Determining transaminase activity in bacterial libraries by time-lapse imaging

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Supplementary information

Chemicals

Substrates used for the transaminase reactions were the following: 2-(4-nitrophenyl)ethylamine hydrochloride from TCI Chemicals (Japan), benzaldehyde from Fisher Scientific (Thermo Fisher Scientific, USA); and the co-factor pyridoxal-5-phosphate hydrate from Alfa Aesar (Thermo Fisher Scientific, USA). Tris-HCl buffer was made with tris(hydroxymethyl)aminomethane from Eurobio (France) dissolved with Milli-Q® water and the pH was adjusted to 8 with HCl. Solvents used were acetonitrile and ethyl acetate both from Fisher Scientific.

Microbial growth and biocatalyst preparation

The bacterial strains used in this study were isolated from samples collected near active volcanic systems in the island of São Miguel, The Azores (Portugal) by cultivation-based methods, as described previously[1]. They are currently deposited and maintained at iBB-Institute for Bioengineering and Biosciences, Instituto Superior Técnico (Lisbon, Portugal), as library "IST-INMARE".

Isolates from IST-INMARE collection were grown in 100 mL of tryptic soy broth (Sigma-Aldrich, USA) for 24h at 200 rpm and 30°C, in an orbital incubator (Agitorb 200, Aralab, Portugal). After incubation, the cells were harvested by centrifugation at 6,000 rpm and 4°C for 5 min (Centrifuge 5810R, from Eppendorf, Germany). Cells were washed with Tris-HCl buffer and centrifugated again under the same conditions. The supernatant was discharged, and a cell

suspension was prepared with 5 mL of buffer or with the appropriate volume of buffer to reach the desired optical density. A calibration curve relating the optical density of the cell suspensions measured at 600 nm and the cell dry weight, measured after placing the cell pellets at 65^{o}C for 24h, was made.

Transaminase reactions

The whole cells from the IST-INMARE collection were used as biocatalysts in the transaminase reaction which was carried out as suggested by Baud *et al.* (2015)[2]. In summary, the reactions using the different biocatalysts were carried out in 96-well plates (from Sarstedt, Germany) containing 200 µL of reaction medium per well comprising the following compounds: 25 mM of 2-(4-nitrophenyl)ethylamine hydrochloride; 1 mM of pyrodoxal-5-phosphate hydrate; 10 mM of benzaldehyde (stock solution: 7.5% v/v benzaldehyde in acetonitrile); cell suspension (concentration provided below); and, 100 mM Tris-HCl buffer pH 8. Each plate was covered with sealing tape (from Sarstedt, Germany) to prevent evaporation. All experiments were done at least in triplicate.

Reactor and Time-Lapse Imaging System

A cube-shaped frame was built in pinewood covered with plywood to hold the following parts: a square 205x205 mm LED panel (4000K, 1800 lumen, LED 16.5W; Adeo, France) placed over a light diffuser on the top of the box; a 96-well plate, placed in the middle of a hollow shelf; and, a HD webcam placed at the bottom of the box (Figure S1). The webcam was connected to a computer for the acquisition of RGB digital images of the bottom of the 96-well plate. The system was placed inside an orbital incubator (Agitorb 200, Aralab, Portugal) at 200 rpm and 30°C. Automatic image acquisition was carried out using the platform Fiji[3] of the open-source

ImageJ software, by a Logitech® HD webcam c525 using a webcam plugin [4]. Images were saved as Tiff files. Improved accuracy and reproducibility was achieved by replacing the initial webcam by this HD model.

Validation of the system

The biotransformation and image acquisition system was validated by correlating the benzaldehyde concentration determined by i. gas chromatography-mass spectrometry (GC-MS) and ii. image analysis. The plates contained the reaction media previously described and the transamination reaction was started by addition of a cell extract of a transaminase producer. The plate was covered with sealing tape (from Sarstedt, Germany) to prevent evaporation and incubated immediately inside the box. Image acquisition was also initiated.

Using a suicidal strategy, the whole content of three wells was removed from the plate and extracted immediately with ethyl acetate in Eppendorf tubes®. After phase separation, the benzaldehyde concentration in the ethyl acetate layer was determined by GC-MS. The concentration determined by GC-MS was compared to the benzaldehyde concentration obtained by image analysis, which related the luminance of the colour observed in each well and the concentration of benzaldehyde, as described below.

Application of the developed system for determining transaminase activity

Two strains, IST-521 and IST-523, were selected during the screening assays and used for further process development using time-lapse imaging. The reactions to determine the specific activity of transaminase producers were conducted using the same reactants and conditions as the reactions described above. Different initial aldehyde/amine concentrations were used while maintaining the relative ratio: 2/5, 4/10, 6/15, 8/20, 10/25, 14/35, 20/50, and 26/65 mM. Seventy-five µL of concentrated cell suspension were deposited in each well of a 96-well

plate. The plate was centrifuged (4000 rpm, 4°C during 30 min on a Centrifuge 5810R, from Eppendorf), and the excess buffer was removed. The reaction was started by resuspending the cell pellets in reaction medium containing the substrates listed above, including benzaldehyde and 2-(4-nitrophenyl)ethylamine hydrochloride. All experiments were done at least in triplicate.

Calculations

The intensity values of red-green-blue (RGB) channels (varying from 0 to 255) from the digital images acquired during the transaminase reactions were determined using the platform Fiji from ImageJ. In summary, RGB digital images were imported in sequence, and the channels were split into separate images. After assigning the region of the image corresponding to each well, using the region of interest (ROI) tool, the intensity values of each RGB channel were measured. The webcam used had an error determined to be lesser than 1%, calculated based on the standard deviation and sample mean of the intensity values of 26 independent images, quoted for a confidence interval of 95%.

Calibration of the system

The intensity values acquired from the image analysis were transformed in luminance by the equation:

$$L = rR + gG + bB \qquad \text{(Equation 1)}$$

where *R*, *G* and *B* are the intensity values from each colour channel and the *r*, *g* and *b* are the contribution factors to the luminance (*L*) of each colour channel. The *r*, *g* and *b* parameters were determined by the least square method by adjusting the intensity values of luminance and the benzaldehyde concentration, determined by GC-MS, for each time point in the transaminase reaction. A linear regression was made relating the calculated luminance and the

conversion of benzaldehyde. By definition the conversion is the ratio between the consumed and initial substrate concentrations. This allows the determination of the benzaldehyde concentration in each image taken at a time *t*, even for different initial benzaldehyde concentrations (Equation 2):

$$C_{t,\,benzaldehyde} = C_{0,\,benzaldehyde}(1 - Conversion) \qquad \text{(Equation 2)}$$

where C_t is the benzaldehyde concentration at time t and C_0 is the initial benzaldehyde concentration.

Analytical Methods

After extraction of unreacted substrates with ethyl acetate, as previously mentioned, the concentration of benzaldehyde was determined by GC-MS on an Agilent 7820A GC equipped with a 7693A autoinjector, and an Agilent 5977E quadrupole MS detector (all from Agilent Technologies, USA). The capillary column was an Agilent J&W Ultra-2 which worked at a constant flow of 1 mL/min. The GC injector was set at 200°C, the MS source at 230°C, the MS quad at 150°C and the MSD transfer line at 280°C. The separation of substrates and products was achieved by setting the oven at an initial temperature of 40°C and increasing the temperature to 240°C at 38°C/min. Peak identification was carried out by comparison of MS data with those of injected standards using the software Qualitative Analysis, whilst peak quantification was done using Quantitative Analysis, both part of the MassHunter Workstation from Agilent.

Figures

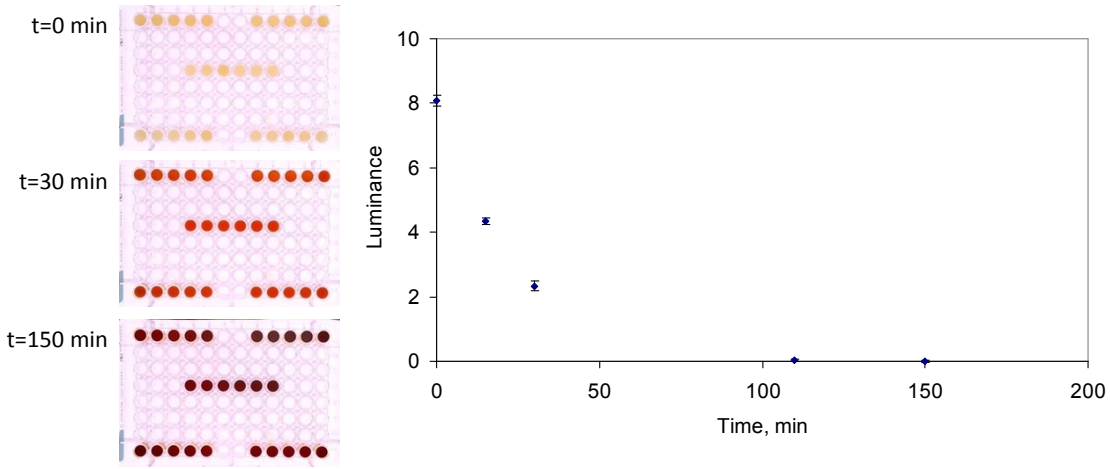

Figure S1. Assessment of the variation of luminance observed between wells located at different positions in a 96-well plate. Left-hand side: photographs acquired by the developed imaging system at 0, 30 and 150 min. Right-hand side: average luminance values determined along time, with error bars indicating the corresponding standard deviation.

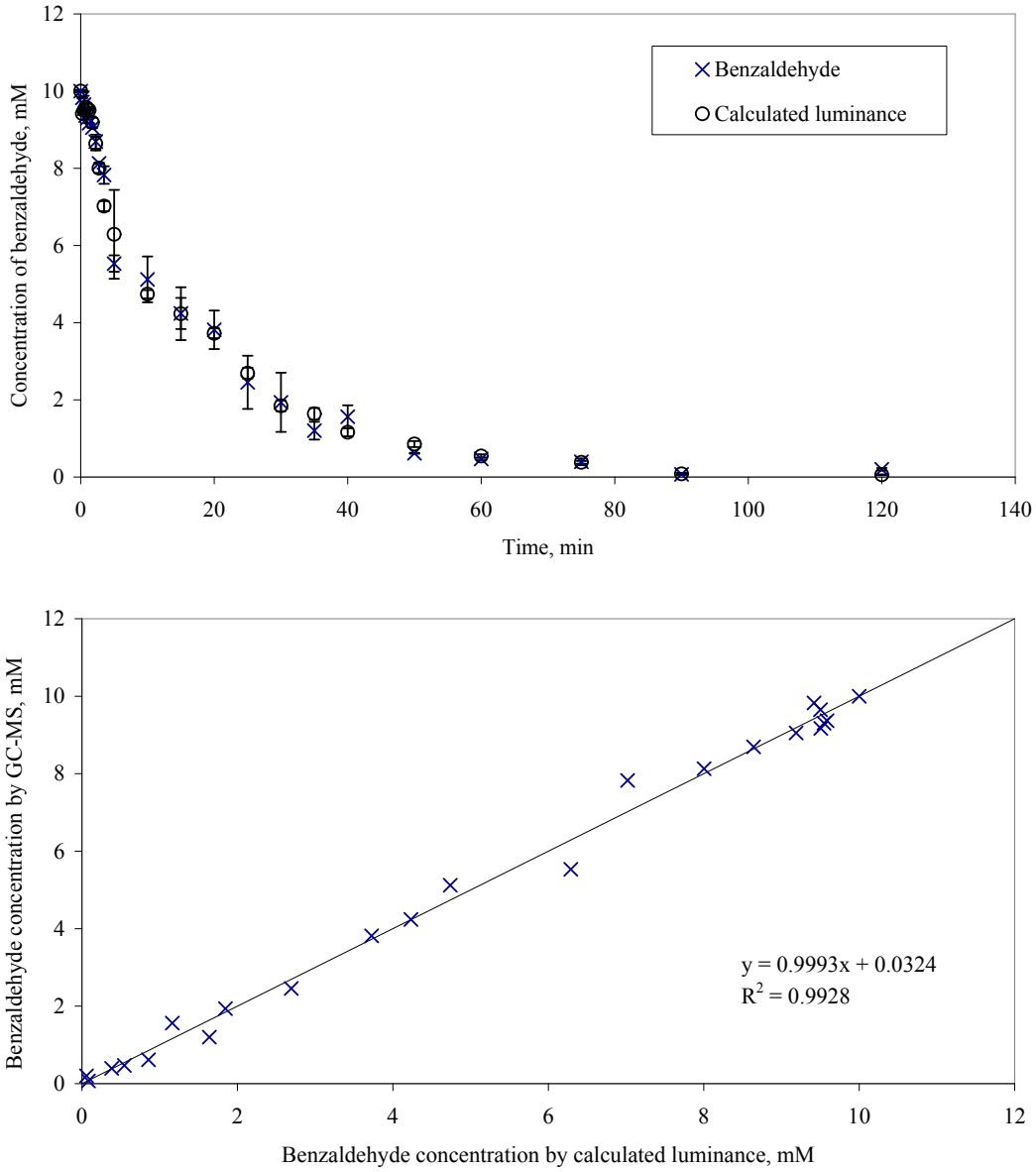

Figure S2. Data fitting of the luminance to the concentration of benzaldehyde using the least squares method (A). Correlation between the concentrations of benzaldehyde determined by GC-MS and by the calculated luminance (B).

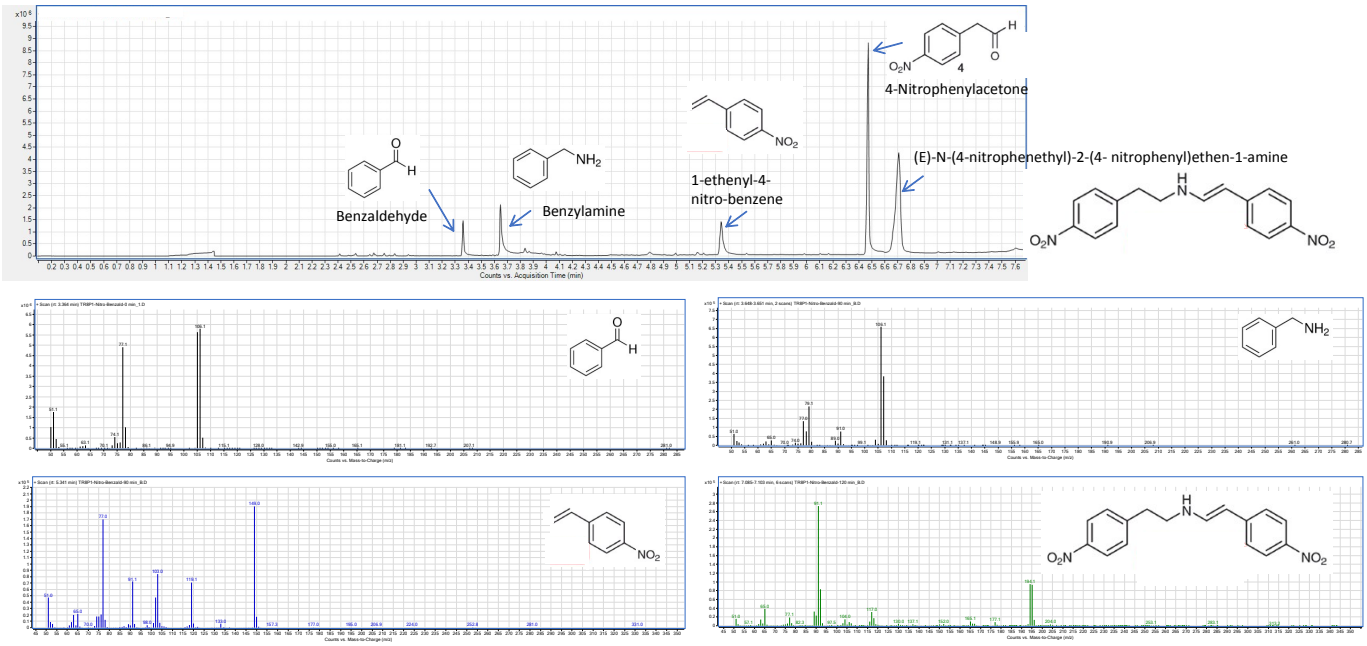

Figure S3. Total ion chromatogram (upper row) and mass spectrum of the peaks shown in the analysis (lower rows) obtained by GC-MS after 120 min of reaction.

References

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