Supporting Information

1 Experimental procedures

1.1 Immobilization of glucoamylase on PMMA carrier

10 g of carrier ReliZyme HA403/M are suspended in 16 mL of potassium phosphate buffer (25 mM, pH 7, 1.6 mL $g^{-1}_{carrier}$). Then, 200 $\mu mol_{dialdehyde}$ $g^{-1}_{carrier}$) are added to the mixture. The mixture is shaken at 25°C for 2 hours. The supernatant is then removed, and the activated carrier is rinsed 3 times with 20 mL of demineralized water.

The activated carrier is resuspended in 16 mL of potassium phosphate buffer (25 mM, pH 7, 1.6 mL $g^{-1}_{carrier}$). 5 g of Dextrozyme GA (commercial glucoamylase solution; 120 U $g^{-1}_{carrier}$ referred to the amount of carrier as provided by the manufacturer) are added to the reaction mixture. The mixture is kept shaking at 25°C for 24 hours. The supernatant is then removed and tested for residual enzyme activity. The immobilized enzyme is rinsed 3 times with 20 mL of demineralized water.

The immobilized enzyme is stored at 4°C in potassium phosphate buffer (25 mM, pH 7).

1.2 Glucoamylase activity assay

The applied glucoamylase activity assay is based on the enzymatic hydrolysis of maltose to two units of glucose:

$$Maltose + H_2O \xrightarrow{glucoamylase} 2\ Glucose$$

One Immobilized Glucoamylase Unit (IGU) is defined as the amount of enzyme that hydrolyses one µmol of maltose at the following conditions: 25% maltose in 10 mM citrate buffer, pH 4.5, at room temperature (20°C).

The maltose solution used in the assay is obtained by dissolving 26.3 g of D-maltose monohydrate and 0.19 g of citric acid in about 70 mL of demineralized water; the pH is then adjusted to 4.5 using 1M NaOH, and water is added to a final volume of 100 mL.

For the assay, 50 mg of immobilized glucoamylase are suspended in 5 mL of the maltose solution; the mixture is shaken at room temperature for 1 hour. Every 10 minutes, a sample (50 µL) of the supernatant is taken and diluted 10 times with 0.1 M HCl before glucose analysis.

1.2.1 Glucose concentration analysis

The glucose concentration in the samples is measured using an enzymatic glucose assay. The glucose assay solution is obtained by dissolving 55 mg of ABTS in 50 mL of 25 mM Kpi (potassium phosphate buffer); then, to the solution are added 0.2 mL of a glucose oxidase aqueous solution (1 mg mL^{-1}) and 0.2 mL of a horse radish peroxidase aqueous solution.

50 µL of the 10× diluted activity assay sample are added, in a 4 mL UV-Vis cuvette, to 2.95 mL of the glucose assay solution. The cuvette is mixed by inversion, and the absorbance at 405 nm is monitored for 5 minutes. The increase in absorbance over time is compared to that of a reference solution of 5 mM D-glucose in water. The glucose concentration in each sample is obtained by:

$$[glucose](mM) = \frac{\Delta A_{405,\ sample}\ (min^{-1})}{\Delta A_{405,\ ref}\ (min^{-1})} \times \frac{[glucose]_{ref}\ (mM)\ \times\ V_{ref}\ (mL)}{V_{sample}(mL)\ \times\ df}$$

Where:

- $\Delta A_{405,\ sample}$ and $\Delta A_{405,\ ref}$ are the variations in absorbance of the sample and the reference solution, respectively.
- $[glucose]_{ref}$ is the glucose concentration of the reference solution, here 5 mM.
- V_{ref} is the volume of reference solution used, here 0.05 mL.
- V_{sample} is the volume of the glucoamylase solution sample, here 0.05 mL.
- df is the dilution factor, here 10.

A negative measure is also performed by introducing 50 µL of 10× diluted maltose assay solution to 2.95 mL of the glucose assay solution and analysing it in the same way as the collected samples. The glucose concentration in the negative is then subtracted from that of all measured samples.

1.2.2 Activity determination

The glucose concentration data is then plotted in a [glucose] *vs* time graph. Using the difference in glucose concentration over time, the glucoamylase activity is calculated by:

$$Activity\ (U\ g^{-1}) = \frac{\frac{\Delta[glucose]\ (mM\ min^{-1})}{2} \times V_{assay}\ (L)\ \times\ 1000}{g_{sample}}$$

Where:

- $\frac{\Delta[glucose]\ (mM\ min^{-1})}{2}$ is the rate of maltose hydrolysis.
  - $\Delta[glucose]\ (mM\ min^{-1})$ is the rate of glucose formation, that is the slope of the [glucose] vs time curve.
- $V_{assay}\ (L)$ is the volume of the assay, here 0.01 L.
- 1000 is the conversion factor from mmol to µmol.
- g_{sample} is the weight of anhydrous immobilized enzyme used for the assay.

1.3 Determination of dried immobilized enzyme

After the last activity assay cycle, the supernatant maltose solution is removed by decanting, and the immobilized enzyme sample is rinsed with 3×10 mL H_2O. The water is then removed, and the enzyme preparation is dried in the vacuum oven (100°C, 6 h). The weight of the dried immobilized enzyme is used for the activity calculations.

1.4 Continuous flow experiment

A continuous flow column was set up by introducing 150 mg_{wet} of immobilized enzyme in a 10 mL glass column. A continuous flow of glucoamylase assay solution (see 1.2) was pumped through the column at a rate of 0.15 mL min^{-1} over the course of 14 days. Every 24 hours, a sample of effluent (3-4 mL) was collected; for each effluent sample:

- 50 µL were immediately diluted 10 times with 450 µL of HCl 0.1 M, to inactivate potential leached enzyme. This diluted sample was used for glucose concentration analysis (see 1.2.1)
- The remaining sample was used for an evaluation of enzyme leaching from the carrier (see 1.4.1)

1.4.1 Glucoamylase leaching evaluation – p-nitrophenyl-L-D-glucopyranoside assay

The assay used for the determination of protein leaching in the continuous flow experiment is a colorimetric activity assay, based on the hydrolysis of *p*-nitrophenyl-L-D-glucopyranoside to glucose and *p*-nitrophenol.

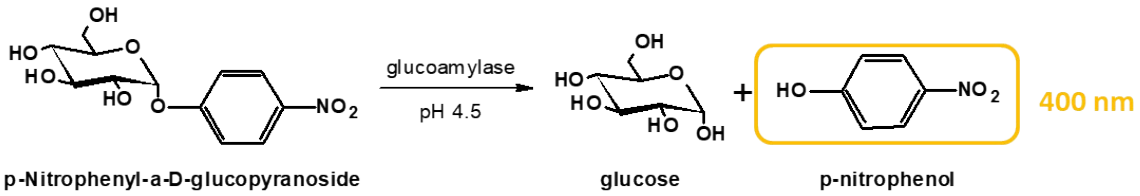

Figure 1: Hydrolysis of *p*-nitrophenyl-L-D-glucopyranoside to glucose and *p*-nitrophenol by glucoamylase

The formation of *p*-nitrophenol in the samples can be monitored by measuring the absorbance of the solution at 400 nm.

For the assay, 600 µL of effluent from the continuous flow column are concentrated to about 60 µL using a Roti-Spin Mini-10 column (Carl Roth) and centrifuging the sample at 7000 rpm for about 10 minutes. 20 µL of the concentrated sample are then mixed with 500 µL of a 1 mg/mL solution of *p*-nitrophenyl-L-D-glucopyranoside in sodium citrate buffer (0.1M, pH 4.5); the mixture is then incubated at 56°C for 4h. After incubation, 500 µL of a 0.5M potassium carbonate solution are added to the mixture. The absorbance of the final mixture at 400 nm is directly measured and compared with a *p*-nitrophenol calibration curve, to calculate the product concentration in the samples

1.4.2 *p*-nitrophenol calibration curve

A p-nitrophenol (Sigma-Aldrich) calibration curve was set up by measuring the absorbance at 400 nm (A_{400}) of p-nitrophenol standards with concentrations ranging from 0.2-3.0 mM. In each cuvette were introduced:

- 20 µL of p-nitrophenol standard solution
- 500 µL of 0.1 M citrate buffer (pH 4.5)
- 500 µL of 0.5M sodium carbonate solution

The measured absorbance values at 400 nm are plotted in a A_{400} *vs* [*p*-nitrophenol] (mM) graph. The slope of the graph can be used to extrapolate the *p*-nitrophenol concentration in the analyzed samples.

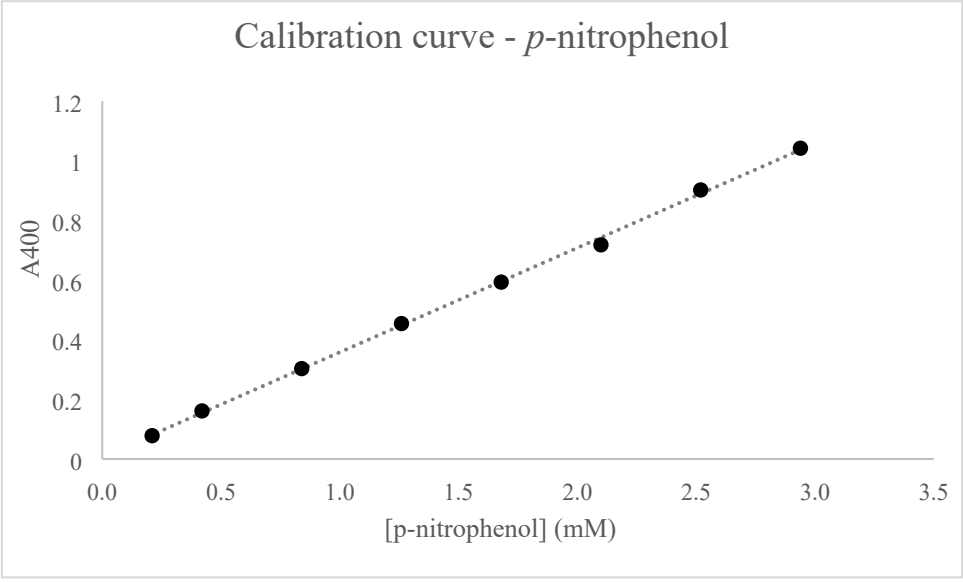

Figure 2: *p*-nitrophenol calibration curve

1.5 Synthesis of DFF

5 g of HMF were solubilized in 80 mL of DMSO. After complete solubilization, 1.42 g of KBr were added to the reaction mixture. The mixture was heated at 150°C in an oil bath for 6 hours. The course of the reaction was monitored *via* TLC analysis (petroleum ether/ethyl acetate 50:50).

The reaction mixture was colled down to room temperature, then diluted 1:1 with water. The mixture was subsequently extracted with diethyl ether 6 times. The reunited organic phases were washed with small quantities of brine, then dried on Na_2SO_4. After removal of the sodium sulphate, the organic phase was evaporated under reduced pressure. The product was obtained as a white solid.

NMR spectra

DFF

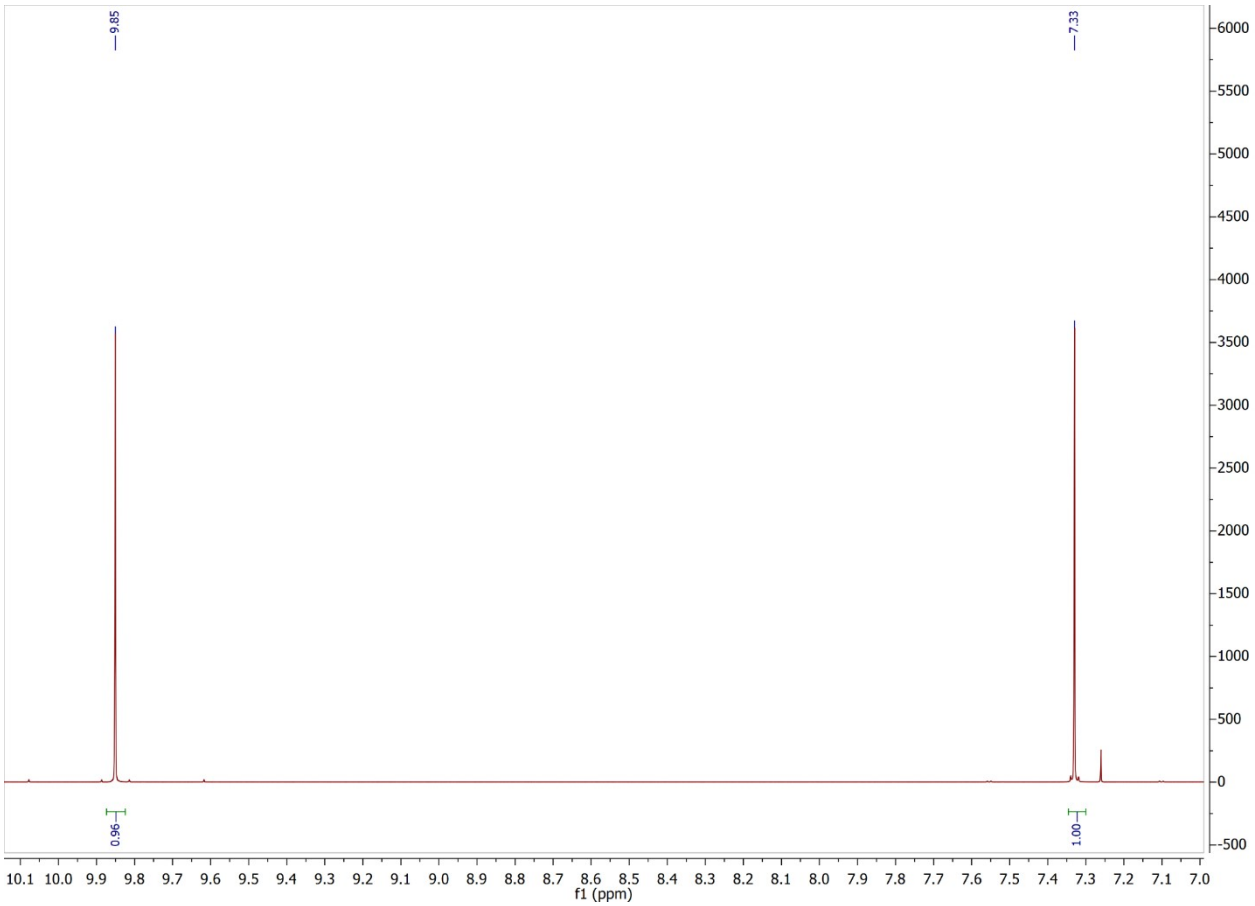

Figure 3: ^{1}H NMR in $CDCl_3$ of 2,5-diformylfuran (DFF). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.85 (s, 2H), 7.33 (s, 2H).

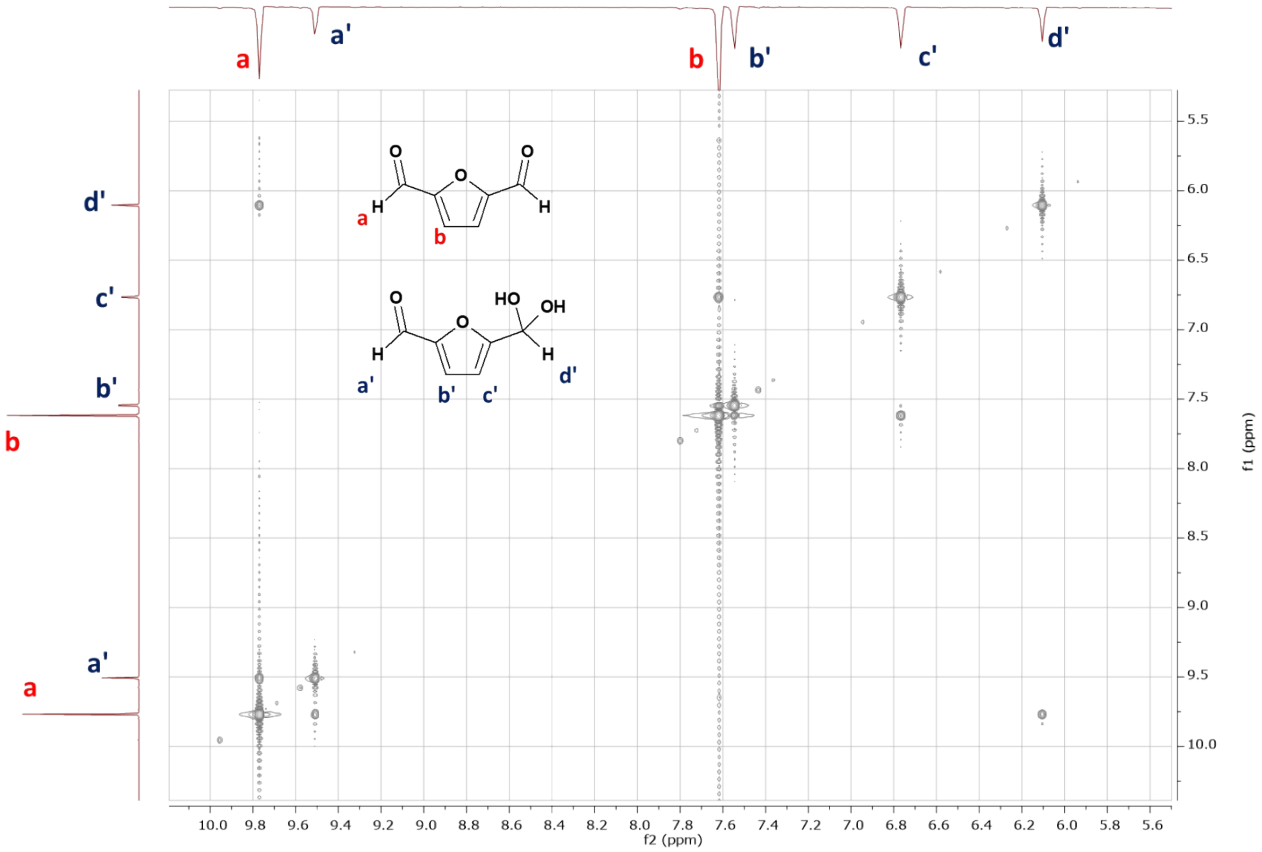

Figure 4: EXSY NMR spectrum (400 MHz, D_2O) of DFF and its mono-hydrated form.

Di-imine derivative

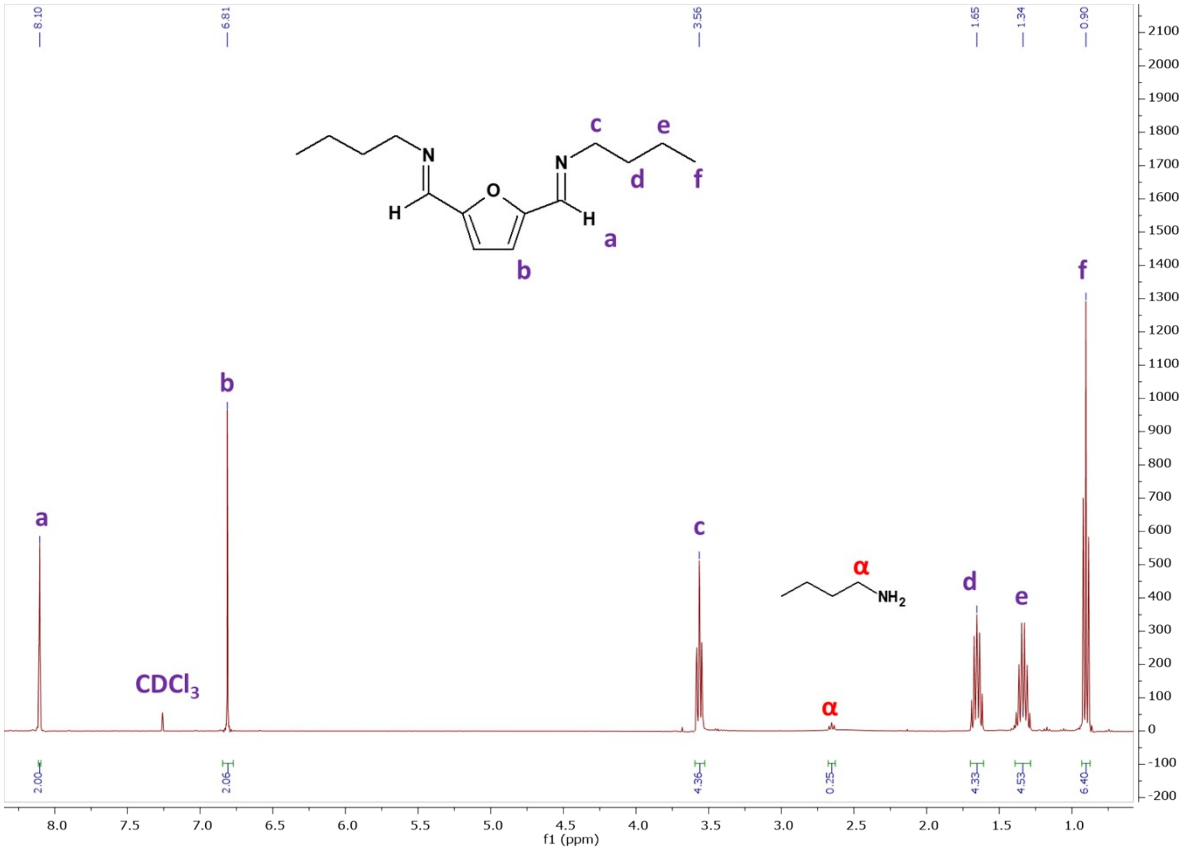

Figure 5: ^{1}H NMR spectrum of the product of reaction between DFF and *n*-butylamine. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (d, J = 0.7 Hz, 2H), 6.81 (d, J = 0.7 Hz, 2H), 3.56 (t, J = 6.8 Hz, 4H), 1.70 – 1.61 (m, 4H), 1.34 (h, J = 7.4 Hz, 4H), 0.90 (t, J = 7.4 Hz, 6H).

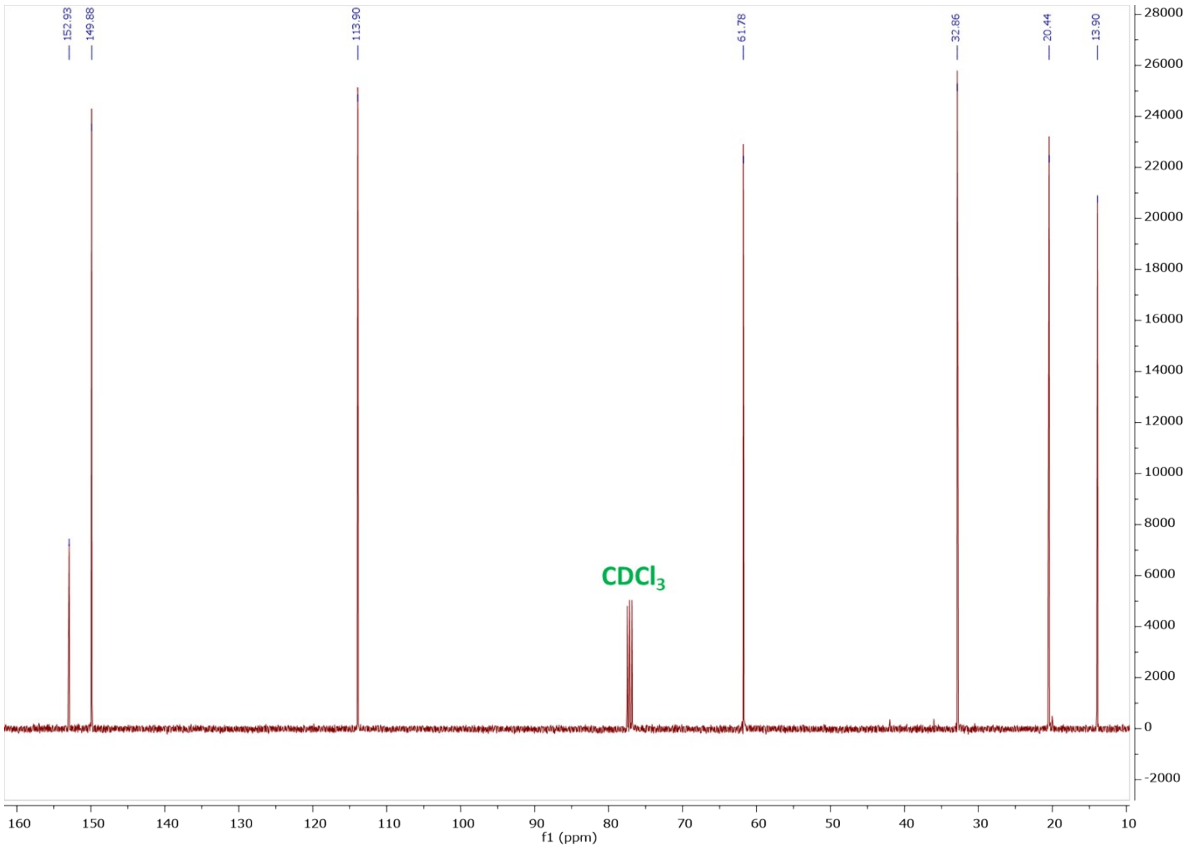

Figure 6: ^{13}C NMR spectrum of the product of reaction between DFF and *n*-butylamine. ^{13}C NMR (101 MHz, cdcl$_3$) δ 152.93, 149.88, 113.90, 61.78, 32.86, 20.44, 13.90.

DFF + isopropylamine

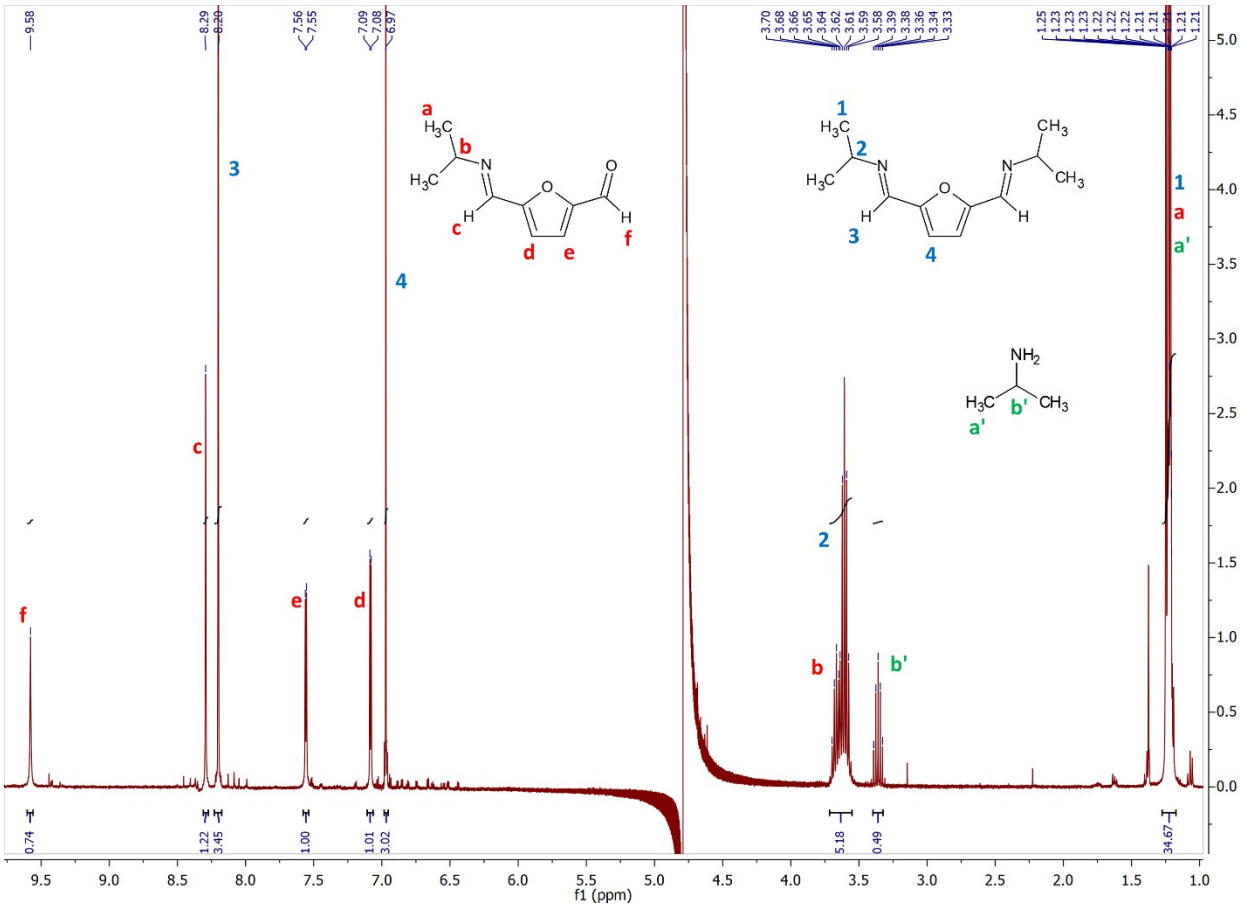

Figure 7: Presaturated ^{1}H NMR spectrum in D_2O of the reaction between DFF and isopropylamine, after 1 hour of reaction. 1H NMR (400 MHz, Deuterium Oxide) δ 9,58 (s, CHO), 8.29 (s, CH=N), 8.20 (s, CH=N), 7.56 (d), 7.08 (d), 6.97 (s), 3.70-3.58 (m), 3.36 (p),1.25-1.21 (m, CH_3)

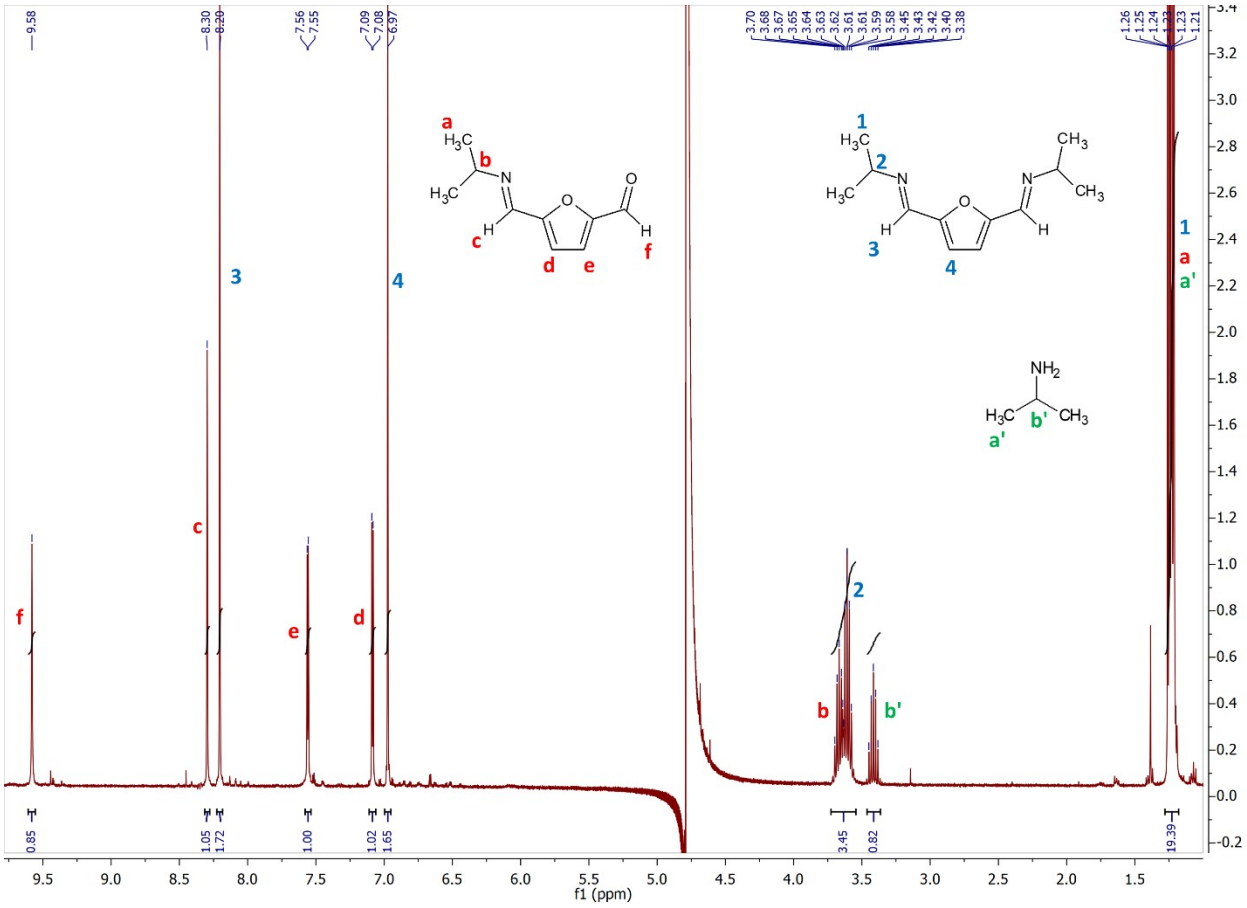

Figure 8: Presaturated ^{1}H NMR spectrum in D_2O of the reaction between DFF and isopropylamine, after 3 days of reaction. ^{1}H NMR (400 MHz, Deuterium Oxide) δ 9,58 (s, CHO), 8.30 (s, CH=N), 8.20 (s, CH=N), 7.56 (d), 7.09 (d), 6.97 (s), 3.70-3.58 (m), 3.36 (p),1.26-1.21 (m, CH_3)

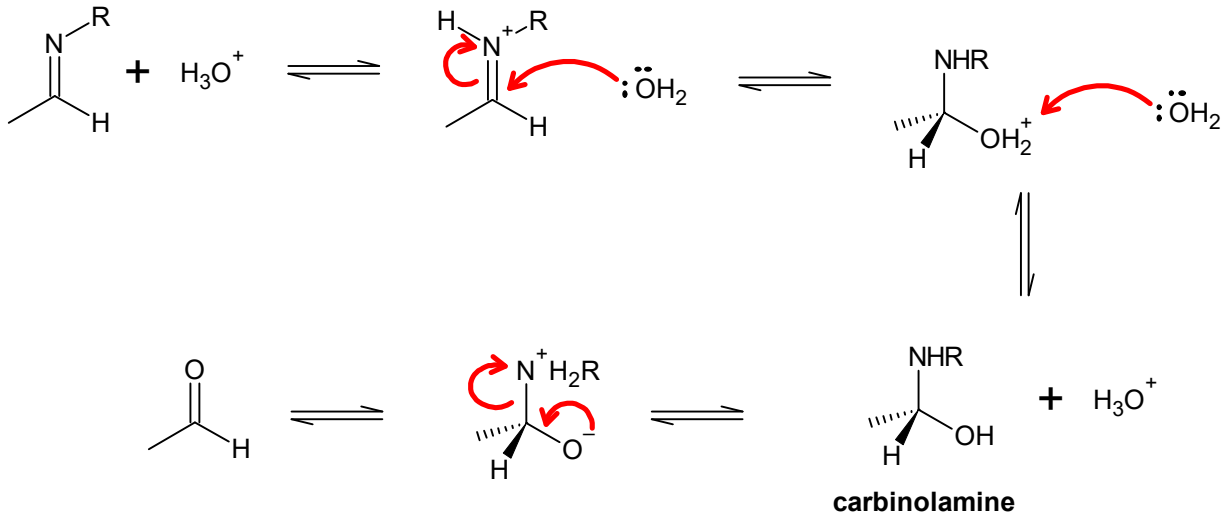

Figure 9: Mechanism of imine hydrolysis

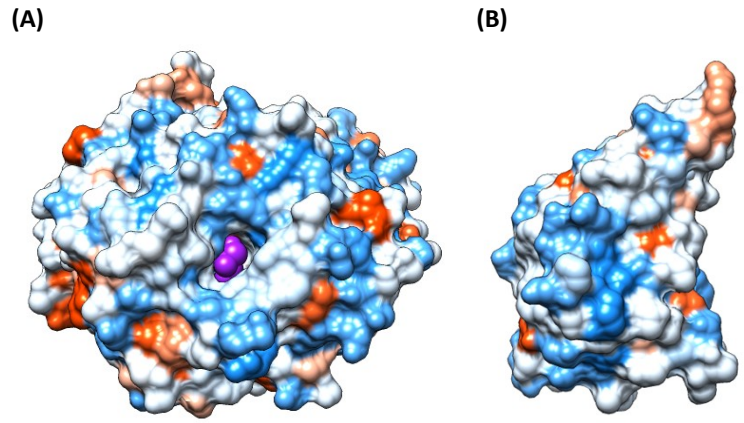

Figure 10: Tridimensional models of the structure of the surface of glucoamylase from *Aspergillus niger*, with hydrophobic surfaces highlighted in red and hydrophilic surfaces highlighted in blue; in purple: TRIS inhibitor in the catalytic pocket. **(A)** Catalytic domain (PDB ID: 3EQA) **(B)** Starch binding domain (PDB ID: 5GHL)