Harnessing Deep Eutectic Solvents and Mesoporous Silica Nanospheres Immobilization for Supercharged Amine Dehydrogenase Catalysis in Continuous Flow

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Contents

General Information

All chemicals and reagents were purchased from Aladdin (Shanhai, China) unless specified otherwise. Nicotinamide adenine dinucleotide oxidised (NAD^+) and reduced (NADH) forms were purchased from Macklin (Shanhai, China). *Escherichia coli* BL21(DE3) Star competent cells were preserved in our laboratory. Specific activity and kinetic parameters of *Cal*AmDH and GDH were measured using SpectraMax iD3 (Molecular Devices, USA). GC analysis was performed on a Shimadzu-2030 gas chromatograph with a flame ionization detector (FID) and nitrogen as the carrier gas. Agilent J&W CP-Chiralsil-DEX CB capillary column (25 m × 0.25 mm × 0.25 µm) was used for determining the conversions and enantiomeric excess (ee).

Method section

Protein Expression and Purification.

The synthesized *Cal*AmDH and GDH genes (Sangon Biotech, Shanghai) were inserted into the expressio n vector pET-28a (+) and then transformed into *Escherichia coli* BL21 (DE3) for heterologous expression. A 6×His-tag was added to the N-terminal of the target protein for subsequent. These cells were cultured in Terrific Broth (TB) medium containing 50 μg mL^{-1} kanamycin at 37 °C until reaching an OD600 of 0.6-0.8. IPTG was added at 0.5mM final concentration to start the protein induction and the cells were further grown 20 h at 20 °C. The cells were then harvested by centrifugation (5,000 × g, 4 °C, 10 min) and washed several times with precooolng potassium phosphate buffer (50 mM, pH 7.5). After resuspension, the cells were disrupted by high-pressure homogenization. The lysate was subsequently centrifuged at 13,000 × g for 45 min to remove cell debris. The target enzyme was purified from the clarified lysate using Ni-NTA affinity chromatography. The Ni-NTA column was first equilibrated with binding buffer (50 mM K_2HPO_4, 500 mM NaCl, 10 mM imidazole, pH 7.5). After loading the filtered lysate, the column was washed stepwise with washing buffers of the same base composition but containing 30 mM and 60 mM imidazole, respectively. Finally, the enzyme was eluted with elution buffers containing 300 mM and 500 mM imidazole. Each washing and elution step used 5-10 column volumes of the respective buffer. At last, the Purificated protein was stored at −80 °C. Total protein concentrations were determined by the Bradford method with bovine serum albumin as the standard. The samples were analyzed by SDS-PAGE (Supplementary Figure S1).

***Cal*AmDH activity assay.**

The activity of *Cal*AmDH was measured by monitoring the decrease in the absorbance of NADH at 340 nm using SpectraMax iD3 (Molecular Devices, USA). The standard 1 mL assay mixture contained 2.0 M NH_4Cl/NH_4OH buffer (pH 10.0), 1 mM NADH, 10 mM **1s**, and 1 mg/mL purefied *Cal*AmDH. The reaction was conducted at 37 °C for 1.0 min. One unit (U) of enzyme activity was defined as the amount of enzyme required to consume 1 μmol of NADH per minute under the assay conditions. All measurements were performed in triplicate.

GDH activity assay.

The activity of GDH was measured by monitoring the increase in the absorbance of NADH at 340 nm using SpectraMax iD3 (Molecular Devices, USA). The standard 1 mL assay mixture contained 2.0 M NH_4Cl/NH_4OH buffer (pH 10.0), 1 mM NAD^+, 10 mM glucose and 1 mg/mL purefied GDH. The reaction was conducted at 37 °C for 1.0 min. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH per minute under the assay conditions. All measurements were performed in triplicate.

Preparation of DESs

DESs were synthesized via a heating method using the molar ratios specified in Table S1. All starting materials were dried prior to use. ChCl/Bet and the corresponding hydrogen bond donors (HBDs) were weighed in the designated molar ratios, combined in a dry beaker, and sealed with parafilm to exclude moisture. The mixture was heated at 90 °C with continuous magnetic stirring for 2–6 hours until a homogeneous, clear liquid formed. After cooling to room temperature, the DESs were stored for subsequent use.

Determination of kinetic parameters

Kinetic parameters of purified *Cal*AmDH mutants against substrates (**1s**) were measured. The substrate concentrations were varied from 1-50 mM (1.0, 2.0, 3.0, 4.0, 5.0, 7.5, 10.0, 15.0, 20.0, 30.0, 40.0, 50.0 mM). The activities were measured in a 1 mL reaction system (NH_4Cl/NH_4OH, 2 M, pH 10.0), 1-50 mM substrate, 1 mg/mL purified *Cal*AmDH, 1.0 mM NADH by monitoring the change in the absorbance of NADH at 340 nm using SpectraMax iD3 (Molecular Devices, USA) for 1.0 min at 37 °C. All reactions were replicated three times and the data were fitted to the *Michaelis–Menten* equation using Origin.

Molecular docking

The structure of *Cal*AmDH protein was obtained using Swiss-Model based on the structure of leucine dehydrogenase from *Bacillus sphaericus* (PDB code: 1LEH_A) with a protein sequence identity of 41.6%.

The molecular docking experiments were conducted using AutoDock4.2 software[1], and the optimal binding site was predicted using SiteMap software[2], and used as the docking center. Firstly, NADH was docked into the protein, and then the complex structure was used for substrate docking. The docking center coordinates X, Y, and Z of NADH are -11.70, 35.41, and 21.55, The docking center coordinates X, Y, and Z of the substrate are -9.34, 25.40, and 20.80, respectively. respectively. Set the box size to a cube with a side length of 22.5 Å and set the Spacing step to 0.375. Set the maximum limit for searching conformations to 10000, use a genetic algorithm for conformational sampling and scoring, and select the optimal conformation based on the docking score for conformational sorting. Construct complexes containing both substrate and coenzyme NADH separately, and then conduct subsequent kinetic simulation studies.

Molecular dynamics simulation of *Cal*AmDH in buffer system

The simulations comprised *Cal*AmDH which were all bound with the cofactor NADH and **1s**. Throughout the simulations, this system were referred to as the pure buffer system. MD simulations and analyses were performed with the GROMACS 5.15 simulation package[3]. The AMBEff14SB forcefield[4] was used for the simulations of *Cal*AmDH in pure buffer system. The protonation state of ionizable residues was defined corresponding to pH 10.0 by Protein Prepare[5]. The NADH and **1s** model was taken from the literature with the optimized parameter set of the GAFF forcefield[6], which reproduced the properties of the water in perfect agreement with the experimental data. Structures were solvated into a cubic box of TIP3P water molecules[7] with a minimal distance of 0.1 nm from the edge of the box to the protein. The electrostatic interactions were calculated by applying the particle mesh Ewald (PME) method. Counterions Na^+ and Cl^- were used to neutralize the total net charge of the systems[8], and all resulting systems have a net charge of zero. Before MD simulations, energy minimization using the steepest descent method was performed to avoid the most unfavorable interactions. A 1000 ps NVT ensemble was performed by temperatures kept close to 298 K and followed by 1000 ps equilibration in the NPT ensemble with position restraints on the *Cal*AmDH. The production simulation time was chosen to be 50 ns at 298 K and 1 bar (time step of 2 fs). During the MD simulation, coordinates, energies, and velocities were stored every 0.05 ns for further analysis. All the observables were calculated by GROMACS analysis tools.

Molecular dynamics simulation of *Cal*AmDH in Bet-HAc-Buffer (12.5%, v/v) system

MD simulations and analysis were performed with the GROMACS 5.15 simulation package[3]. The AMBEff14SB forcefield[4] was used for the simulations of *Cal*AmDH in Bet-HAc-Buffer (12.5%, v/v) system. The protonation state of ionizable residues was defined corresponding to pH 10.0 by Protein Prepare[5]. The Bet and HAc model were taken from the literature with the optimized parameter set of the GAFF forcefield[6], which reproduced the properties of the buffer/Bet-HAc mixture in perfect agreement with the experimental data. Structures were solvated into a cubic box of TIP3P water molecules[7] with a minimal distance of 0.1 nm from the edge of the box to the protein. The simulation systems were filled with 2112 water molecules, 30 Bet molecules and 60 HAc molecules, according to the experimental conditions. The electrostatic interactions were calculated by applying the particle mesh Ewald (PME) method. Counterions Na^+ and Cl^- were used to neutralize the total net charge of the systems[8], and all resulting systems have a net charge of zero. Before MD simulations, energy minimization using the steepest descent method was performed to avoid the most unfavorable interactions. A 1000 ps NVT ensemble was performed by temperatures kept close to 298 K and followed by 1000 ps equilibration in the NPT ensemble with position restraints on the *Cal*AmDH. The production simulation time was chosen to be 50 ns at 298 K and 1 bar (time step of 2 fs). During the MD simulation, coordinates, energies, and velocities were stored every 0.05 ns for further analysis. All the observables were calculated by GROMACS analysis tools. Pymol and were applied for visualization.

Procedure for the synthesis of racemic products

Ammonium acetate (2.691 g, 35 mmol) and sodium cyanoborohydride (438 mg, 7 mmol) were added to a solution of the corresponding ketone (3.5 mmol) in anhydrous methanol (11 mL, 0.32 M). The reaction was stirred for 24 h at room temperature. After this time the solvent was evaporated under reduced pressure and the remaining residue was dissolved in distilled water (20 mL). The solution was acidified to pH 1 with concentrated HCl and then washed with EtOAc (3 × 20 mL), discarding the organic layer. Then, the aqueous phase was basified to pH 10 with an aqueous NaOH 10 M solution (5 mL) and extracted with CH_2Cl_2 (3 × 20 mL). The organic layers were collected, dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. The crude amine was purified by column chromatography on silica gel (dichloromethane/methanol 90:10)[9].

Preparation and modification of MSN.

A microemulsion containing aqueous urea (0.4 M, 100 g), CTAB (2.5 g), n-butanol (2.5 g), and cyclohexane (25 g) was formed by ultrasonication (30 kHz) in a water bath at room temperature for 30 min. With vigorous stirring, TEOS (2.5 g) was then added dropwise. The mixture was stirred at room temperature for 30 min and then reacted at 70 °C for 24 h. The synthesized MSN were centrifuged (12,000 rpm, 15 min), washed with ethanol and water, redispersed in acetone (250 mL), and refluxed at 80 °C for 48 h to remove the template. The final product (MSN) was dried at 70 °C overnight. The MSN was amino-functionalized by post-modification with APTES. In a typical procedure, 1.0 g of support was mixed with 100 mL n-hexane and 0.3 mL APTES, and the mixture was reacted at 70 °C for 24 h. The product (AMSN) was washed with ethanol and water to remove excess APTES, dried at 60 °C, and then activated with 1 wt% glutaraldehyde at room temperature for 2 h. The final glutaraldehyde-activated AMSN was washed, dried at 60 °C, and stored at 4 °C.

Preparation of *Cal*AmDH&GDH@AMSN

100 mg of glutaraldehyde-activated AMSNwas added to 3 mL of PBS buffer (50 mM, pH 7.5) and ultrasonicated (30 kHz) for 30 min at room temperature. Then 2 mL mixture of free *Cal*AmDH and GDH were added, the final protein concentration of *Cal*AmDH was 2.5 mg/mL, and GDH was 2.5 mg/mL. The mixture was shaken (150 rpm) at 25 °C. During this process, samples of 0.1 mL were withdrawn at different

intervals for protein concentration determination by Bradford assay until equilibrium was reached. At last, the obtained *Cal*AmDH&GDH@AMSN were centrifuged (6000 rpm for 15 min) and washed with PBS buffer (50 mM, pH 7.5, 5.0 mL) three times to ensure the removal of all the noncovalently-attached or loosely-bounded *Cal*AmDH and GDH.

$$\text{Protein loading yield (wt\%)}=\frac{(\mathrm{P_i}-\mathrm{P_f})\ (\mathrm{mg})}{\mathrm{P_i}\ (\mathrm{mg})}\times 100\% \qquad (1)$$

$$\text{Protein loading }(\mathrm{mg_{protein}/g_{support}})=\frac{(\mathrm{P_i}-\mathrm{P_f})\ (\mathrm{mg})}{\mathrm{w}\ (\mathrm{g})} \qquad (2)$$

$$\text{Activity recovery (\%)}=\frac{\mathrm{A_s}\ (\mathrm{U})}{\mathrm{A_i}\ (\mathrm{U})}\times 100\% \qquad (3)$$

Stability of *Cal*AmDH

The pH stability of free and immobilized *Cal*AmDH in pure-buffer

Incubation conditions: [free *Cal*AmDH] = 10 mg/mL (or [*Cal*AmDH@AMSN (254 $\mathrm{mg}_{Cal\mathrm{AmDH}}/\mathrm{g}_{\mathrm{support}}$)] = 100mg/mL), NH_4Cl/NH_4OH buffer (2.0 M, pH 10, 50 mL), or HAc-NaAc buffer (0.1 M, pH 4, 50 mL)T = 25 °C, 200 rpm. During the incubation, samples of 0.5 mL were withdrawn at different intervals for residual activity determination. The initial activity is defined as 100%.

The pH stability of immobilized *Cal*AmDH in Bet-HAc DES

Incubation conditions: [*Cal*AmDH@AMSN (254 $\mathrm{mg}_{Cal\mathrm{AmDH}}/\mathrm{g}_{\mathrm{support}}$)] = 100 mg/mL, Bet-HAc DES 6.25 mL, NH_4Cl/NH_4OH buffer (2.0 M, pH 10, 43.75 mL), or HAc-NaAc buffer (0.1 M, pH 4, 43.75 mL)T = 25 °C, 200 rpm. During the incubation, samples of 0.5 mL were withdrawn at different intervals for residual activity determination. The initial activity is defined as 100%.

The thermostability of free and immobilized *Cal*AmDH in pure-buffer

Incubation conditions: [free *Cal*AmDH] = 10 mg/mL (or [*Cal*AmDH@AMSN (254 $\mathrm{mg}_{Cal\mathrm{AmDH}}/\mathrm{g}_{\mathrm{support}}$)] = 100 mg/mL), PBS buffer (50 mM, pH 7.5, 50 mL), T = 50 °C, 200 rpm. During the incubation, samples of 0.5 mL were withdrawn at different intervals for residual activity determination. The initial activity is defined as 100%.

The thermostability of immobilized *Cal*AmDH in Bet-HAc DES

Incubation conditions: [*Cal*AmDH@AMSN (254 $\mathrm{mg}_{Cal\mathrm{AmDH}}/\mathrm{g}_{\mathrm{support}}$)] = 100 mg/mL, Bet-HAc DES 6.25 mL, PBS buffer (50 mM, pH 7.5, 43.75 mL), T = 50 °C, 200 rpm. During the incubation, samples of 0.5 mL were withdrawn at different intervals for residual activity determination. The initial activity is defined as 100%.

The storage stability of free and immobilized CalAmDH in pure-buffer at 4 °C

Incubation conditions: [free *Cal*AmDH] = 10 mg/mL (or [*Cal*AmDH@AMSN (254 $\mathrm{mg}_{Cal\mathrm{AmDH}}/\mathrm{g}_{\mathrm{support}}$)] = 100 mg/mL), PBS buffer (50 mM, pH 7.5, 50 mL), T = 4 °C.

During the incubation, samples of 0.5 mL were withdrawn at different intervals for residual activity determination. The initial activity is defined as 100%.

The storage stability of immobilized CalAmDH Bet-HAc DES at 4 °C

Incubation conditions: [*Cal*AmDH@AMSN (254 $mg_{CalAmDH}/g_{support}$)] = 100 mg/mL, Bet-HAc DES 6.25 mL, PBS buffer (50 mM, pH 7.5, 43.75 mL), T = 4 °C. During the incubation, samples of 0.5 mL were withdrawn at different intervals for residual activity determination. The initial activity is defined as 100%.

Stability of GDH

The pH stability of free GDH in pure-buffer

Incubation conditions: [free GDH] = 10 mg/mL in NH_4Cl/NH_4OH buffer (2.0 M, pH 10, 50 mL), or HAc-NaAc buffer (0.1 M, pH 4, 50 mL), T = 25 °C, 200 rpm. During the incubation, samples of 0.5 mL were withdrawn at different intervals for residual activity determination. The initial activity is defined as 100%.

The pH stability of free GDH in Bet-HAc DES

Incubation conditions: [free GDH] = 10 mg/mL, Bet-HAc DES 6.25 mL, NH_4Cl/NH_4OH buffer (2.0 M, pH 10, 43.75 mL), or HAc-NaAc buffer (0.1 M, pH 4, 43.75 mL)T = 25 °C, 200 rpm. During the incubation, samples of 0.5 mL were withdrawn at different intervals for residual activity determination. The initial activity is defined as 100%.

The thermostability of free GDH in pure-buffer

Incubation conditions: [free GDH] = 10 mg/mL, PBS buffer (50 mM, pH 7.5, 50 mL), T = 50 °C, 200 rpm. During the incubation, samples of 0.5 mL were withdrawn at different intervals for residual activity determination. The initial activity is defined as 100%.

The thermostability of free GDH in Bet-HAc DES

Incubation conditions:[free GDH] = 10 mg/mL, Bet-HAc DES 6.25 mL, PBS buffer (50 mM, pH 7.5, 43.75 mL), T = 50 °C, 200 rpm. During the incubation, samples of 0.5 mL were withdrawn at different intervals for residual activity determination. The initial activity is defined as 100%.

The storage stability of free GDH in pure-buffer at 4 °C

Incubation conditions: [free GDH] = 10 mg/mL, PBS buffer (50 mM, pH 7.5, 50 mL), T = 4 °C. During the incubation, samples of 0.5 mL were withdrawn at different intervals for residual activity determination. The initial activity is defined as 100%.

The storage stability of free GDH Bet-HAc DES at 4 °C

Incubation conditions: [free GDH] = 10 mg/mL, Bet-HAc DES 6.25 mL, PBS buffer (50 mM, pH 7.5, 43.75 mL), T = 4 °C. During the incubation, samples of 0.5 mL were withdrawn at different intervals for residual activity determination. The initial activity is defined as 100%.

Calculation Formulas

$$\text{ee (\%)}=\frac{\text{major enantiomer} - \text{minor enantiomer}}{\text{major enantiomer} + \text{minor enantiomer}}\times 100\%$$

$$\text{yield (\%)}=\frac{\text{reaction product (mM)}}{\text{starting meterial (mM)}}\times 100\%$$

The turnover number (TON) for a single batch reaction was calculated as the moles of product generated per mole of enzyme:

$$\text{TON}=\frac{\text{reaction product (mM)}}{\text{enzyme (mM)}}$$

The total turnover number (TTN) was obtained by summing the TON values over all reuse cycles:

$$\text{TTN} = \sum_{i=1}^{n} \text{TON}_i \text{ (n is the total number of reaction cycles)}$$

The space time yield (STY) in batch reactions was calculated as follows:

$$\text{STY (g/L/h)}=\frac{\text{substrate concentration (M)}\times\text{yiled (\%)}\times\text{MW(g/mol)}}{\text{time (h)}\times 100}$$

The space time yield (STY) in continuous flow reactions was calculated as follows:

$$\text{STY (g/L/h)}=\frac{\text{substrate concentration (M)}\times\text{yiled (\%)}\times\text{flow rate (L/h)}\times\text{MW(g/mol)}}{\text{reactor volume (L)}\times 100}$$

Determination of aqueous-phase substrate concentration in the presence of DES

To quantitatively evaluate the effect of Bet-HAc DES on the availability of hydrophobic substrates, the equilibrium concentrations of model substrates **1s** and **11s** in the aqueous phase were determined under conditions consistent with the enzymatic reactions. Briefly, excess amounts (200 mg) of **1s** or **11s** were added to 1.0 mL of either pure buffer (NH_4Cl/NH_4OH, 2 M, pH 9.0) or DES-containing buffer (12.5% Bet-HAc DES in NH_4Cl/NH_4OH, 2 M, pH 9.0) in 2 mL centrifuge tubes. The samples were incubated at 35 °C in a thermostatic shaker at 250 rpm for 24 h to reach equilibrium, followed by centrifugation at 10,000 × g for 10 min to achieve complete separation of the aqueous and organic phases. An aliquot (500 μL) of the aqueous phase was carefully withdrawn and extracted with an equal volume of ethyl acetate by vigorous shaking for 5 min. The mixture was then centrifuged at 10,000 × g for 5 min to separate the organic layer. The organic phase was collected, and the substrate concentration was quantified by GC. For **1s**, the GC program was as follows: initial temperature 130 °C, hold for 5 min, then increase at 5 °C/min to 200 °C, hold for 5 min. For **11s**, the GC program was: initial temperature 130 °C, then increase at 5 °C/min to 200 °C, hold for 5 min. Calibration curves were established by extracting known concentrations of **1s** and **11s** in the corresponding buffer matrices with ethyl acetate under identical conditions to account for extraction efficiency and any matrix effects. All measurements were performed in triplicate, and the results are summarized in Table **S2.**

Extraction recovery of 1p in pure buffer and DES-buffer

To evaluate the impact of Bet-HAc DES on product recovery, the extraction efficiency of the amine product was determined in both the absence and presence of DES using a controlled experimental setup. For the control group, the 1-(4-fluorophenyl)propan-2-amine (**1p**, product derived from substrate **1s**) was added to 1.0 mL of pure buffer (NH_4Cl/NH_4OH, 2 M, pH 9.0) at a final concentration of 10 mg/mL. For the experimental group, the **1p** was added to 1.0 mL of DES-buffer (12.5% Bet-HAc DES in NH_4Cl/NH_4OH, 2 M, pH 9.0) at the same final concentration of 10 mg/mL. An equal volume of ethyl acetate (1.0 mL) was added to each sample, followed by vigorous shaking for 5 min. The mixture was then centrifuged at 10,000 × g for 5 min to achieve phase separation. The organic layer was carefully collected, and the extraction was repeated twice more with ethyl acetate (three extractions in total). The combined organic phases were dried over anhydrous Na_2SO_4, and the product concentration in the organic phase was quantified by gas chromatography (GC). The extraction recovery was calculated as the percentage of the product detected in the organic phase relative to the initial amount added. All measurements were performed in triplicate.

The method for detecting the yield in the continuous flow reactor

Specifically, samples (1.0 mL) were collected from the reactor outlet after the system reached steady state (typically after three residence times). The collected samples were subjected to extraction with equal volumes of ethyl acetate (three times). The organic layer was collected, and 50 μL of acetic anhydride was added to perform derivatization. The conversion and ee values were determined by chiral gas chromatography (GC) analysis after derivatization. Detailed GC conditions are summarized in Table S5 (Supporting Information).

Figures

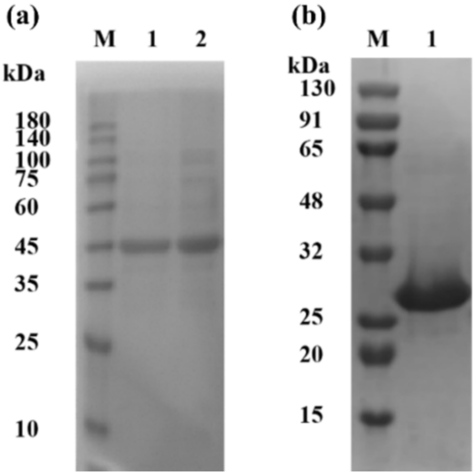


Figure S1. SDS-PAGE of (a) *Cal*AmDH (b) and GDH.

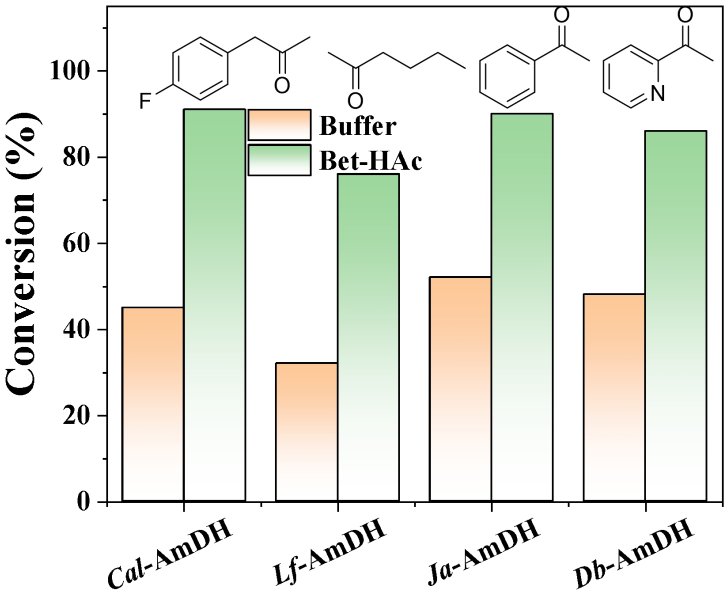


Figure S2. AmDHs-catalyzed ARA in pure buffer and Bet-HAc system.

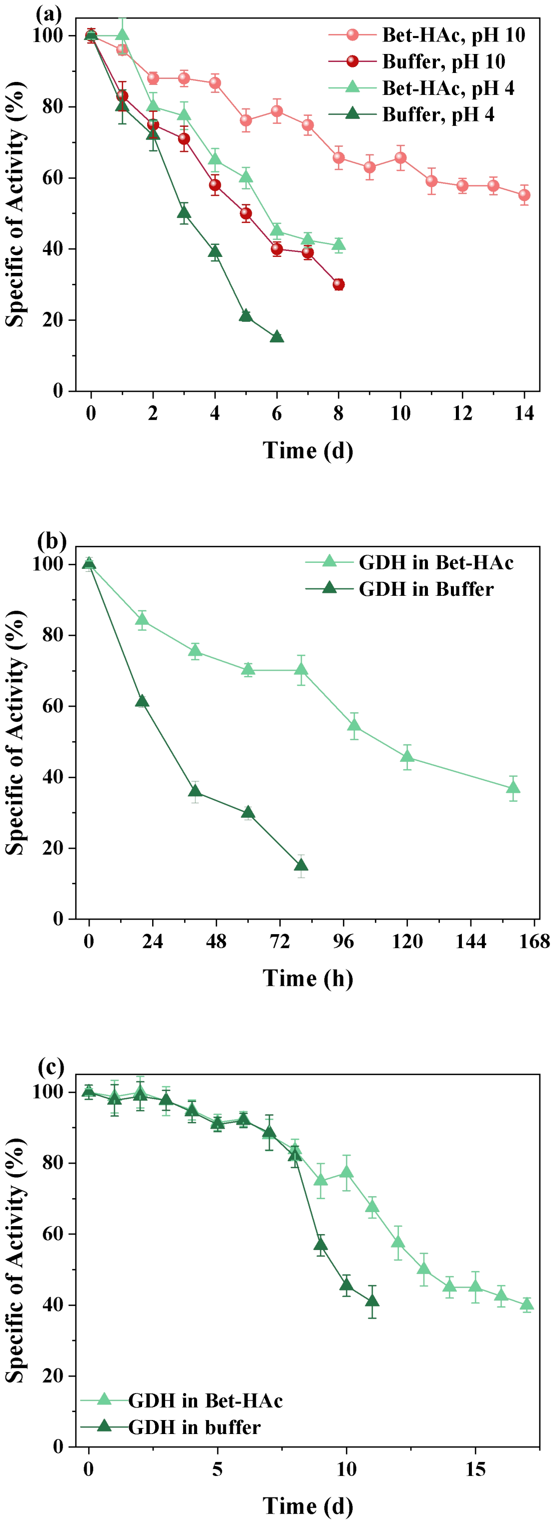
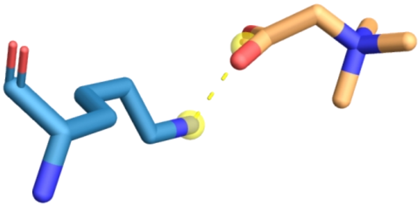
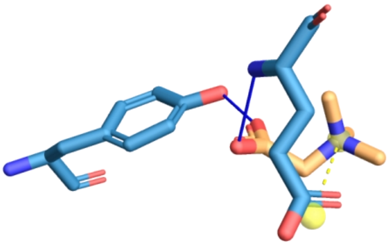


Figure S3. Stability profiles of GDH: (a) pH stability of AmDH at pH 4 and 10; (b) thermal stability at 50 °C; (c) storage stability at 4 °C. Enzyme activity before incubation was defined as 100%; error bars indicate the standard deviation of at least triplicate measurements.



Salt Bridges ····

Index	Residue	AA	Distance	Protein positive?	Ligand Group	Ligand Atoms
1	308C	LYS	2.60	✓	Carboxylate	3366, 3367

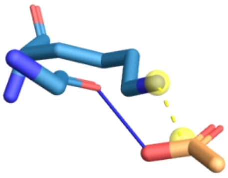


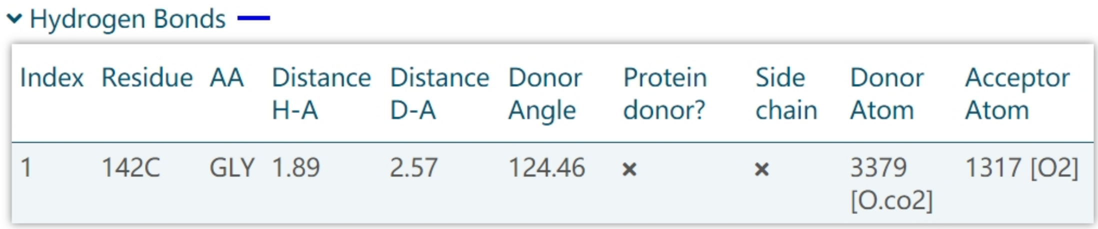
Hydrogen Bonds —

Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein donor?	Side chain	Donor Atom	Acceptor Atom
1	127C	TYR	1.75	2.70	175.59	✓	✓	1184 [O3]	3374 [O3]
2	138C	GLU	2.00	2.96	158.79	✓	×	1275 [Nam]	3375 [O3]

Salt Bridges ····

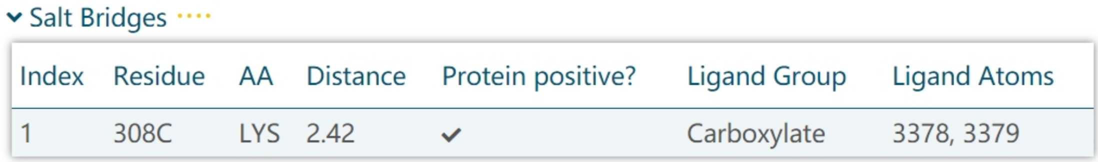
Index	Residue	AA	Distance	Protein positive?	Ligand Group	Ligand Atoms
1	138C	GLU	4.54	×	Quartamine	3368





Hydrogen Bonds —

Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein donor?	Side chain	Donor Atom	Acceptor Atom
1	142C	GLY	1.89	2.57	124.46	×	×	3379 [O.co2]	1317 [O2]



Salt Bridges ····

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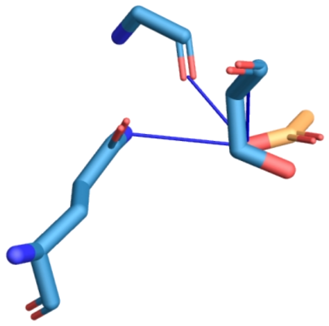
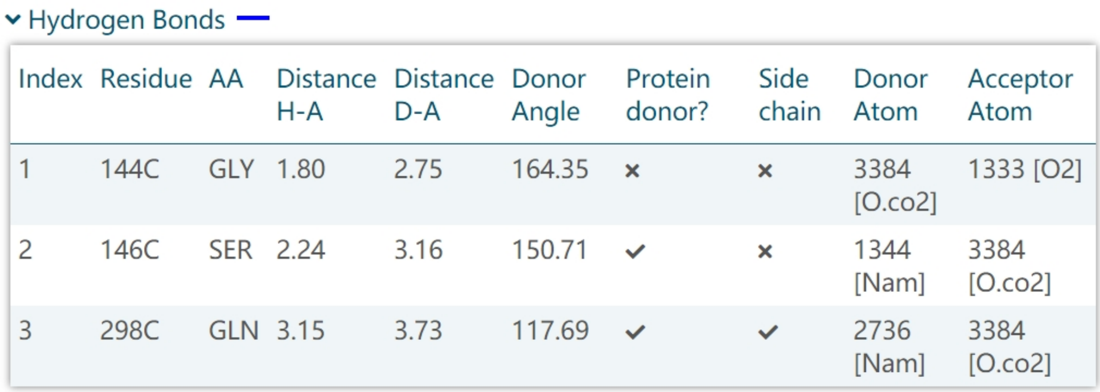
1	308C	LYS	2.42	✓	Carboxylate	3378, 3379





Hydrogen Bonds —

Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein donor?	Side chain	Donor Atom	Acceptor Atom
1	144C	GLY	1.80	2.75	164.35	×	×	3384 [O.co2]	1333 [O2]
2	146C	SER	2.24	3.16	150.71	✓	×	1344 [Nam]	3384 [O.co2]
3	298C	GLN	3.15	3.73	117.69	✓	✓	2736 [Nam]	3384 [O.co2]

Figure S4. Interactions of *Cal*AmDH with (a-b) two Bet molecules and (c-d) two HAc molecules.

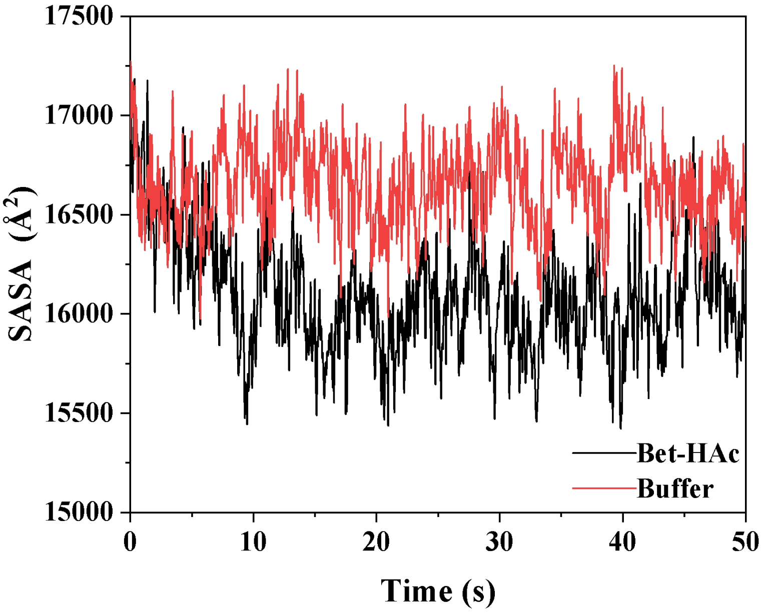


Figure S5. Solvent accessible surface area (SASA).

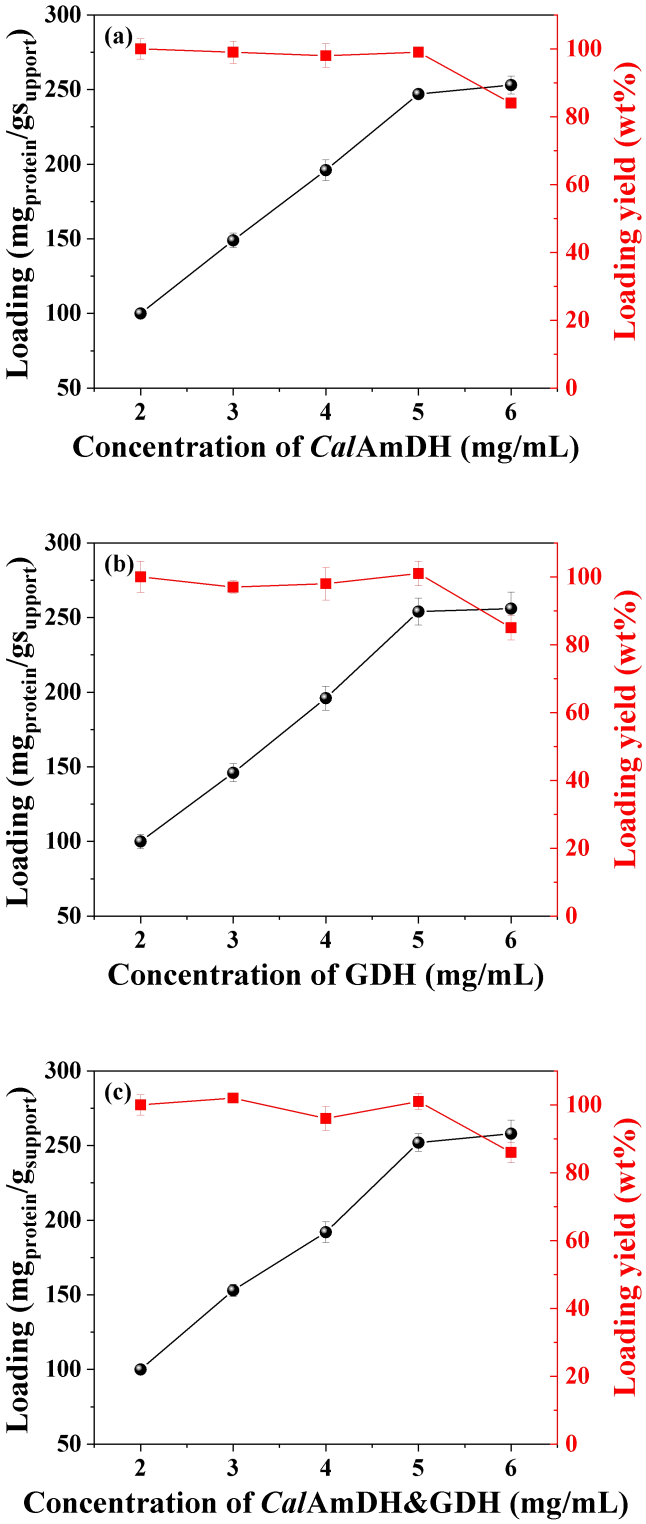
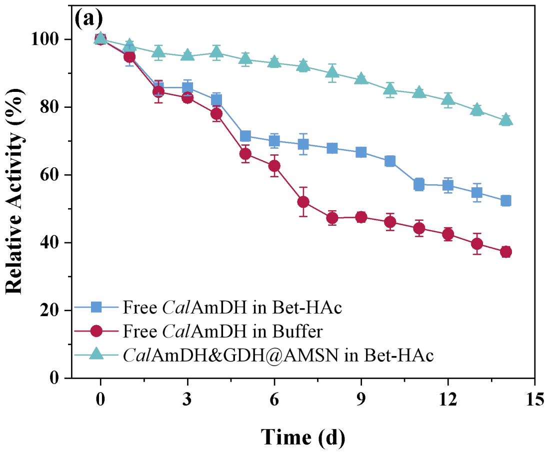
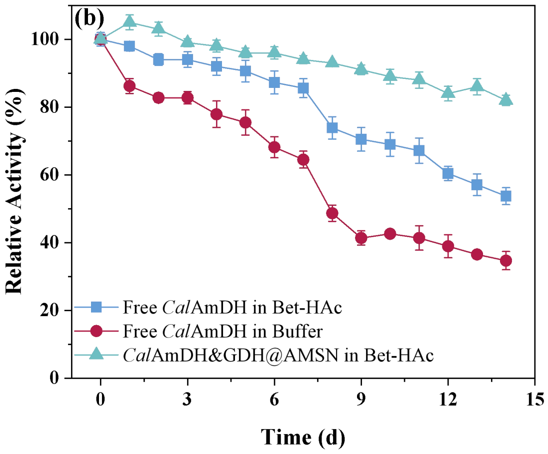
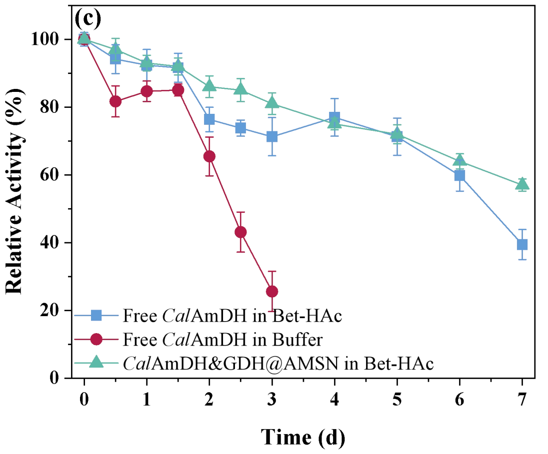


Figure S6. Protein loadings of *Cal*ADH (a), GDH (b) and *Cal*ADH&GDH (m/m = 1:1) (c) on AMSN at different enzyme concentrations. The data represent the means of three experimental replicates and error bars the standard deviation (n=3).


(a)
Relative Activity (%)
0
20
40
60
80
100
Time (d)
0
3
6
9
12
15
Free *Cal*AmDH in Bet-HAc
Free *Cal*AmDH in Buffer
*Cal*AmDH&GDH@AMSN in Bet-HAc


(b)
Relative Activity (%)
0
20
40
60
80
100
Time (d)
0
3
6
9
12
15
Free *Cal*AmDH in Bet-HAc
Free *Cal*AmDH in Buffer
*Cal*AmDH&GDH@AMSN in Bet-HAc


(c)
Relative Activity (%)
0
20
40
60
80
100
Time (d)
0
1
2
3
4
5
6
7
Free *Cal*AmDH in Bet-HAc
Free *Cal*AmDH in Buffer
*Cal*AmDH&GDH@AMSN in Bet-HAc

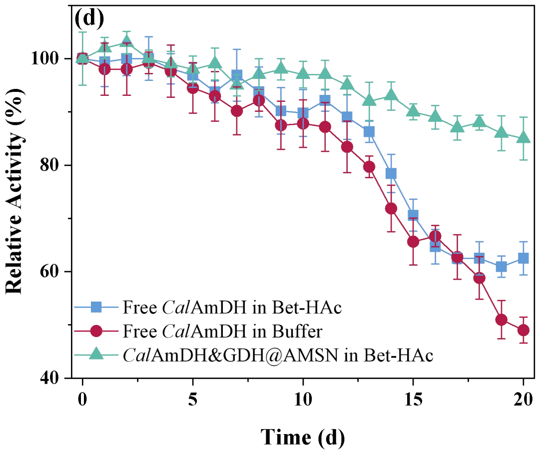


Figure S7. Stability profiles of CalAmDH: pH stability of AmDH at (a) pH 4 and (b) pH 10; (c) thermal stability at 50 °C; (d) storage stability at 4 °C. Enzyme activity before incubation was defined as 100%; error bars indicate the standard deviation of at least triplicate measurements.

Table

Table S1. Different composition of DESs.

Entry	Hydrogen Bond Donors (HBDs)	Hydrogen bond Acceptors (HBAs)	Name	Mole Ratio
1	Choline Chloride （ChCl）	Ethylene glycol （EG）	ChCl-EG	1:2
2		Diethylene glycol （DEG）	ChCl-DEG	1:2
3		Formic acid（FA）	ChCl-FA	1:2
4		Levulinic acid（Lva）	ChCl-Lva	1:2
5		Acetic acid（HAc）	ChCl-HAc	1:2
6		Propionic acid（PA）	ChCl-PA	1:2
7		Triethylene glycol （TEG）	ChCl-TEG	1:2
8		Glycerol（G）	ChCl-G	1:2
9		Urea（U）	ChCl-U	1:2
10		Maltose（Mal）	ChCl-Mal	4:1
11		D-Fructose（Fru）	ChCl-Fru	5:2
12		D-Galactose （Gal）	ChCl-Gal	5:2
13		Lactic acid （LA）	ChCl-LA	1:2
14	Betaine （Bet）	Ammonium formate （FA）	Bet-FA	1:2
15		Acetic acid（HAc）	Bet-HAc	1:2
16		ammonium propionate （PA）	Bet-PA	1:2
17		Lactic acid（LA）	Bet-LA	1:2
18		D-Galactose（Gal）	Bet-Gal	5:2
19		Maltose（Mal）	Bet-Mal	4:1
20		Levulinic acid（Lva）	Bet-Lva	1:2
21		Glycerol（G）	Bet-G	1:2
22		Ethylene glycol（EG）	Bet-EG	1:2
23		D-Fructose（Fru）	Bet-Fru	5:2
24		Glucose（Glu）	Bet-Glu	5:2
25		Citric acid（CA）	Bet-CA	4:1

Table S2. Aqueous-phase concentrations of model substrates 1s and 11s in pure buffer and DES-buffer.

Substrate	Pure buffer (mM)	DES-buffer
1s	33±3.1	62±4.3
11s	5.3±0.3	15.4±0.4

Conditions: Pure buffer (NH_4Cl/NH_4OH, 2 M, pH 9.0), DES-buffer (12.5% Bet-HAc DES in NH_4Cl/NH_4OH, 2 M, pH 9.0), equilibration at 35 °C for 24 h. Concentrations were determined by GC after ethyl acetate extraction.

Table S3 Extraction recovery of 1p in pure buffer and DES-buffer

Substrate	Pure buffer (mM)	DES-buffer
1p	94% ± 1.7%	94% ± 1.3%

Table S4. The protein loading and activity recovery of CalAmDH-GDH (co)-immobilized enzymes.

Entry	Catalyst	Protein loading ($mg_{protein}/g_{support}$)	Activity recovery of ADH (%)	Activity recovery of AmDH (%)
1	GDH@AMSN[a]	247±3	42±0.51	-
2	*Cal*AmDH@AMSN[b]	254±9	-	44±1.56
3	*Cal*AmDH&GDH@AMSN[c]	252±6	44±1.1	43±1.2

[a] Preparation conditions: [AMSN] = 100 mg, [GDH] = 5 mg/mL, 25 °C, 2.0 h; [b] Preparation conditions: [AMSN] = 100 mg, [*Cal*AmDH] = 5 mg/mL, 25 °C, 2.0 h；[c] Preparation conditions: [AMSN] = 100 mg, [*Cal*ADH] = 2.5 mg/mL, [GDH] = 2.5 mg/mL, 25 °C, 2.0 h. More details were shown in Method section. The data represent the average of three independent experiments with the standard deviation (n=3).

Table S5. Analysis methods and retention time of the products

Entry	Products	Amine retention time (min)		
		S	*R*	Methods
1p	(*R*)-1-(4-fluorophenyl)propan-2-amine	10.892	11.144	program 1
2p	(*R*)-1-(3-fluorophenyl)propan-2-amine	10.433	10.712	program 1
3p	(*R*)-1-(2-fluorophenyl)propan-2-amine	10.087	10.362	program 1
4p	(*R*)-1-(4-chlorophenyl)propan-2-amine	15.063	15.281	program 1
5p	(*R*)-1-(3-bromophenyl)propan-2-amine	17.487	17.818	program 1
6p	(*R*)-(4-methoxyphenyl)propan-2-amine	13.622	13.867	program 1
7p	(*R*)-1-(pyridin-3-yl)propan-2-amine	13.383	13.605	program 1
8p	(*R*)-4-phenylbutan-2-amine	8.637	8.809	program 2
9p	(*R*)-1-phenylpentan-3-amine	13.885	14.076	program 1
10p	(*R*)-5-phenylpentan-2-amine	14.805	15.085	program 3
11p	(*R*)-4-(benzo[d][1,3]dioxol-5-yl)butan-2-amine	14.815	15.136	program 4
12p	(*R*)-4-(thiophen-2-yl)butan-2-amine	11.236	11.473	program 5
13p	(*R*)-heptan-2-amine	14.496	14.773	program 6
14p	(*R*)-5-methylhexan-2-amine	14.288	14.475	program 6

program 1: The initial temperature was 130 °C, hold for 5 min, then, 5 °C per minute increase to 200 °C, hold for 5 minutes.

program 2: The initial temperature was 130 °C, then, 5 °C per minute increase to 200 °C.

program 3: The initial temperature was 130 °C, then, 4 °C per minute increase to 200 °C, hold for 5 minutes.

program 4: The initial temperature was 130 °C, then, 5 °C per minute increase to 200 °C, hold for 5 minutes.

program 5: The initial temperature was 130 °C, hold for 3 min, then, 5 °C per minute increase to 200 °C, hold for 5 minutes.

program 6: The initial temperature was 50 °C, hold for 2 min, then, 5 °C per minute increase to 100 °C, 20 °C per minute increase to 200 °C, hold for 5 minutes.

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