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CONVERSION OF TRYPTAMINE TO SEROTONIN BY CELL SUSPENSION CULTURES OF *PEGANUM HARMALA*

DIDIER COURTOIS, DANIEL YVERNEL, BRUNO FLORIN and VINCENT PETIARD

Plant Biotechnology Research, Francereco, B.P.0166, 37001, Tours Cedex, France

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Key Word Index—*Peganum harmala*; Zygophyllaceae tryptamine; serotonin; biotransformation; bioreactor; cryopreservation; immobilization; Ca-alginate; chitosan.

Abstract—Biotransformation of tryptamine to serotonin by cell cultures of *Peganum harmala* was performed in 250 ml conical flaskes or 10 l bioreactor with high serotonin yields (2.5 g/l of culture and 20% of the biomass dry weight). The specific biotransformation rate reached more than 100 mg/g dry weight/day. The influence of pH, auxin concentration, and temperature were studied. Phenobarbital stimulated the reaction. Immobilized cells showed a lower biotransformation rate than cell suspensions. The stability of the cell line after cryostorage (growth and biotransformation capability) was established.

INTRODUCTION

Plant cell cultures may be able to perform specific biotransformation of exogenous compounds to obtain products usually found in the plant or new compounds [1–4]. Serotonin, presently extracted from coffee waxes, could have an interest as a drug for central nervous system or antileprosy activity [5]. Recently Sasse *et al.* [4, 6, 7] reported the production of 5OH-tryptamine (Serotonin) from tryptamine by cell cultures of *Peganum harmala*. To establish industrial production of a metabolite by plant cell culture, we must investigate a strain collection, select the best producing strain, optimize the culture conditions for growth and production, and scale up in a bioreactor. The preservation of the interesting strains is also an important factor to be considered. Thus some parameters affecting growth and/or the conversion of tryptamine to serotonin (pH, temperature, auxin concentration, feeding of tryptamine and phenobarbital, cell immobilization, cryostorage of cells), are studied. The feasibility of a small-scale production in a bioreactor is also described.

RESULTS AND DISCUSSION

Variability between different cell strains

In screening 25 tissue culture strains of *Peganum harmala* for their ability to produce serotonin from tryptamine after their transfer in liquid medium (Table 1), a wide variation was observed. Serotonin was diffusing into the medium (20–30% of the total serotonin) only for the highest producing strains (No 4,7,16,18 and 20). It must be pointed out that these tissue culture strains were initiated in 1983 and thus considered as stable on solid medium but they may not be stable in liquid medium (experiments were performed after the first subculture in liquid medium). Only cell line no 9 was transferred to liquid medium in 1984. Thus to avoid some variations due to the adaptation to liquid medium, all further experiments described hereafter were performed with cell line no 9. The strain no 9 is not the best producing strain with regard to the biotransformation rate; however it is one of the most vigorous.

Table 1. Specific biotransformation rates of tryptamine in serotonin with 25 *Peganum harmala* cell strains established for three years and transferred in liquid medium

No of strain	Specific biotransformation rate (mg/g dry wt/day)	No of strain	Specific biotransformation rate (mg/g dry wt/day)
5	26.3	3	47.3
10	29.0	24	48.7
8	30.7	23	52.7
1	30.7	19	52.7
13	32.0	14	56.7
12	33.3	17	61.0
9	34.7	21	62.3
25	34.7	4	68.0
2	36.0	7	68.0
6	36.0	16	68.0
11	38.0	18	70.7
15	44.3	20	93.0
22	44.3		

250 ml conical flasks (100 ml medium), 500 mg/l tryptamine added, and three days incubation time.

Conical flask experiments

Initial experiments were performed to describe and optimize the production of serotonin. The cell line (no 9) produces very small amounts of serotonin without added tryptamine. When tryptamine was added, there was a large increase of serotonin content (Table 2A) without significant diffusion into the medium. These results are very similar to those reported by Sasse *et al.* [4].

Table 2. Effect of tryptamine and phenobarbital on the production of serotonin by *Peganum harmala* cell suspensions

	A		*B*	
	Control	+Tryptamine	Control +phenobarbital	+tryptamine +phenobarbital
Fr. wt (g/l.)	91.8±7.8	73.9±2.2	124.2±11.6	116.4±8.9
Dry wt (g/l.)	2.2±0.2	1.8±0.1	3.0±0.2	2.8±0.2
Serotonin (mg/l.)	3.7±0.3	123.0±6.7	8.4±0.8	213.3±19.9
Biotransformation rate (mg/g dry wt/day)	1.0	38.3	1.9	50.3

250 ml conical flasks (100 ml medium), 500 mg/l tryptamine, 100 mg/l phenobarbital, 48 hr incubation time. Values are the means of 3 experiments. Initial cell fr. wt: 70 g/l.

As observed for other hydroxylation reactions, e.g. digitoxin 12B hydroxylase [11, 12], these authors suggested a cytochrome P-450 dependent monooxygenase as the enzyme involved. Phenobarbital was used as an inducer of microsomal cytochrome P-450 in higher plant tissues [13]. With the *Peganum harmala* cell suspension, phenobarbital induced an increase of serotonin production (Table 2B), even without tryptamine. While the specific biotransformation rate of tryptamine to serotonin was 38.3 mg/g dry wt/day with only tryptamine (this value being near the value of 30 mg/g dry wt/day observed by Sasse *et al.* [4]), this rate reached 50.3 mg/g dry wt/day when phenobarbital and tryptamine were simultaneously fed to the cell suspension. This result indicates a very significant effect of phenobarbital but further investigations are required to demonstrate a direct relation with a cytochrome P-450 dependent enzyme.

Serotonin degradation. Using cell line no 9, there was a constant increase of serotonin when tryptamine was added every two days (Fig.1). The sum (tryptamine + serotonin) was stable during the two days between every addition of tryptamine, showing that all the tryptamine was converted to serotonin, without any important degradation.

Furthermore, to check the possible degradation of serotonin during a culture cycle, serotonin was added to the suspension at various concentrations (without addition of tryptamine). At least until day six of culture time, growth was not modified by the serotonin added, even at a concentration of 750 mg/l (Table 3). There was an important uptake of serotonin by the cell and no degradation of it (total serotonin recovery was not significantly different from the total added serotonin).

These results differ from those of Sasse *et al.*[4] and led us to check the analytical procedure used by these authors (thin layer chromatography followed by the quantification of *A* at 280 nm using a Camag-TLC Scanner). No difference was found between the techniques. The differences observed may be due to the nature of the cell line. It is possible that a graduation in the capability of degradation occurs between strains as it does for the biotransformation rate (Table 1).

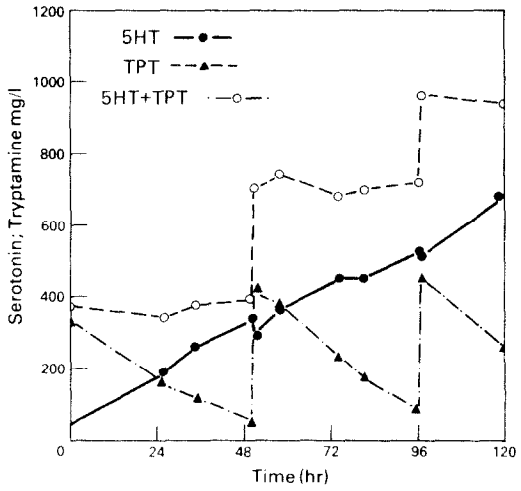

Fig. 1. Biotransformation of tryptamine to serotonin by cell suspension of *Peganum harmala* cultivated in conical flasks. 200 g fr. wt/l (initial cell biomass).

Effect of pH on the biotransformation. pH could have an effect on the yield, for example by changing the permeability of tonoplast or plasmalema membrane (i.e. the compartmentation of tryptamine and serotonin) and thus on the uptake of the substrate or the diffusion of serotonin out of the cells.

pH 5.6 MES (2C*N*-morpholinolethanesulphonic acid) and pH 6.6 MOPS (3C*N*-morpholinolpropanesulphonic acid) 50 mM buffers were used in a short-time experiment (Fig.2). The highest serotonin concentration was obtained at pH 6.6. Very high specific biotransformation rates were obtained, from 89 mg/g dry wt/day (control, pH medium 4.6) to 160 mg/g dry wt/day (pH 6.6 buffer). No significant diffusion of serotonin in the culture medium was observed. pH values above 7.2 induced the death of the cells.

Temperature and hormonal effects on the growth of the cell suspension. The biotransformation seems to be inde-

Table 3. Effect of various concentrations of serotonin on the growth of cell suspension and the level of recovered serotonin

Initial serotonin added (mg/l)	Harvest time (days)	Serotonin cells (mg/l)	Serotonin medium (mg/l)	Serotonin total (mg/l)	Fresh weight (g/l)
0.0	3	0.0	43.0	3.0	98.2
	6	0.0	7.0	7.0	489.0
150.0	3	97.0	47.0	143.0	102.0
	6	126.0	27.0	153.0	441.0
225.0	3	165.0	60.0	225.0	98.0
	6	197.0	25.0	222.0	482.0
400.0	3	144.0	208.0	351.0	95.0
	6	360.0	34.3	394.0	445.0
750.0	3	339.0	451.0	789.0	100.0
	6	705.0	40.0	745.0	491.0

Serotonin was added at $t=0$, in conical flasks containing 50 g of cell fresh weight per l.

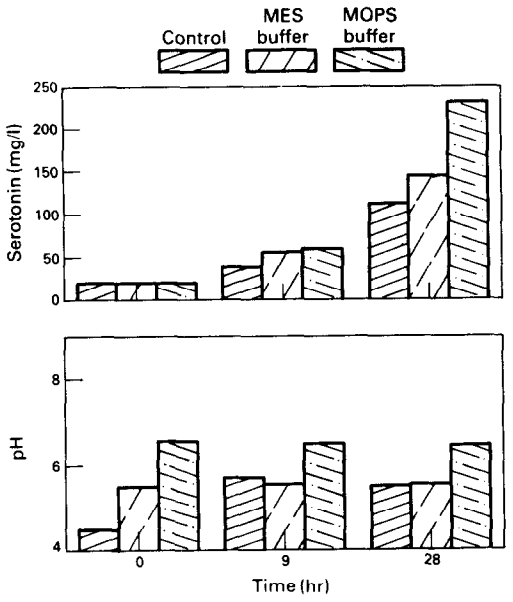

Fig. 2. pH effect on the biotransformation of tryptamine to serotonin. Short-term experiment in 250 ml conical flasks (100 ml medium and 1.25 g cell dry wt). Tryptamine: 500 mg/l.

pendent of the growth stages [4]. From the industrial point of view, it is useful to increase the growth rate to reach a sufficient biomass for the production step in a short time. Figure 3 summarizes the best results obtained as a function of temperature and/or NAA concentration. The standard conditions (control) in MS medium were K1, A10 (kinetin 1 μM, NAA 10 μM) for the medium, at 26°. Reduced NAA (1 μM) induced the greatest fresh and dry weights, whereas at 30° the lag phase was reduced. The best result was obtained in MS medium with 1 μM K,

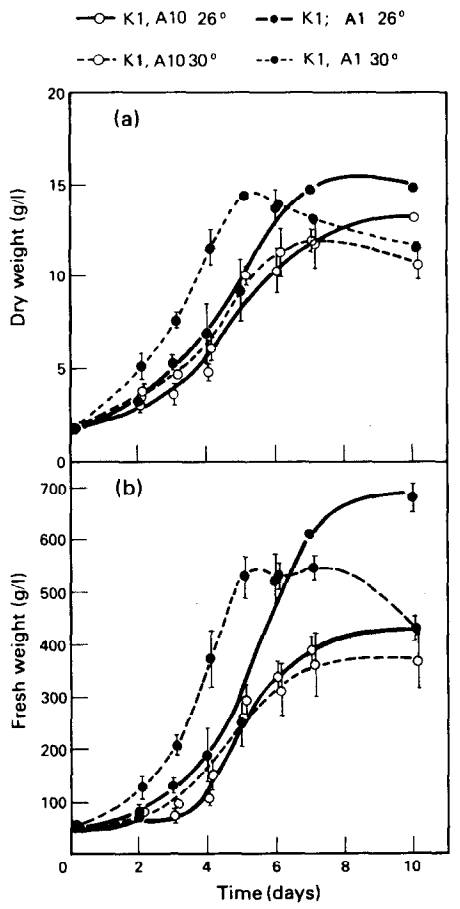

Fig. 3. Effect of the temperature and auxin concentration on the growth of the cell suspension in conical flasks. MS medium. Experimental points were triplicated. a dry wt; b fr. wt.

1 μM NAA at 30°. In this case, cell doubling time was 42 hr (48 hr in control culture).

Bioreactor experiments

A standard batch culture in a 10 l. stirred tank bioreactor is illustrated in Figs 4 and 5 (standard culture medium). Tryptamine was fed regularly as a function of the serotonin and tryptamine analysis (The basis was to maintain the total tryptamine concentration near 100 mg/g dry wt). The cell doubling time was three days and the maximum specific growth rate u_m = 0.0098/hr, with 1.82 g cell dry weight/g O_2 (Y_x/O_2). The maximum growth yield reached 0.6 g cell dry wt/g sugar. At the end of the culture, the carbon source was totally consumed. The fact that the cell doubling time was longer in bioreactor (72 hr) than in conical flasks (48 hr with the same medium, Fig. 3) suggests an oxygen limitation in the bioreactor, or at least problems of mass transfer. The mean biotransformation rate was 36 mg/g dry wt/day, very similar to those obtained in conical flasks (Table 2), and the maximum serotonin content into the cells was 20% of cell dry wt. Serotonin diffusion remained insignificant (results not shown).

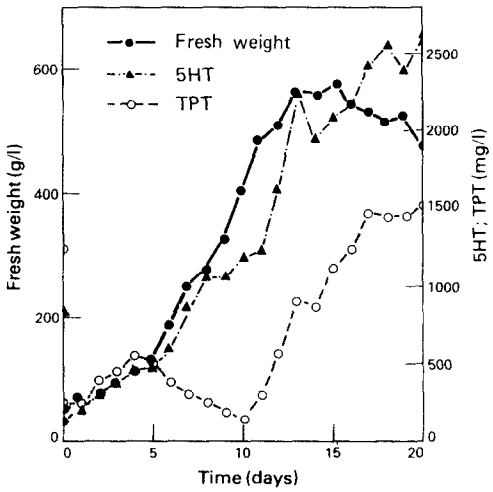

Fig. 4. Biotransformation of tryptamine to serotonin by cell suspension in a 10 l bioreactor. Tryptamine was fed continuously.

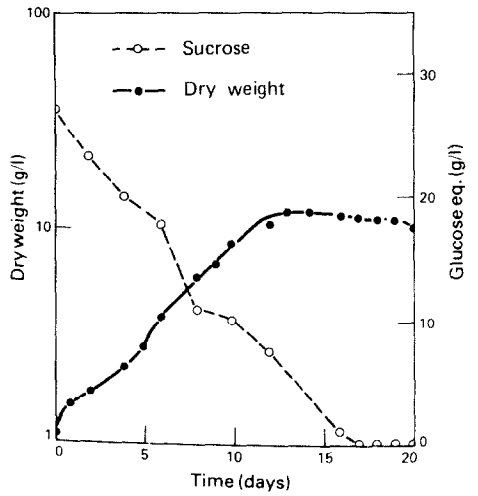

Fig. 5. Growth of cell suspension in a 10 l bioreactor. Sucrose concentration expressed as glucose equivalent.

In such a system, it was possible to reach a maximum of 2.5 g serotonin/l in 20 days from an inoculum of 1 g cell dry wt. A theoretical productivity of 400 mg/l/day was reached on the basis of 6 g constant dry biomass. For an industrial development, the feasibility of a continuous production must be demonstrated. Figure 6 describes an experiment performed in a 10 l stirred tank reactor with three successive semi-continuous culture conditions. The culture was carried out during two months with five subcultures every three days (200 mg/l tryptamine added at each subculture), then four subcultures every five days (200 mg/l tryptamine), then one after seven days with a large addition of tryptamine (up to 1.5 g/l). Each subculture was performed by harvesting half of the culture volume (5 l).

Until day 45 the highest serotonin accumulation per cell dry mass was not reached, due to insufficient concentration of tryptamine that was totally transformed at the end of each subculture (serotonin: 7% of cell dry weight with a mean biotransformation rate of 21 mg/g dry wt/day and a constant productivity of *ca* 200 mg/l/day). From days 45 to 61, the highest serotonin accumulation was reached: 20% of cell dry wt (as in the previous experiment, Fig.4) with a biotransformation rate of 35.7 mg/g dry wt/day. The productivity reached about 300 mg/l/day. These results show that semicontinuous cultures, with various tryptamine feeding conditions, can be performed in at least a 10 l bioreactor with no changes in the parameters of growth and serotonin production during at least two months.

Immobilized cells in continuous flow reactor. The results obtained with Ca-alginate gel entrapped cells following the procedure described in experimental are showing in (Table 4). The culture time was 31 days without renewal of the medium.

If we assume a constant biotransformation rate, this one reached *ca* 6 mg/g dry wt/day, i.e. five times less than with cell suspension. However 80% of serotonin was

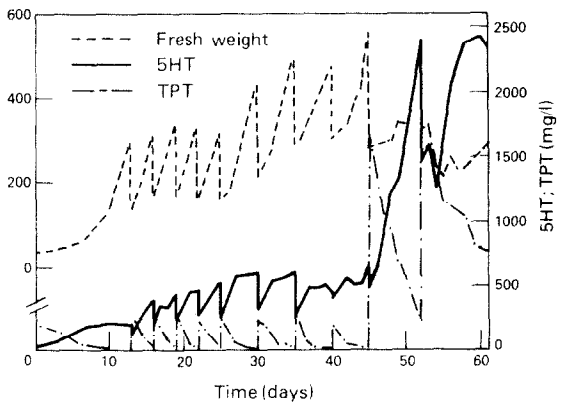

Fig. 6. Semicontinuous cultures in 10 l bioreactor during two months with feeding of tryptamine. Fresh weight:------; serotonin(SHT): ——; tryptamine(TPT):- - - - -.

Table 4. Entrapped cells in Ca alginate, serotonin and tryptamine content (mg/l) in the working volume (51) after 31 days of culture

	Serotonin medium 104mg	Serotonin cells 25mg	Tryptamine medium 65mg	Tryptamine cells 3mg
Total serotonin	129 mg			
Total tryptamine			68 mg	

1g tryptamine added (200 mg/l); 3.3 g cell dry weight (140 g fr. wt) inoculated.

recovered in the culture medium, and the sum of tryptamine and serotonin was 197 mg/l (200 mg/l added tryptamine), showing that the specificity of the reaction is maintained.

If a regular renewal of the medium is performed (Fig.7) every three or four days, 95% of serotonin is recovered in the total harvested media and the biotransformation rate reached 5.38 mg/g dry wt/day. Thus the renewal of the medium did not allow a better serotonin production, which was still much lower than in the cell suspensions Biotransformation does not seem to be limited by the concentration of the product inside the cells, because even when 95% of the product diffused into the medium the production is not enhanced. The entrapment of cells is probably not in favour of hydroxylation. It could be due to a poor oxygen supply to the cells.

Similar results were obtained by Sasse *et al.* [4]. However, as was already discussed, the cell line we used is distinguished by the fact that it has some capability to diffuse serotonin outside the cells and no capability to degrade it. No positive results were obtained with chitosan entrapped cells (no biotransformation, nor diffusion) may be due to the high pH value (8) required to stabilize the beads before inoculation into the culture medium, that induced the cell death.

Cryopreservation of Peganum harmala *cell suspension.* The growth evolution (dry wt) of the cell suspension before and after cryopreservation (18 subcultures after thawing) is shown in Fig.8. The growth was not modified by the cryopreservation procedure, Table 5 summarizes the results concerning the biotransformation capability before and after cryopreservation and shows that the cell line kept this biochemical capability after cryostorage. The specific biotransformation rate was around 30 mg/g dry wt/day in both cases and no significant diffusion of serotonin was observed (*ca* 6% of serotonin in the medium for the control and 10% after cryostorage). Without tryptamine added (results not shown), there was no serotonin production. At the present time, there are only few reports on the metabolite production after cryostorage of a cell line [14], whereas this technique is an important tool in the field of production of natural compounds by plant cell culture for the maintenance of strains and their stability.

EXPERIMENTAL

Material. Tissue culture strains of *Peganum harmala* L. were initiated in Sept. 1983 from stem and leaf explants on a MS medium [8] supplemented with sucrose 20 g/l, agar 7 g/l and various hormonal conditions. They were subcultured monthly at 26° in darkness. One of these strains (no. 9) was transferred two years ago in liquid MS medium (kinetin 1 μM, naphthalene acetic acid[NAA] 10 μM). The suspension was subcultured

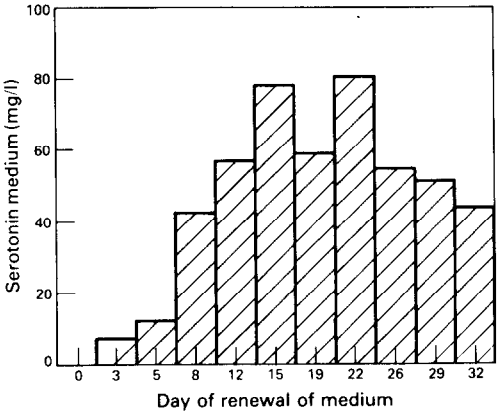

Fig. 7. Biotransformation of tryptamine to serotonin by immobilized cells in Ca-alginate. Serotonin concentration in each renewed medium.

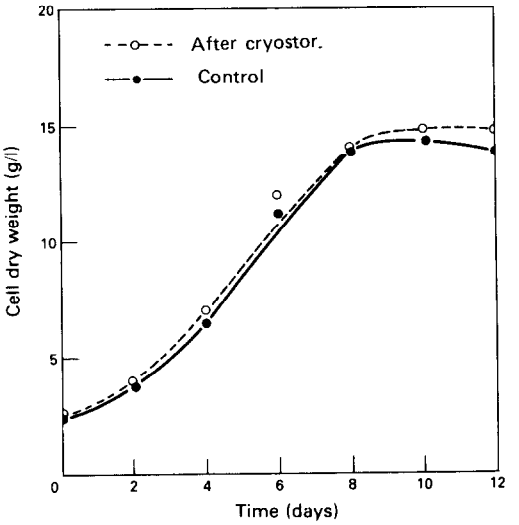

Fig. 8. Growth of the cell suspension in conical flasks after cryostorage in liquid nitrogen.

Table 5. Concentration of serotonin and tryptamine (mg/l culture) in medium and cells in control and after cryostorage

	Serotonin(mg/l)		Tryptamine(mg/l)	
	Medium	Cells	Medium	Cells
Control	8.5 ± 2.3	136.0 ± 21.1	232.0 ± 23.6	53.3 ± 9.5
	(total: 144.5)		(total: 285.3)	
After cryostorage	17.3 ± 4.6	141.0 ± 6.4	232.5 ± 28.1	38.1 ± 6.2
	(total: 158.3)		(total: 270.6)	

Incubation time: 48 hr. 500 mg/l tryptamine. 2.5 g cell dry weight inoculated. Values are the means of three experiments.

weekly (5 g fr. wt into 100 ml medium) and incubated in 250 ml conical flasks agitated at 100 rpm and 26° in darkness.

Bioreactor. A 10 l stirrer tank reactor (New Brunswick Inc.) was used for batch culture. The aeration rate varied, depending on culture time and cell density, from 0.04 VVM (150 rpm, Kla = 2.88/hr) to 0.25 VVM (200 rpm, Kla = 15.23/hr). Temperature was regulated at 26°.

Immobilization experiments. Two procedures were used to obtain gel-entrapped cells in a continuous flow reactor: calcium alginate: cells (200 g wet wt) were suspended in 300 ml MS medium with reduced content in $CaCl_2$ (1.4 mM) and 3% alginic acid (Sigma ref. 2158). The suspension was dripped into the same medium with 50 mM $CaCl_2$. The beads were collected and washed with MS medium containing 5 mM $CaCl_2$. Chitosan: the procedure of ref. [9] as described in ref. [10] for plant cells was used.

Continuous flow reactor: the working volume (500 ml) was inoculated with beads (3 g cell dry wt). An aerated (0.5 l/min) medium storage vessel (5 l) was connected to the reactor to allow a continuous circulation of the medium on the beads (750 ml/hr).

Cryopreservation. The procedure of ref.[15] was used to perform the cryopreservation of cells. Cells at the early stage of the growth phase were suspended in culture medium supplemented with 6% mannitol. The cryoprotectants (0.5M DMSO 0.5 M glycerol and 1 M sucrose) were added after 3 days [16], and the cell suspension shaken for 1 hr (100 rpm) at 4°. The cell suspension (1 ml) was dispensed into sterile polypropylene tubes (2 ml), then frozen in a programmable freezer (Minicool LC 40, Compagnie Francaise de produits oxygenes) at 0.5°/min until −40°. After 1 hr at this temp., the tubes were immersed directly in liquid nitrogen.

After one month storage, the cell suspension was thawed at temp.40°, then immediately spread on 5 ml solid culture medium (8 g/l agar) in Petri dishes (5 cm diam.). Once growth had been re-established, the cells were transferred into liquid medium and cultivated in standard conditions.

Incubation experiments. Tryptamine chloride and phenobarbital were added to the medium before autoclaving. Phenobarbital was used at 100 mg/l. Depending on the experiment and on the cell mass, tryptamine was used from 200 to 1000 mg/l. Details are given in the text and Figures.

Analytical procedures. Sugar concn, expressed as glucose equivalent, was determined by an enzymatic assay (Boehringer Manheim, ref. 716260). Tryptamine and serotonin quantification: cell or medium samples were extracted with hot EtOH, filtered then diluted and analysed by HPLC using a Waters apparatus (Nova pack 5 μm reverse phase C_{18} column) at 280 nm. 410 ml of 0.2 M HOAc and 90 ml of 0.2 M NaOAc supplemented with H_2O to 1l. pH4 buffer was used as the mobile phase after addition of EtOH (20%).

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