* 72W nitrilase in the present application. The production of **1** as the ammonium salt allows for both the facile removal of unreacted **5** by extraction, and for recovery of **1** in the acid form in high yield and purity. Although not demonstrated in the present work, it should be possible to recover unreacted **5** in high purity, and convert it to **2** using an appropriate biological or chemical catalyst.

### 2.3. Regioselective hydrolysis of 6 using microbial nitrile hydratase/amidase catalysts

Microbial cell catalysts having a combination of nitrile hydratase and amidase activities can also be used to separate **6** into a mixture of products that are readily separated by extraction. The nitrile hydratase of *Comamonas testosteroni* 5-MGAM-4D,[14] *C. testosteroni* S5C, and *C. testosteroni* S2B-1 were each found to have significantly greater specific activity for hydration of **4** to (*E*)-2-methyl-2-butenamide (**17**) than for hydration of **5** to (*Z*)-2-methyl-2-butenamide (**18**). Similarly, the amidase of these three microbial catalysts were each found to have significantly greater specific activity for hydrolysis of **17** to **1** than for hydrolysis of **18** to **2** (Table 3). A time course for the reaction of **6** (*E*/*Z*=72:28) with *C. testosteroni* S2B-1 is presented in Figure 2. S2B-1 completely hydrolyzed both geometric isomers of **8** (*E*/*Z*=22:78) to the corresponding acids, so regioselectivity of these nitrile hydratase/amidase biocatalysts was limited to (*E*)-and (*Z*)-2-methyl-substituted α,β-unsaturated nitriles.

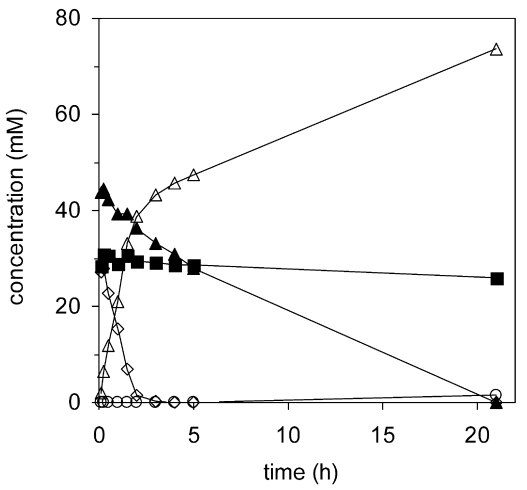


**Figure 2.** Time course for reaction of **6** ([**4**]=74 mM, [**5**]=29 mM) with *C. testosteroni* S2B-1 (1.2 mg dcw/mL) at 25 °C; **4** (▲), **5** (■), **1** (△), **18** (○), **17** (◇).

*C. testosteroni* S2B-1 and *C. testosteroni* S5C were each isolated and characterized for the first time in this application. The nitrile hydratase and amidase activities of *C. testosteroni* 5-MGAM-4D have previously been reported as catalysts for the regioselective hydrolysis of aliphatic α,ω-dinitriles to the corresponding ω-cyanocarboxylic acids.[14] In that application, *C. testosteroni* 5-MGAM-4D exhibited both non-regioselective and regioselective nitrile hydratase activities, and heating a suspension of *C. testosteroni* 5-MGAM-4D at 50 °C for 30–60 min was required to inactivate the non-regioselective nitrile hydratase activity of the microbial cell catalyst. For regioselective production of **1** by hydrolysis of **6**, no heat-treatment of *C. testosteroni* 5-MGAM-4D cells was required.

## 3. Conclusions

A method has been developed for the facile preparation of **1** from **6**, where **6** was first prepared by the facile isomerization of commercially-available **3**. Although *C. testosteroni* microbial cell catalysts had higher specific activities for hydrolysis of **4**, regioselective hydrolysis of **6** by *A. facilis* 72W nitrilase was preferred over the combination of nitrile hydratase and amidase activities of *C. testosteroni* 5-MGAM-4D, S2B-1 or S5C, since the latter catalysts each produced significant conversion of **5** to the corresponding amide at complete conversion of **4** to **1**, whereas there was no detectable conversion of **5** by the nitrilase at extended reaction times. The immobilized-cell nitrilase catalysts were robust under reaction conditions employing high concentration of **6** and catalyst recycle, and high yields of **1** were obtained with the added advantages of low temperature and energy requirements, and low waste production when compared to chemical methods of nitrile hydrolysis.

## 4. Experimental

### 4.1. General

Chemicals were obtained from commercial sources unless otherwise noted, and used as received. Isolated yields are unoptimized and melting points uncorrected. **3**[18] and **4**[18] were each isolated from a mixture of **6** by fractional distillation under vacuum using a 10-plate Oldershaw column and a reflux ratio of >10:1. The calculated recovery of nitriles and yields of the hydrolysis products were based on initial nitrile concentration, and determined by HPLC using a refractive index detector and either a Supelcosil LC-18 DB column (30 cm×4.6 mm dia.) and 10 mM acetic acid/10 mM sodium acetate in 2.5% methanol/water as mobile phase (for hydrolysis of **4** and **6**), or a Supelcosil LC-18 DB column (15 cm×4.6 mm dia.) and 10 mM acetic acid/10 mM sodium acetate in 7.5% methanol/water as mobile phase (for hydrolysis of **3**, **7**, and **8**). Gas chromatographic analysis of nitriles was performed on a J&W Scientific DB1701 column (30 m, 0.53 mm ID, 1 μm film thickness). Chemical shifts for $^{1}H$ and $^{13}C$ NMR spectra are expressed in parts per million positive values downfield from internal TMS. Identification of hydrolysis products of **6**, **3** and **8** were made by comparison of HPLC retention times to commercially available samples.

The isolation and growth of *A. facilis* 72W,[14,19] *E. coli* SS1001,[13] and *C. testosteroni* 5-MGAM-4D[14] has been reported. *C. testosteroni* S2B and S5C were isolated from soil samples using standard enrichment procedures and S12-N medium (S12 medium with ammonium sulfate replaced with sodium sulfate),[20] then grown aerobically in E2 medium containing 0.2% (w/v) 3-hydroxyvaleronitrile at 30 °C. Cell paste isolated from fermentation was frozen at −80 °C without pre-treatment with glycerol or DMSO. Wet cell weights (wcw) of microbial catalysts were obtained from cell pellets prepared by centrifugation of fermentation broth or cell suspensions in buffer. Dry cell weights (dcw) were determined by microwave drying of wet cells. Microbial cell enzyme activity was measured by stirring a suspension of 8.5–12.5 mg dry cell weight/mL in 25 mM phosphate buffer (pH 7.0) and 0.14 M substrate at 25 °C. *E. coli* SS1001 cells were immobilized in both alginate[15] and carrageenan beads,[16] and *A. facilis* 72 W cells were immobilized in alginate beads,[15] using previously reported procedures.

### 4.2. Isomerization of 2-Methyl-3-butenenitrile (3)

A mixture of **3** (50 g, 0.62 mol) and activity I basic alumina (5 g) was heated with stirring at 85 °C. After 18 h, the mixture was cooled to ambient temperature and filtered to yield 49 g (98% isolated yield) of a 72:28 (mole/mole) mixture of (*E*)- and (*Z*)-2-methyl-2-butenenitrile, determined by analysis of the reaction product by gas chromatography.

### 4.3. Isomerization of (*Z*)-2-pentenitrile (7)

A mixture of **7** (402.7 g, 4.96 mol), triphenylphosphine (16.0 g, 61.1 mmol), and zinc chloride (8.4 g, 61.6 mmol) (anhydrous) was heated at 70 °C for 22 h. The resulting mixture was cooled to ambient temperature, the insoluble zinc chloride filtered from the mixture, and the mixture vacuum distilled at 82 °C and 130 Torr to separate the mixture of (*Z*)- and (*E*)-2-pentenitriles from triphenylphosphine and soluble zinc chloride. The resulting distillate was analyzed by gas chromatography, using authentic samples of (*Z*)- and (*E*)-2-pentenitrile as standards; the ratio of (*Z*)- and (*E*)-isomers in the distillate was 78:22 (mole:mole).

### 4.4. General procedure for reactions using unimmobilized cells

In a typical procedure, an aqueous solution (10.0) mL containing 0.10 M nitrile, cell paste (0.50 g wcw) and 50 mM potassium phosphate buffer (pH 7.0) was stirred at 35 °C At pre-determined times, a 0.100 mL aliquot of the reaction was removed and mixed with 0.900 mL of 60 mM *N*-ethylacetamide (HPLC external standard) in 1:1 acetonitrile:methanol, the sample centrifuged, and the supernatant analyzed by HPLC. Results are tabulated in Tables 1 and 3.

### 4.5. Immobilized-cell catalyst recycle reactions

In a typical procedure, distilled, deionized water (15.0 mL),

0.20 M calcium acetate buffer (0.2 mL) (pH 7.0, 2.0 mM final calcium ion concentration in reaction mixture), glutaraldehyde/polyethylenimine-crosslinked *E. coli* SS1001 cell/alginate beads (4.0 g), and **6** (0.802 mL, 0.656 g) of (*E*/*Z*=72:28, 0.404 M total concentration) were placed in a 50 mL jacketed reaction vessel equipped with an overhead stirrer (temperature-controlled at 35 °C with a recirculating temperature bath), and the mixture stirred at 35 °C. After 20 h, the conversions of **4** and **5** were 100 and 0%, respectively, and the yields of **1** and **2** were 100 and 0%, respectively. The product mixture was decanted from the catalyst beads, and distilled, deionized water (15.1 mL), 0.20 M calcium acetate buffer (pH 7.0) (0.2 mL) and **6** (0.809 mL, 0.662 g, 0.407 M) (*E*/*Z*=72:28) was again mixed with the immobilized-cell catalyst at 35 °C. After 20 h, the conversions of **4** and **5** were 100 and 0%, respectively, and the yields of **1** and **2** were 97 and 0%, respectively. At the completion of the second reaction with catalyst recycle, the final concentrations of **1** and **5** were 357 and 78.9 mM, respectively.

**4.5.1. Isolation of (*E*)-2-methyl-2-butenoic acid (1) from hydrolysis of (*E*,*Z*)-2-methyl-2-butenenitrile (6) with alginate-immobilized *E. coli* SS1001 cells.** Into a 250 mL jacketed reaction vessel equipped with an overhead stirrer (temperature-controlled at 35 °C with a recirculating temperature bath) was placed GA/PEI-crosslinked *E. coli* SS1001 cell/alginate beads (30 g). To the reaction vessel was added distilled, deionized water (98.5 mL), 0.20 M calcium chloride (2.0 mM final calcium ion concentration) (1.5 mL) and **6** (20.0 mL, 16.32 g, 0.201 mole) (*E*/*Z*=72:28, 1.34 M total concentration), and the mixture stirred at 35 °C. After 22 h, the conversions of **4** and **5** were 100 and 0%, respectively, and the yields of **1** and **2** were 100 and 0%, respectively. The product mixture was decanted from the immobilized cell catalyst, and the catalyst washed twice with aqueous 2 mM calcium chloride (75 mL). The combined aqueous washes and decantate were extracted twice with ethyl ether (100 mL) to remove unreacted **5**, then the aqueous phase was adjusted to pH 1.5 with concentrated hydrochloric acid. After saturation with sodium chloride, the aqueous phase was extracted four times with ethyl ether (100 mL), the organic extracts combined, dried with magnesium sulfate, and the solvent removed by rotary evaporation. The resulting white solid was dried to constant weight under vacuum to yield (*E*)-2-methyl-2-butenoic acid (13.2 g, 90% isolated yield based on **4**): mp 63.6–64.8 °C (reported 64.5–65.5 °C);[4] $^1$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 1.81–1.87 (6 H, m, $H_3CCCO_2H$, $HCCH_3$), 6.99–7.03 (1 H, d of quartets, $J$=7.0, 1.3 Hz, $HCCH_3$), 12.26 (1 H, bs, $CO_2H$); $^{13}$C NMR (126 MHz, $CDCl_3$) $\delta_C$ 11.6, 14.6, 128.2, 140.0, 173.9; MS (CI) *m*/*z* 101 ($MH^+$, 100), 100 (30), 83 (72), 69 (58); HRMS calcd for $C_5H_9O_2$ ($MH^+$) 101.0603, found 101.0605.

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