Supporting information

Flow-based enzymatic synthesis of melatonin and other high value tryptamine derivatives: a five-minute intensified process

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1. General information

NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer using the residual signal of the deuterated solvent as internal standard. ^{1}H chemical shifts (δ) are expressed in ppm, and coupling constants (*J*) in hertz (Hz). Merck Silica gel 60 F254 plates were used for analytical TLC. Visualization was then achieved using UV light**,** ninhydrin or ceric ammonium molybdate. Organic solutions were concentrated using a Buchi rotary evaporator below 40 °C at 25 torr. Morphological investigation was carried out with a LEO 1430 (Carl Zeiss, Oberkochen, Germany) Scanning Electron Microscope operating at at 20 kV and 3×10^{-3} Torr, SEI detection mode. Before analysis, samples were sputter-coated with gold to a thickness of approximately 10 nm using a Semprep 2 sputter coater (Nanotech Ltd, Prestwick, UK) at 10 mA. Enzyme localization was observed using a Nikon A1 laser scanning confocal microscope with an excitation laser (λ: 488 nm) and the emission filter 500-550 nm. HPLC Analysis were performed using a Merck-Hitachi 655 A-12 Liquid Cromatograph with L-5000 LC controller and 655A Variable Wavelength UV monitor detector. Column: Chiralpack IC (250 x 4.6 mm x 3 µm). Eluent: *n*-hexane/EtOH 70:30 + 0.1% TEA, λ = 250 or 280 nm, flow rate: 1 mL/min). Continuous flow biotransformations were performed using a $R2^{+}$/R4 Vapourtec flow reactor equipped with an Omnifit® glass column (6.6 mm i.d. × 100 mm length).

2. Chemicals

All reagents and solvents were obtained from commercial suppliers and were used without further purification.

3. Cloning, Overexpression and Purification of MsAcT

Protein expression and purification was performed following previously reported protocols by Contente *et al.*[1]

4. Activity assay of the MsAcT free enzyme

Free enzyme activity measurements were performed following previously reported protocols by Contente *et al.*[1]

5. Activity assay of the MsAcT immobilized enzyme

The activity of immobilized MsAcT was determined by weighing an appropriate amount of imm-MsAcT (5-10 mg) into a 15 mL reaction tube with cap, followed by the addition of 10 mL reaction mixture (0.1 M phosphate buffer pH 8.0, containing 0.1 mg/mL *p*-nitrophenylacetate, 0.1% v/v EtOH). The immobilized enzyme reaction mixture was shaken at 25 °C, 150 rpm and the absorbance at 400 nm was recorded every minute as single readings using half-microcuvettes (total volume 1 mL). The imm-MsAcT specific activity (U/g) is defined as µmol of *p*-nitrophenol formed for minute for grams of immobilized enzyme.

6. Immobilization onto agarose matrix

Aldehyde agarose immobilization was performed as previously described by Dall'Oglio *et al.*[2] The immobilization results in terms of retained activity and stability (after a week at 4 °C) at different concentration of enzyme (1, 5, 10 mg/g_{matrix}) are reported in Table S1. Data are collected in duplicates and the results are expressed as average of the obtained data.

Table S1.MsAcT immobilization onto glyoxyl agarose

1 mg/g_{matrix}	Average activity (%)	Average stability (%)	**5 mg/g_{matrix}**	Average activity (%)	Average stability (%)	**10 mg/g_{matrix}**	Average activity (%)	Average stability (%)
Agarose	73	74	Agarose	20	19	Agarose	8	8

7. Immobilization onto epoxy resins

Immobilization was conducted according to the procedure reported by Planchestainer *et al.*[2] Four different types of matrix were used: EP403/S, HFA403/S, EC-HFA/S and EC-EP/S (Figure S1). The immobilization results in terms of retained activity and stability (after a week at 4 °C) at different concentration of enzyme (1, 5, 10 mg/g_{matrix}) are reported in Table S2. Data are collected in duplicates and the results are expressed as average of obtained the data.

Epoxy resins

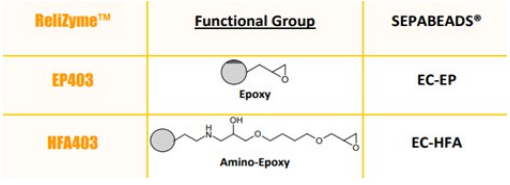


Figure S1. Epoxy resins used for the immobilization of MsAcT. S grade: particle size 100-300 nm. Series 403: average pore diameter 40-60 nm. Series EC: average pore diameter: 20-40 nm.

Table S2.MsAcT immobilization onto epoxy resins

1 mg/g_{matrix}	Average activity (%)	Average stability (%)	**5 mg/g_{matrix}**	Average activity (%)	Average stability (%)	**10 mg/g_{matrix}**	Average activity (%)	Average stability (%)
EP403/S	28	26	EP403/S	5	6	EP403/S	3	3
HFA403/S	30	28	HFA403/S	9	6	HFA403/S	6	5
EC-HFA/S	24	26	EC-HFA/S	6	5	EC-HFA/S	3	4
EC-EP/S	23	25	EC-EP/S	6	6	EC-EP/S	3	3

8. Immobilization onto cellulose

1 g of cellulose was suspended in 100 mL of THF and subsequently 10 mL of APTMS (3-aminopropyl)trimethoxysilane) were added. After 3 h under magnetic stirring, it was filtered and carefully washed with fresh THF to remove the unreacted APTMS. The solvent was then evaporated while the dry cellulose was re-suspended in phosphate buffer 0.05 M pH 7.0 at room temperature. A solution of 25% glutaraldehyde (7 mL) was added and the mixture was left for 24 h under magnetic stirring. The derivatized cellulose was then filtered, abundantly washed with deionized water and dried at room temperature over night (Figure S2).

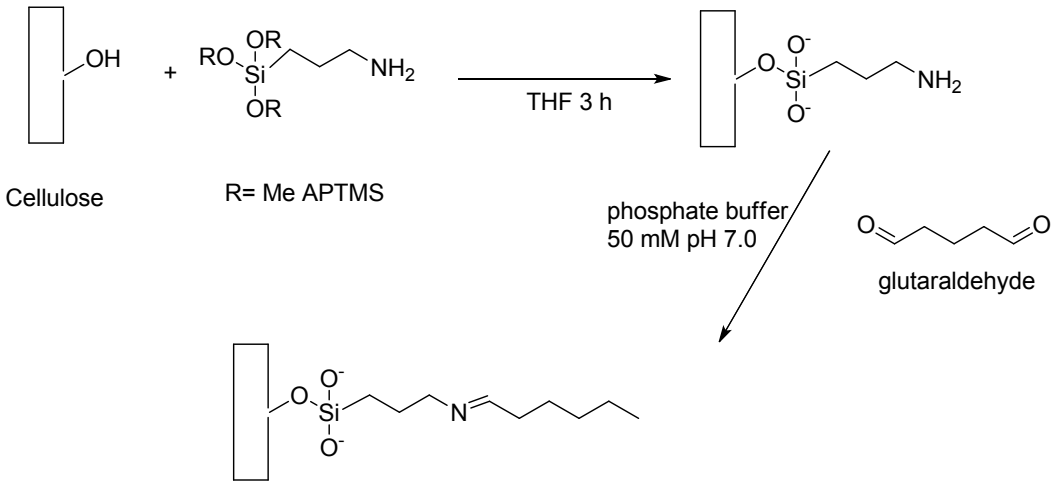


Figure S2. Activation of cellulose matrix for the enzyme binding

In a 15 mL vial, 6.4 mL of phosphate buffer (0.05 M pH 7.0) containing the desired concentration of MsAcT (1, 5, 10 mg/g_{matrix}) and 0.5 g of activated cellulose were added. The mixture was kept under gently shaking for 24 h. After checking the absence of the protein in the supernatant, 1 mg/mL of $NaBH_4$ was added and

left reacting for 30 min. The cellulose-immobilized MsAcT was washed abundantly with water and then stored in buffer phosphate 0.1 M pH 8.0 at 4 °C.

The immobilization results in terms of retained activity and stability (after a week at 4 °C) at different concentration of enzyme (1, 5, 10 mg/g_{matrix}) are reported in Table S3. Data are collected in duplicates and the results are expressed as average of the obtained data.

Table S3. MsAcT immobilization onto cellulose

1 mg/g_{matrix}	Average activity (%)	Average stability (%)	**5 mg/g_{matrix}**	Average activity (%)	Average stability (%)	**10 mg/g_{matrix}**	Average activity (%)	Average stability (%)
Cellulose	26	30	Cellulose	17	17	Cellulose	8	8

9. Immobilization onto 3-aminopropyl-functionalized silica

Glutaraldehyde activation and immobilization onto 3-aminopropyl-functionalized silica were performed according to the procedure reported above (pararagraph 7). The immobilization results in terms of retained activity and stability (after a week at 4 °C) at different concentration of enzyme (1, 5, 10 mg/g_{matrix}) are reported in Table S4. Data are collected in duplicates and the results are expressed as average of the obtained data.

Table S4. MsAcT immobilization onto silica gel

1 mg/g_{matrix}	Average activity (%)	Average stability (%)	**5 mg/g_{matrix}**	Average activity (%)	Average stability (%)	**10 mg/g_{matrix}**	Average activity (%)	Average stability (%)
Silica	15	13	Silica	6	6	Silica	4	3

10. SEM (Scanning Electron Microscope) analysis

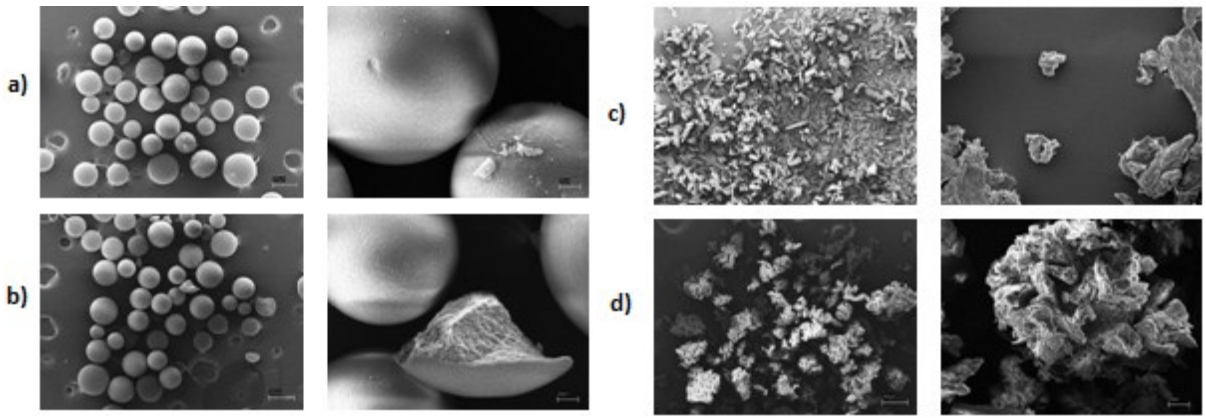


Figure S3. SEM images of HFA403/S beads. Line a: activated epoxy resin before MsAcT immobilization 60x; 500x magnification respectively. Line b: activated epoxy resin after MsAcT immobilization (1 mg/g_{matrix}) 60x; 500x magnification respectively. Line c: activated cellulose before MsAcT

immobilization 60x; 500x magnification respectively. Line d: activated cellulose after MsAcT immobilization (1 mg/g_{matrix}) 60x; 500x magnification respectively.

11. Fluorescein labeling of MsAcT

Fluorescent label was done according with reported methodologies.[4] An enzyme solution was mixed (1:1 molar ratio) with fluorescein isothiocyanate (FITC) (from a stock solution 10 mg/mL in DMSO) in 0.1 M potassium phosphate pH 8.0 and incubated for 1 h with gentle agitation at 25 °C in darkness. The excess of FITC was removed *via* dialysis against phosphate buffer 0.1 M pH 8.0 until the flow trough becomes colorless.

12. CLSM (Confocal Laser Scanning Microscopy) Imaging

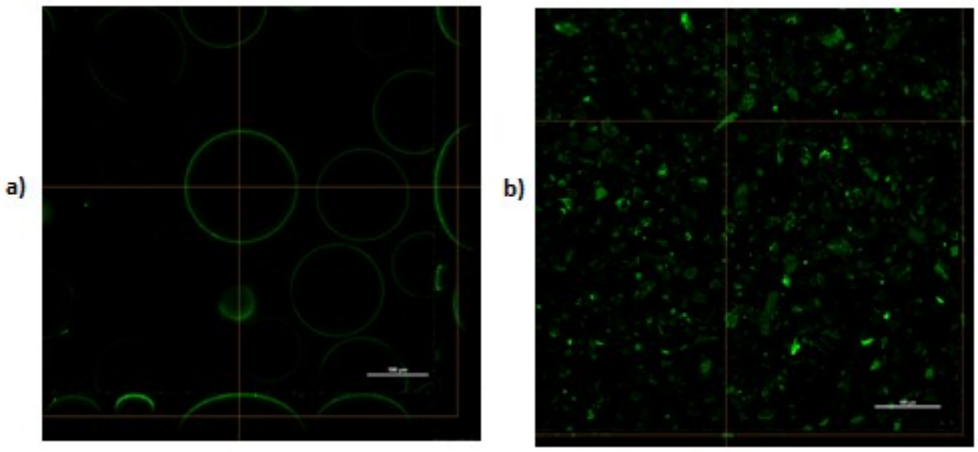


Figure S4. a) Confocal microscopy of HFA403/S particles immobilizing MsAcT (1 mg/g_{matrix}) 20x magnification b) Confocal microscopy of cellulose microfibrils immobilizing MsAcT (1 mg/g_{matrix}) 20x magnification.

13. Characterization of the immobilized MsAct *vs* free form

Stability of the immobilized and free enzyme in a solution of phosphate buffer (0.1 M, pH 8.0) and 10%v/v immiscible acyl donors was assayed. Following incubation at different time points, the residual activity was determined spectrophotometrically following the procedure described in paragraph 9 (100% corresponds to the activity before the beginning of the incubation).

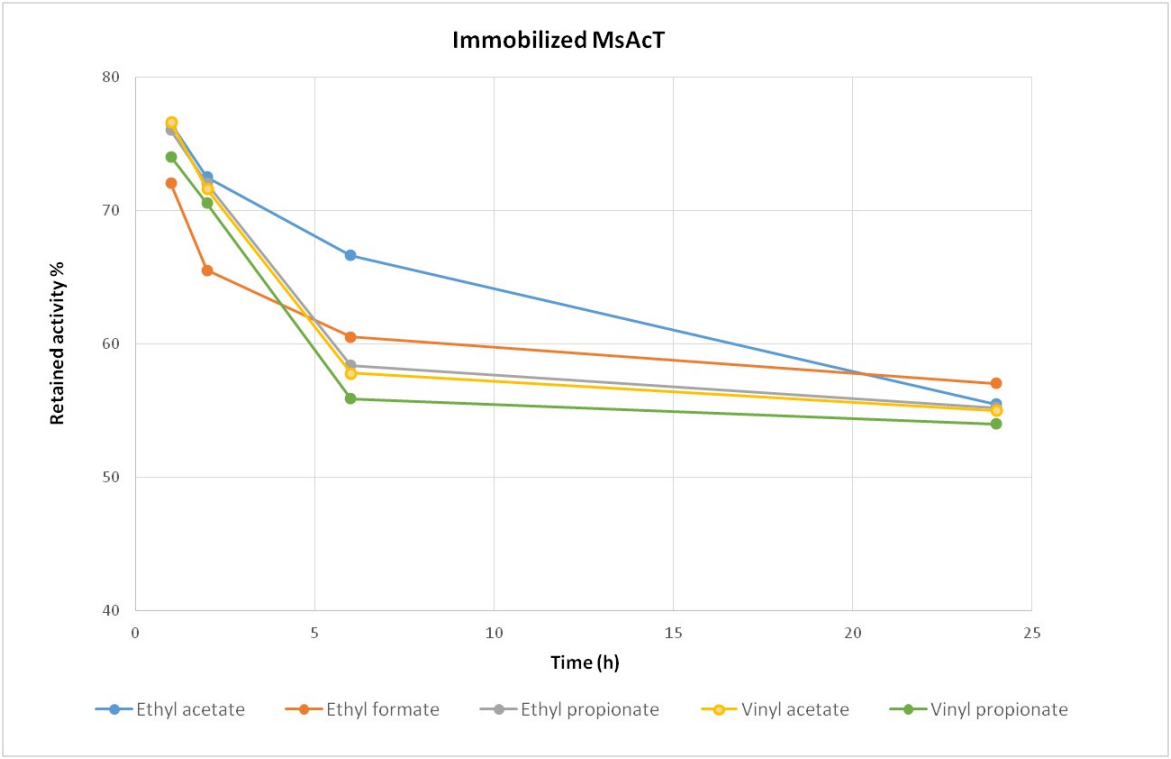


Figure S5. Solvent effect on immobilized MsAcT stability

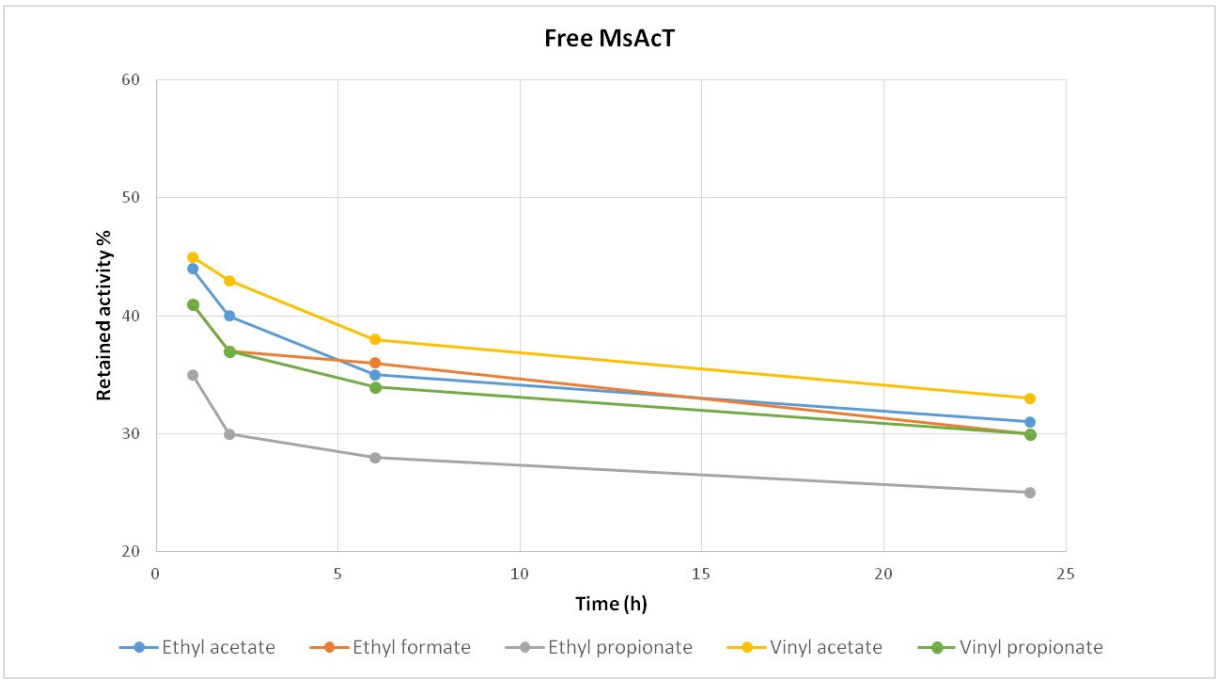


Figure S6. Solvent effect on free MsAcT stability

Temperature effect on immobilized and free enzyme stability was also tested. The enzyme was incubated in phosphate buffer (0.1 M, pH 8.0) at different temperatures over a period of time. The residual activity was determined spectrophotometrically following the procedure described in paragraph 9 (100% corresponds to the activity before the beginning of the incubation).

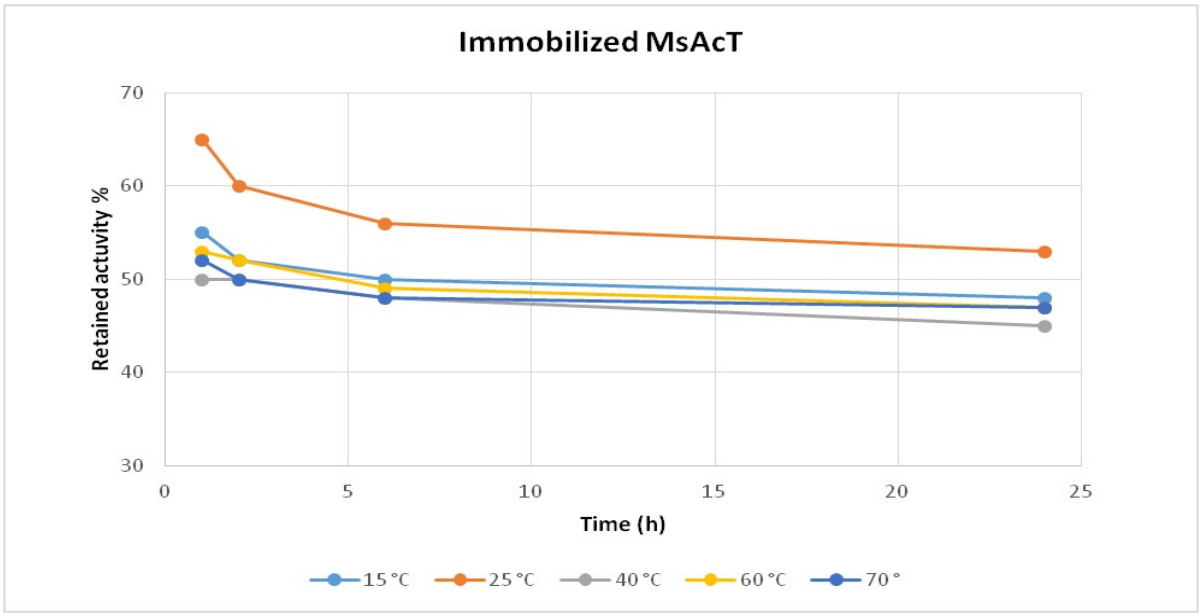


Figure S7. Temperature effect on free MsAcT stability

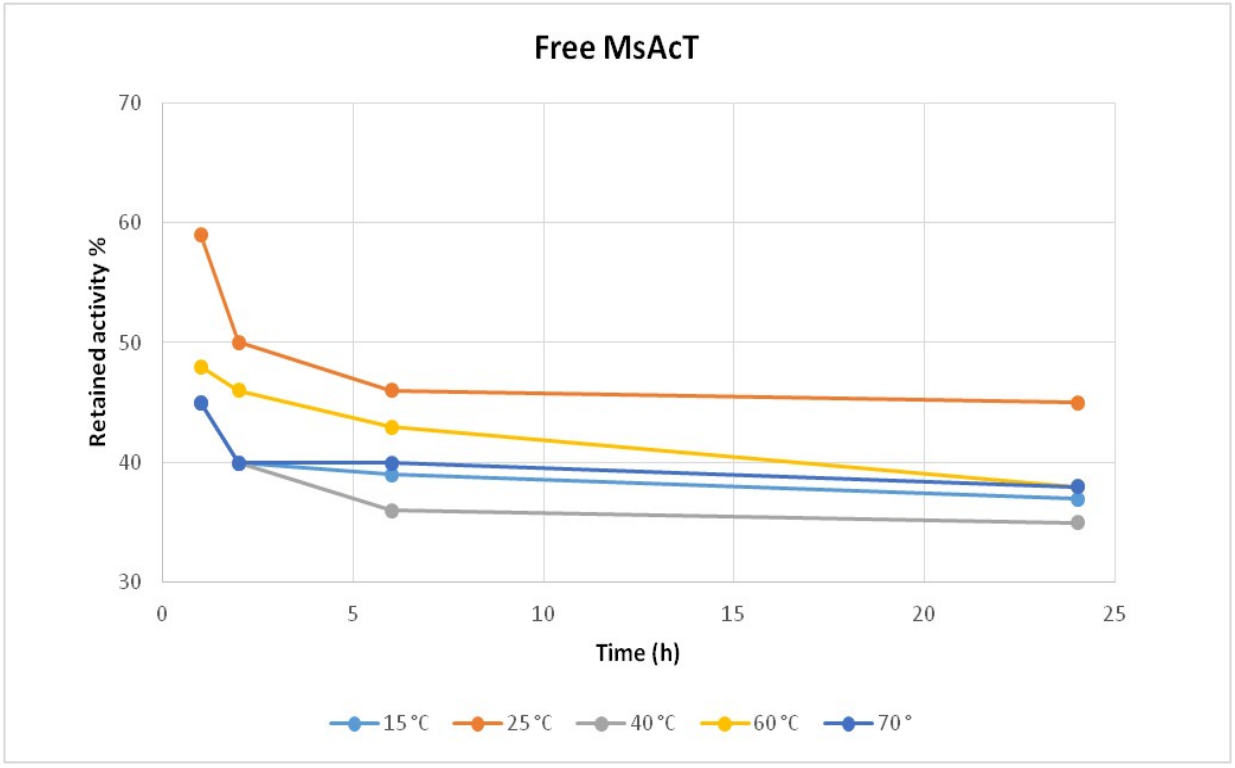


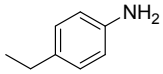
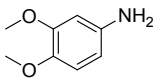
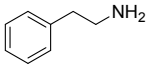
Figure S8. Temperature effect on free MsAcT stability

14. Small scale batch reactions

Batch reactions using agarose immobilized MsAcT (Table S4) were performed in 10 mL screw cap tubes; 1 mL reaction mixture in 0.1 M phosphate buffer pH 8.0, containing 0.25 M amine, 200 mg (1 mg/$g_{agarose}$) of imm-enzyme, and 10% v/v of acyl donor were left under gentle agitation at 25 °C. 50 µL aliquots were

collected at different reaction times (30 min, 1 h, 2 h, 4 h, 6 h, 24 h) quenched with NaOH 1 M and extracted with 100 µL of EtOAc for TLC (CH_2Cl_2/MeOH 9:1 + 0.1% TEA). After evaporation, the samples were re-suspended in the mobile phase for HPLC analysis. The retention times were: *p*-ethylaniline 4.7 min, *N*-(*p*-ethylbenzyl)formamide 5.0 min, *N*-(*p*-ethylbenzyl)acetamide 4.0 min, *N*-(*p*-ethylbenzyl)propionamide 4.1 min; 3,4-dimethoxybenzylamine 17.6 min, *N*-(3,4-dimethoxybenzyl)formamide 12.3 min, *N*-(3,4-dimethoxybenzyl)acetamide 10.0 min, *N*-(3,4-dimethoxybenzyl)propionamide 9.8 min; 2-phenylehtylamine 5.2 min, *N*-(phenetyl)formamide 4.0 min, *N*-(phenetyl)acetamide 5.2 min, *N*-(phenetyl)propionamide 4.5 min confirmed by comparison with chemically prepared standards obtained following the procedure reported in Contente *et al*.[1]

Table S4. Batch biotransformations with agarose immobilized MsAcT (phosphate buffer 0.1 M, pH 8.0, 200 mg immobilized enzyme (1 mg/$g_{agarose}$), 25 °C.

Substrate (0.25 M)	Acyl donor (10% v/v)	Time[a] (h)	m. c.[b] (%)
 1a	Ethyl formate	0.5	90
	Ethyl acetate	1	90
	Ethyl propionate	2	90
	Vinyl acetate	1	95
	Vinyl propionate	1	90
 1b	Ethyl formate	1	75
	Ethyl acetate	1	82
	Ethyl propionate	2	90
	Vinyl acetate	0.5	92
	Vinyl propionate	0.5	90
 1c	Ethyl formate	1	90
	Ethyl acetate	1	90
	Ethyl propionate	1	80
	Vinyl acetate	0.5	>99
	Vinyl propionate	0.5	>99

[a] Time corresponding to maximum conversion

[b] As determined by HPLC

15. HPLC analysis of melatonin derivatives

For determining the molar conversion 50 µL samples were re-suspended in the mobile phase for HPLC analysis. The retention times were: tryptamine 7.7 min, *N*-acetyl tryptamine 4.5 min; 5-methoxytryptamine 9.1 min, melatonin 5.4; setonin 9.8 min, *N*-acetyl serotonin 5.9 min.

16. Flow synthesis of amides

A glass column (6.6 mm i.d.) was filled with 1.6 g of imm-MsAcT (1 mg/g). A 0.25, 0.5, 1 M amine solution in phosphate buffer (0.1 M, pH 8.0) with 5 or 10 % of DMSO, and pure EtOAc or VinylOAc were prepared. The two solutions were mixed in a T-piece and the resulting segmented flow stream (90:10 or 85:15 in the case of 1 M amine solution) was directed into the column packed with the biocatalyst (packed bed reactor volume: 1.2 mL). The flow rate was varied and optimized. An in-line extraction was performed using a Zaiput liquid/liquid separator and an inlet of EtOAc (flow rate: 0.19 mL min^{-1}) that was mixed to the exiting reaction flow stream using a T-junction. Both the organic and aqueous phase were analyzed by HPLC using

the above reported conditions (paragraph 13-14). The organic phase, containing the amide, was evaporated to yield the desired product.

17. NMR spectra of the flow prepared amides*N*-acetyl tryptamine:

^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.16 (br s, 1H), 7.60 (d, *J* = 7.7 Hz, 1H), 7.40 (d, *J* = 7.9 Hz, 1H), 7.21 (dd, *J* = 7.9, 7.1 Hz, 1H), 7.11 (dd, *J* = 7.7, 7.1 Hz, 1H), 7.00 (d, J = 1.8 Hz, 1H), 5.80 (br s, 1H), 3.60 (dt, *J* = 6.6, 6.8 Hz, 2H), 3.00 (t, *J* = 6.8 Hz, 2H), 1.95 (s, 3H) according to previously reported spectra.[1]

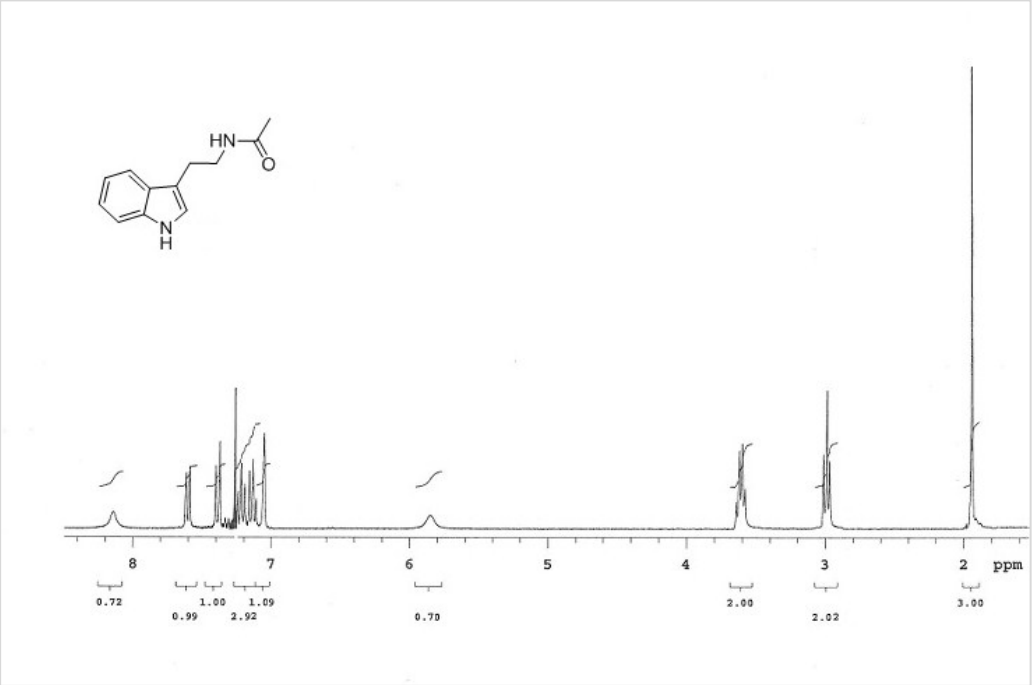


Figure S9. ^{1}H NMR spectrum of *N*-acetyl tryptamine

Melatonin: ^{1}H NMR (300 MHz, CD_3OD) δ (ppm): 7.20 (d, *J* = 8.5 Hz, 1H), 7.05 (d, *J* = 2.2 Hz, 1H), 7.02 (s, 1H), 6.75 (dd, *J* = 8.5, 2.2 Hz, 1H), 3.83 (s, 3H), 3.45 (t, *J* = 7.2 Hz, 2H), 2.86 (t, *J* = 7.2 Hz, 2H), 1.94 (s, 3H) according to previously reported spectra.[5]

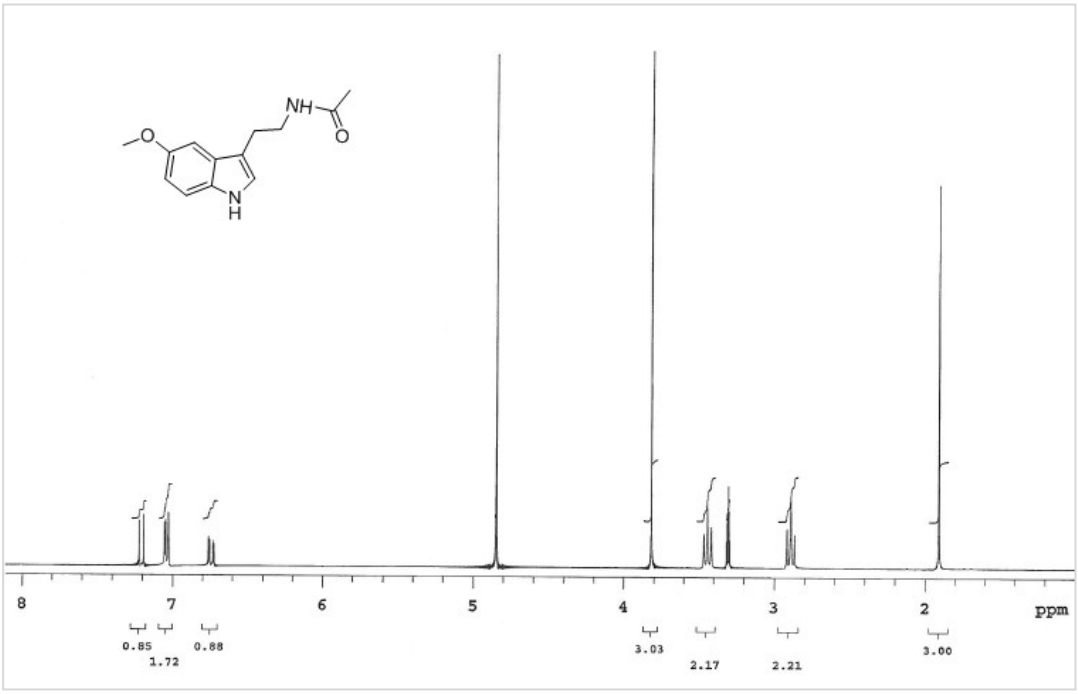


Figure S9. ^{1}H NMR spectrum of melatonin

***N*-acetyl serotonin**: ^{1}H NMR (300 MHz, CD_3OD) δ (ppm): 7.16 (d, *J* = 8.6 Hz, 1H), 7.01 (s, 1H), 6.95 (d, *J* = 2.3 Hz, 1H), 6.66 (dd, *J* = 8.6, 2.3 Hz, 1H), 3.44 (t, *J* = 7.3 Hz, 2H), 2.85 (t, *J* = 7.3 Hz, 2H), 1.93 (s, 3H) according to previously reported spectra.[6]

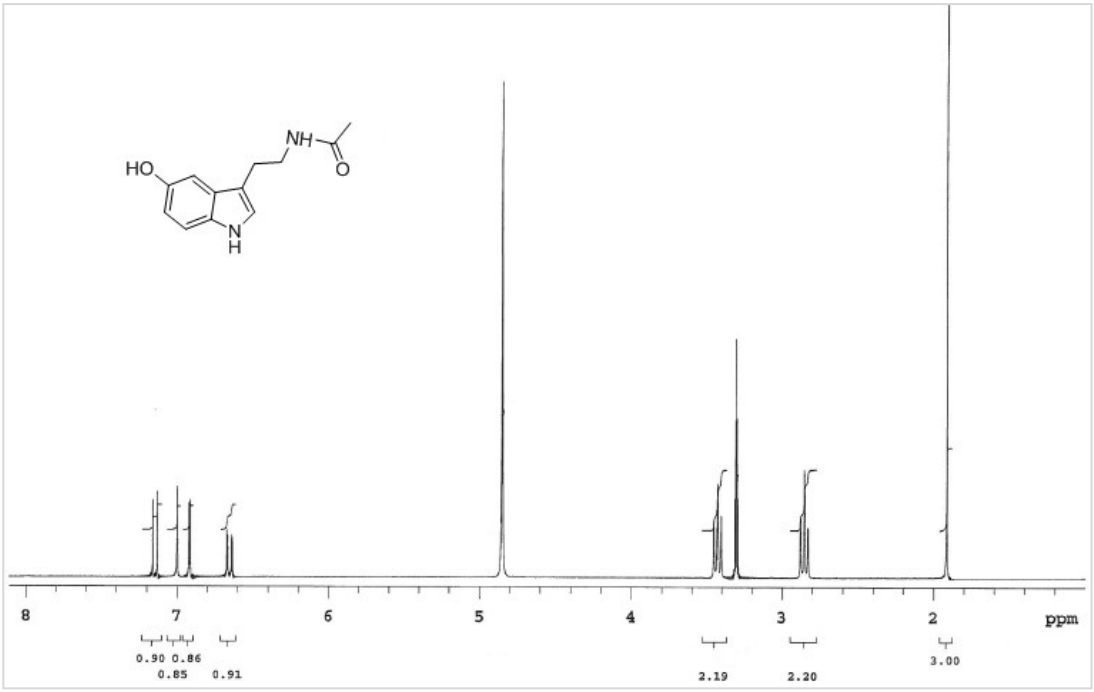


Figure S11. ^{1}H NMR spectrum of *N*-acetyl serotonin

18. References

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