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Research article

Rhodococcus erythropolis ATCC 25544 as a suitable source of cholesterol oxidase: cell-linked and extracellular enzyme synthesis, purification and concentration

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Abstract

Background: The suitability of the strain *Rhodococcus erythropolis* ATCC 25544 grown in a two-liter fermentor as a source of cholesterol oxidase has been investigated. The strain produces both cell-linked and extracellular cholesterol oxidase in a high amount, that can be extracted, purified and concentrated by using the detergent Triton X-114.

Results: A spray-dry method of preparation of the enzyme inducer cholesterol in Tween 20 was found to be superior in both convenience and enzyme synthesis yield to one of heat-mixing. Both were similar as far as biomass yield is concerned. Cell-linked cholesterol oxidase was extracted with Triton X-114, and this detergent was also used for purification and concentration, following temperature-induced detergent phase separation. Triton X-114 was utilized to purify and to concentrate the cell-linked and the extracellular enzyme. Cholesterol oxidase was found mainly in the resulting detergent-rich phase. When Triton X-114 concentration was set to 6% w/v the extracellular, but not the cell-extracted enzyme, underwent a 3.4-fold activation after the phase separation process. This result is interpreted in the light of interconvertible forms of the enzyme that do not seem to be in equilibrium. Fermentation yielded 360 U/ml (672 U/ml after activation), 36% of which was extracellular (65% after activation). The Triton X-114 phase separation step yielded 11.6-fold purification and 20.3-fold concentration.

Conclusions: The results of this work may make attractive and cost-effective the implementation of this bacterial strain and this detergent in a purification-based industrial production scheme of commercial cholesterol oxidase.

Background

Microbial cholesterol oxidases (EC 1.1.3.6) (COX) catalyze the oxidation and isomerization of cholesterol to 4-cholesten-3-one. Interest in these enzymes mostly relies in their utility in the determination of cholesterol in biological samples such as serum and foods [1], and also in the

bioconversion of a number of 3β-hydroxysteroids in organic solvents [2] and in reverse micelles [3] (for a recent review see [5]). Since earliest reports on crude preparations from *Mycobacterium sp.*[4], cholesterol oxidases have been described in a number of bacteria and fungi [5]. Enzymatic properties of cholesterol oxidase from *Rhodococcus* strains (some of which named formerly as *Nocardid*) are particularly suitable for use in the analytical determination of cholesterol, in which the hydrogen peroxide formed is used in a chromogenic reaction catalyzed by horseradish peroxidase [6].

The *Rhodococcus* enzyme has been usually reported to be membrane bound, extractable from whole cells by treatment with detergents or trypsin, although no phospholipids are detected in the enzyme extracts [7]. More recent reports have demonstrated the production of both extracellular and cell-bound cholesterol oxidase by strains of this genus such as *Rhodococcus* sp. GK1 [8], *R. erythropolis* ATCC 25544 [9] and the pathogenic specie *R. equi*[10,11].

The kinetics of enzyme synthesis at both bench and large scale by *Nocardia rhodochrous* (renamed as *Rhodococcus rhodochrous*), a strain that produces only a cell-bound COX, has been studied and the growing conditions for bacterial enzyme synthesis in fermentor were defined [12].

Due to the high cell-bound to extracellular ratio of cholesterol oxidase produced by *Rhodococcus*, even in those strains that also produce extracellular enzyme, the use of detergents is compulsory in the cost-effective extraction of this enzyme. The properties of protein extraction and purification combine in polyoxyethylene type detergents whose cloud point is in the biocompatible range [13]. For instance, Triton X-114 is as effective as Triton X-100 to extract membrane proteins, but its cloud point in semidiluted solutions (temperature at which the detergent solubility decreases sharply and a liquid-liquid phase separation is produced) is 23°C as compared to 65°C of Triton X-100. Therefore, extracted proteins partition between a detergent-rich phase and a detergent-depleted phase thus occurring protein purification. Protein purification has been accomplished successfully from either animal cells and organelles, plant tissues and microbial cells [13–15]. Triton X-114 at 1%w/v in buffers has been utilized to study the partitioning behavior of commercial cholesterol oxidase from several bacterial sources, resulting in partitioning toward the detergent rich-phase in all cases [16]. The polyoxyethylene detergent C12EO5 added the to a non-clarified culture of *Nocardia rhodochrous* was used for direct solubilization and extraction of the cell-bound cholesterol oxidase followed by phase separation [17]. A four-fold preconcentration and five-fold purification were achieved in optimal conditions. Due to the high cost of C12E05 these authors tried the cheaper four narrow range ethylene oxide surfactant C12-C18E05 [18] which was found equally suitable for direct solubilization and extraction of cell-bound cholesterol oxidase, thus this system was expanded to pilot scale [19].

In a previous work [9] we described the cell-bound and extracellular cholesterol oxidase activities from *R. erythropolis* ATCC 25544, achieving in optimal conditions 55% cell-bound and 45% extracellular activity. Their enzymatic properties strongly supported the idea that the particulate and the extracellular cholesterol oxidases are two different forms of the same enzyme with an estimated molecular mass of 55 kDa. In this work we optimize the culture conditions in a 2-liter fermentor of this extracellular cholesterol oxidase producer strain and carry out the extraction, partial purification and concentration of both types of cholesterol oxidase by using Triton X-114 phase separation. The results obtained are very promising for the use of this strain and this technique in the industrial processing of bacteria to obtain cholesterol oxidase.

Results and discussion

Batch cultivation of R. erythropolis (ATCC 25544)

The bacteria were grown on the GYS medium in a 2-liter scale fermentor in batch mode operation under pH and temperature controlled conditions. Under this conditions the cell yield was doubled (9.5 mg/ml vs. 4.8 mg/ml dry cell weight) and the cultivation time was reduced to one third (60 vs. 180 hours) as compared with shaken flasks. These results are in good agreement with the literature [12]. We found that addition of 2 g/l cholesterol to the culture broth [12], prepared as an aqueous emulsion with the aid of Tween 80 at a weight ratio 2:1 results in a high yield of COX production [9], but the preparation procedure of that emulsion had a marked influence in the final enzyme yield, although not on the cell weight, as seen in Table 1. The spray-dry method resulted advantageous because the cholesterol :Tween 80 emulsion formed readily and COX production increased in overall by three times with respect to the preparation of the cholesterol:Tween 80 mixture at the flame. Enzyme production improvement resulted larger as cell-linked (3.8-fold) than as extracellular (2.3-fold). This overall increase of COX production can be due to a better availability of cholesterol to the cell since particle size obtained by spray-dry is smaller.

Results from a typical batch fermentation are shown in Figure 1. Three stages can be differentiated during the fermentation process, (i) A first stage (0–16 h), in which 02 consumption increases continuously and HCl is consumed to keep the pH constant to 6.75. Buckland et al. [12] found that pH of the culture rose by 0.5 units in the first part of growth, and then fell. An exponential increase of cell mass is observed and low levels of COX activity ap-

Table 1: Effect of the cholesterol emulsification method on the production of COX.

	COX activity (U/ml)*		
Emulsification cholesterol method	**Cell-linked**	**extracellular**	**Dry weight (mg/ml)**
Spray-dry	230	140	8.75
At the flame	60	60	9.05
Improvement	3.8	2.3	0.97

*Enzymatic activity figures correspond to 70 hours of fermentation.

pear linked to cells, (ii) The second stage (16–45 h) is characterized by a strong 02 consumption and a consumption of base. In this phase aerobic metabolism drives the cell growth but the growth rate is certainly limited by the O_2 availability – note pO_2 is nearly zero under continuous stirring and air supply-. This stage is also characterized by a high rate of COX production of both types, cell-linked and extracellular, (iii) The third stage (45 h to the end of the process) a second phase of consumption of acid was recorded whereas pO_2 increased again to reach saturating levels. The greatest increase of cell-linked COX production was observed in this stage whilst extracellular COX production stopped.

The profile of fermentation was very similar to that obtained by Buckland et al. [12] but differed in the accumulation of extracellular COX: the strain of Nocardia (NCIB 10554) used by these authors produced very low levels of extracellular enzyme while the strain tested in this work produces high levels. They also tested the effect of dissolved oxygen tension on the production of COX and found that in limiting conditions of oxygen supply the production of cell-linked COX was low. As seen in Figure 1, when oxygen supply is limiting (in the second stage) the rate of cell-linked COX production decreases, however is in these conditions when extracellular COX production takes place. Thus, there seem to be some relation between dissolved oxygen tension and extracellular COX production by the strain used in this work.

The results obtained are coherent with those presented in a previous study in shaken flasks [9], where extracellular COX production is large and arises from the partial solubilization of the cell-linked enzyme [20,21]. After 70 hours of fermentation the total enzyme activity obtained was ca. 360 U/ml, being 230 U/ml cell-linked and 130 U/ml extracellular, thus the cell-linked to extracellular ratio

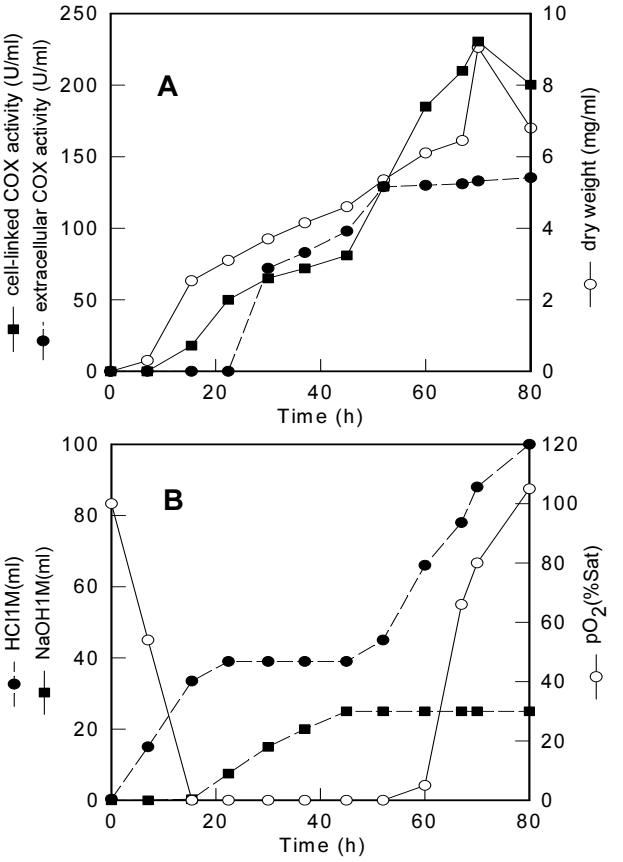

Figure 1
Characterization of the *R. erythropolis* fermentation process: biomass and production of cell-linked and extracellular COX. Enzyme activities are given as units/ml of cell culture. The data shown are from a single experiment but are representative of three separate replicates.

is 1.26. This ratio in shaken flasks ranged from 1.26, using the same amount of Tween 80 as in this work (0.1%), to 1.38, using 1% Tween 80, but in the latter the overall yields of COX production were 7-fold smaller [9]. The overall yield obtained in this work is comparable to that obtained by Buckland et al. [12] and by Minut et al. [17] but larger than that of Cheetham et al. [22]. Watanabe et al [24] compared the cell-linked and extracellular COX production of 31 strains of the genus *Rhodococcus* and *Nocardia* and found that among the best extracellular COX producers, the strains *Rhodococcus* sp. N° 31 and *R. equi* N° 24, displayed the highest cell-linked to extracellular ratio, 1.32 and 2.68 respectively.

Use of Triton X-114 for extraction and purification of COX

In most of the available wild producer strains, COX behaves as a cell-linked enzyme, which is particularly true in the genus *Rhodococcus*[7,8,23]. Significant levels of extracellular COX have been described to be produced by the pathogenic species *R. equi*[10,11,24,25] and also by *R. erythropolis*[9] and *Rhodococcus* sp. [8,24] in certain culture conditions.

Several authors have investigated the ability of different detergents to disrupt lipid-protein associations and to release cell-linked COX in its native state. The use of Triton X-100 has been largely accepted [7,9,12,26,27] but other polyoxyethylene type non-ionic detergents whose cloud point is in the biocompatible range can be used for COX solubilization and purification [17].

Table 2 shows the amount and the percentage of cell-linked COX extracted by either Triton X-100 or Triton X-114 at several detergent concentrations. COX could be extracted from cells and solubilized by both detergents at 1% w/v. The % of recovery is of the same order as previously reported for these detergents [17]. The increase of the Triton X-114 concentration from 1 to 3% w/v resulted in concomitant increases of 1.5-fold the total activity, 2.6-fold the specific activity and a % of recovery above 90%. A Triton X-114 concentration as high as 6% w/v does not improve significantly the recovery nor the specific activity. Phase separation was further induced to gain COX purity in extracts and in the broth independently.

Table 2: Cell-linked COX extraction by Triton X-100 and Triton X-114 detergents.

DETERGENT	COX activity (UE/ml)	% recovery	COX activity (UE/mg Prot)
TRITON X-1001%	234.2	59.3	1306.9
TRITON X-114 1%	243.4	61.6	566.9
TRITON X-114 2%	274.5	69.5	860.5
TRITON X-114 3%	363.8	92.1	1466.9
TRITON X-114 6%	365.0	92.4	1674.3

The cell-free extract of Triton X-114 was subjected to phase separation as such. The culture broth was first supplemented with Triton X-114, well dissolved at 4°C and then warmed up to induce detergent phase separation. Figures 2a and 3a show the distribution of enzyme activity in each phase (detergent-depleted and -rich) for each of the COX sources (cells extract and culture broth) respectively.

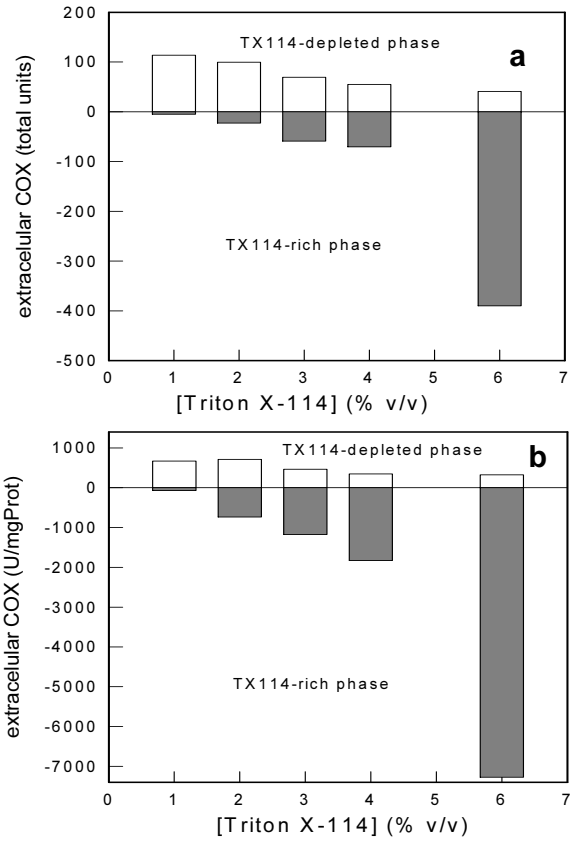

Figure 2
Distribution of COX activity among detergent depleted and detergent rich phases after induction of phase separation of culture broth supplemented with the indicated concentration of Triton X-114. (a) Total activity; (b) Specific activity.

As Triton X-114 concentration is increased, COX partitions towards the detergent rich phase, increasing its specific activity (Figures 2b and 3b) thus resulting in enzyme purification and also in enzyme concentration since the volume of the detergent-rich phase is much lower than the initial volume. The 1% concentration of detergent was an exception to this rule since COX partitioned toward the depleted phase under our working conditions. Partitioning of commercial COX in buffers containing 1% Triton X-114 occurred toward the rich phase and was very influenced by the buffer concentration [16]. Therefore, it seems that the composition of phase separation media is extremely important to the partitioning of particular proteins.

The purification was made evident by running samples of COX from cells and culture broth in SDS-PAGE gels. Figure 4 shows that in both cases the detergent-rich phase

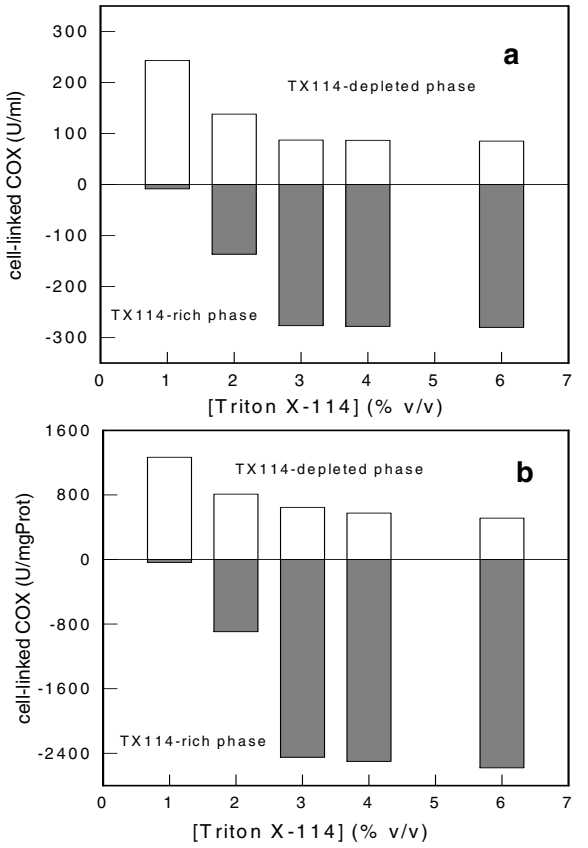

Figure 3
Distribution of COX activity among detergent depleted and detergent rich phases after induction of phase separation of cell extracts done with the indicated concentration of Triton X-114. (a) Total activity; (b) Specific activity.

was enriched in some proteins, including COX, whereas the depleted phase showed other different protein bands.

An exceptional result was obtained when performing COX purification from the culture broth supplemented with a 6% w/v Triton X-114. The total activity recovered after phase separation was ca. 3.5-fold that measured in the broth before phase separation. This result suggests that soluble COX produced by the culture is not fully active and that it can be activated by a treatment with 6% Triton X-114 but not with 4% or less. Further increase of Triton X-114 concentration results in no improvement with respect to 6% (results not shown). This phenomenon was not observed with COX extracted from cells, therefore the enzyme most likely exists in a fully active form in the cells.

We have shown previously that cell-linked and soluble COX from the same strain are almost indistinguishable as

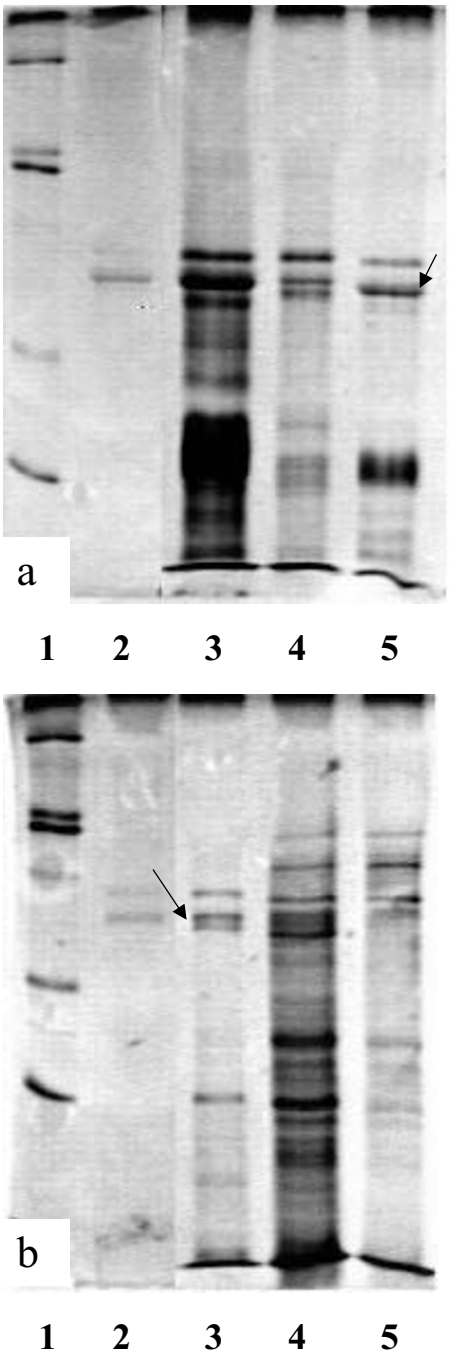

Figure 4
SDS-PAGE of COX fractions using 3% Triton X-114 for extraction, purification and concentration, (a) Cell extracts: lane 1, Mw markers; lane 2, commercial COX; lane 3, total extracted proteins; lane 4, proteins in detergent depleted phase; lane 5, proteins in detergent rich phase, (b) Culture broth: lane 1, Mw markers; lane 2, commercial COX; lane 3, proteins in detergent rich phase; lane 4, total proteins in culture broth; lane 5, proteins in detergent depleted phase. Arrows indicated the COX band.

judged by some enzymatic properties such as kinetic parameters, electrophoretic mobility of the native active enzyme and thermostability [9]. Now we show evidence of a differential characteristic of soluble COX as compared to cell-linked: the activation by 6% Triton X-114.

The observed phenomenon accepts in principle several explanations: (i) all the soluble COX molecules become activated by 3.5-fold due to a detergent effect on the protein conformation, (ii) a fraction of soluble COX is active and a fraction 3.5-fold larger is fully inactive, but can be activated due to a detergent effect on the protein conformation, (iii) an inhibitor is removed as a consequence of detergent treatment. From the first hypothesis it could be expected some difference between both enzyme forms at least at kinetic level, which we did not observe in previous studies, although it cannot be discarded. The inhibitor hypothesis is perhaps less likely since the activation effect might have been observed at all concentrations of Triton X-114 and gradually. The second hypothesis may be the most likely according to our previous results since we characterize only active enzyme and not the enzyme protein. In that case it may be hypothesized that there is an active form of COX able to both interact with components of the cell membrane or the cell wall to remain cell-linked, and to stay soluble in the culture broth, and there is an inactive form which is soluble in the culture broth. Reversion of inactive to active is induced by a high detergent concentration, which may provide an environment resembling that of cell membranes or cell walls. The active soluble form secreted by bacterial cells might eventually and reversibly turn into inactive soluble COX. When detergent concentration of the 6% rich phase was lowered by dilution the specific activity did not change, therefore the conversion of active to inactive must be very slow, that is, the existing enzyme forms are not in equilibrium. In any case, the characterization of this activation phenomenon requires further studies that are now under progress in our laboratory.

The enzymatic properties exhibited by COX have been shown to depend on the method of extraction from cells, either with Triton X-100, with buffer or with trypsin [7]. The extracted enzyme could be interconverted from one to another with appropriated treatments such as addition or removal of 0.5% Triton X-100. So they showed evidence for the existence of different isoforms of COX from *Nocardia rhodochrous* (renamed as *Rhodococcus rhodochrous*). These authors also pointed out that no phospholipids were co-extracted even when using Triton X-100 and suggested that cell-linked enzyme is anchored through a hydrophobic tail that interacts with naturally occurring surfactants of the cell wall.

Taking that work into account and our own results it may be proposed that COX shed from cell walls aided by bacterial surfactant solubilization becomes the extracellular enzyme and so its level might be related with the production of bacterial surfactants.

The effect of Triton X-114 phase separation step on the purification and concentration of COX is summarized in Table 3. Taking together purification, concentration and % of recovery, the best results were obtained when using Triton X-114 at 3% w/v for the cell-linked and at 6% for the extracellular COX. Minuth et al. [17,18] achieved 5-fold purification and 4-fold concentration adding the detergent pentaethyleneglycol mono *n*-dodecyl ether (C12EO5) to an non-clarified culture of *Nocardia rhodochrous*. Our results, 11.6-fold purification and 20.3-fold concentration indicate that Triton X-114 should be highly recommended for use with a clarified culture broth of an extracellular COX producer strain.

Table 3: Purification and concentration of COX during Triton X-114 phase separation.

	Cells extract of 3%Triton X-114 w/v	Culture broth with 6% Triton X-114 w/v
Purification fold	1.67	11.6(2.52)[b]
Concentration factor	2.56	20.3 (4.38)[b]
% recovery	76 (70)[a]	312 (65)[b]

[a]Recovery in this step and, in parenthesis, with respect to cells. [b] Figures in parenthesis correspond to purification, concentration and recovery in this step as if no activation occurred, calculated on the basis of remaining activity in the upper detergent-depleted phase

The quantification of COX partitioning in the Triton X-114 two-phase system was accomplished by determining its partition coefficient on the basis of enzyme activity. To that, concentration of COX activity in each phase has been determined by measuring enzyme activity and phase volumes after phase separation. The partition coefficient is defined as activity concentration in the rich phase over activity concentration in the depleted phase:

$$K = [COX]_{rich} / [COX]_{depleted}$$

Results are shown in Figure 5. Concerning the enzyme extracted from cells, partition coefficient reaches an optimum at 3% w/v detergent, decreasing at higher detergent concentrations. We do not have direct evidence to explain this decrease but a reason for it could be a change in the composition of the rich phase that may contain more components of the bacterial cell wall extracted at high de-

tergent concentration, thus interfering in the partitioning of COX. In the case of the extracellular COX the higher the detergent concentration the larger the partition coefficient. As seen in table 4, the rich phase is quite more compact after phase separation in the culture broth than in the cell-extract and its relative volume is not proportional to the detergent concentration. Thus the rich phase is less hydrated, that is, more hydrophobic, with the culture broth than with the cell-extract and the more hydrophobic the higher the detergent concentration. As a result the partition coefficient for COX in the culture broth increases with the detergent concentration. Phase separation of Triton X-114 is affected by the presence of other surfactants such as Triton X-45 [28] and polyols such as glycerol [29], thus bacterial surfactants extracted during detergent treatment may affect phase separation as well. Natural surfactants may be also present in the broth but at a much lower concentration, therefore having less influence on the phase separation.

Table 4: Partitioning of cell-linked and extracellular COX after phase-separation of Triton X-114

		COX total units		COX activity (U/ml)		K
% Triton X-114	% volume of rich phase	Rich phase	Depleted phase	Rich phase	Depleted phase	
Cell-linked						
1	10.8	8.5	234.8	78.7	263.2	0.30
2	15.9	137.9	136.5	867.3	162.3	5.34
3	29.6	276.5	87.1	934.1	123.7	7.55
4	37.5	278.0	86.5	741.3	138.4	5.35
6	41.8	280.0	85.0	669.8	146.0	4.58
Extracellular						
1	6.4	5.0	113.7	78.2	121.5	0.64
2	9.3	22.8	99.7	245.7	109.7	2.24
3	10.7	58.8	69.4	550.5	77.7	7.08
4	12.8	70.5	55.1	552.3	63.1	8.75
6[a]	15.3	84.0	41	547.9	48.4	11.32
6	15.3	390	41	2544	48.4	52.60

[a]COX units in rich phase calculated as if no activation occurred: total units before partitioning (125) – units in depleted phase (41)

The partitioning of commercial COX from *Nocardia erythropolis* and *Pseudomonas sp.* has been shown to depend on the detergent partitioning, since factors that affected the Triton X-114 distribution, such as temperature of partitioning, pH and phosphate concentration of the buffers, affected in the same way the distribution of enzyme [16]. Thus our results are in agreement with those findings. The reason for the different behavior of the detergent packing in cell extracts and in broth may lie in the fact that detergent partitioning is affected by physicochemical factors such as the presence of polyols, lipids, surfactants, etc [13], which can be present in cell extracts but not in the cell culture.

Conclusions

From a technological point of view it is simpler to handle an extracellular enzyme than a cell-linked one. The amount of extracellular COX of the non-pathogenic strain *R. erythropolis* ATCC 25544 obtained in a 1.5 1 batch fermentation represents a 36% of the activity (130 out of 360 U/ml) when measured directly from the broth, but after the 6% Triton X-114 treatment and phase separation it represents a 65% of the total activity (442 out of 672 U/ml). In addition, active COX becomes 11.6 times purer and 20.3 times more concentrated. These results may make attractive and cost-effective the use of this bacterial strain and the Triton X-114 phase separation in a 6% w/v

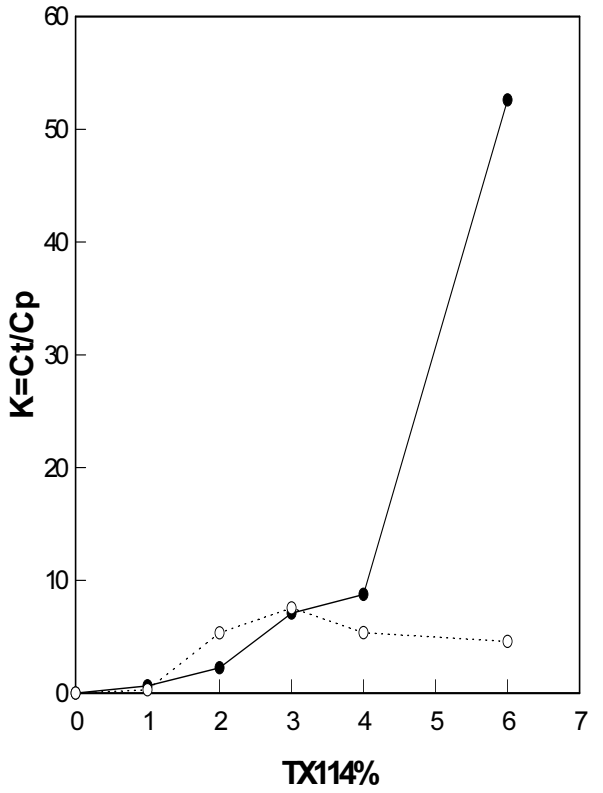

Figure 5
Partition coefficient of cell-linked (•) and extracellular (•) COX in a Triton X-114 phase separation system.

for the industrial production of COX used in serum and food cholesterol analysis. The purification step based on Triton X-114 phase separation should be followed by further steps, such as ion-exchange chromatography, which can combine non-ionic detergent removal and protein purification in one step, in order to obtain a preparation of COX suitable for analytical applications [16].

Investigations to improve the percentage of extracellular COX are currently under progress.

Materials and methods

Commercial cholesterol oxidase from *R. erythropolis* was purchased from Boehringer Mannheim (25 U/mg). Cholesterol from lanolin, cholesterol from human gallstones, Triton X-100 and Triton X-114 were purchased from Fluka. Triton X-114 was condensed three times in 5 mM sodium phosphate buffer pH 7.5 [30,31]. The detergent phase of the third condensation had a concentration of 25% w/v TX-114 and was used as the detergent stock solution for all the experiments. The Triton X-114 concentration was determined from its absorption at 278 nm (A_{278} = 1.25 for 0.05% w/v) [29]

Microorganism and culture conditions

The strain used in this work was *R. erythropolis* ATCC 25544 which was routinely maintained in the laboratory by periodic subculturing in GMP medium [20] consisting of 0.1 g/l glucose, 0.02 g/l yeast extract, 0.04 g/l peptone, 0.04 g/l meat extract, 0.05 g/l NaCl, 0.0025 g/l MgSO4 and 0.25 g/l agar.

The microbial production of cholesterol oxidase was assessed as previously described by us [9].

Cells were grown in GYS medium in a 2 1 reactor (BIOSTAT B from B. Braun Biotech Ltd.) with a working volume of 1.5 1. Air was supplied at 2.6 vol/vol/min; pH was set constant to 6.75 and temperature to 29°C. The GYS medium is a modification of the mineral medium described by Buckland [26] that consisted of 10 g/l glycerol, 20 g/l yeast extract, 2 g/l $(NH_4)_2SO_4$, 2 g/l K_2HPO_4, 0.01 g/l $CaCl_2.2H_2O$, 0.01 g/l $FeSO_4.7H_2O$, 0.1 g/l $MgSO_4.7H_2O$. When this culture reached a dry weight of ca. 1.0 mg/ml, an aqueous suspension of Tween 80/cholesterol was added to a final concentration of 0.2% cholesterol and 0.1% Tween 80.

An aqueous suspension of cholesterol was prepared in two ways; at the flame and by a spray-dry method. (i) In the first method, cholesterol and Tween 80 were mixed by heating at the flame until total dissolution of solids, then water was added to form an emulsion by vigorous shaking for 1 hour. (ii) In the second, cholesterol and Tween 80 were co-dissolved in diethyl ether; the solvent was then removed by spray drying and the solid material was recovered and used to readily prepare an stable aqueous suspension.

Extraction and partial purification of cholesterol oxidase

The extraction of cell-linked COX by using Triton X-100 was as described previously [9]. For the cell linked COX extraction and purification by the Triton X-114 method, the extract obtained in cold after removal of cells by centrifugation was submitted to temperature-induced phase separation. The coalescence of the detergent was facilitated by warming up to 37°C for 15 min that was followed by the sharp separation of the two resulting phases by spinning at 4000 g for 15 min at 25°C. Both phases, the lower detergent-rich and the upper detergent-depleted were assayed for both enzyme activity (see below) and protein [32]. Using Triton X-114 also purified the extracellular COX. The cold culture broth was supplemented with Triton X-114 to the desired final concentration and deter-

gent was completely dissolved at 4°C. Phase separation was induced as above.

Enzyme assay

Cholesterol oxidase activity was assayed by a modification of the method of Allain et al. [1] as described previously [9]. One unit of activity was defined as the amount of enzyme that converts 1 μmole of cholesterol/min at 37°C. All samples were diluted before enzyme assay to a final Triton X-114 concentration of 0.1% to avoid detergent interference with the assay [16].

SDS-PAGE

The protein extracts were prepared as described previously [9]. SDS-PAGE electrophoresis [33] was carried out at 200 volts at 25° in a Mini Protean cell (Bio-Rad, Richmond, California). The gels were developed by using the silver staining technique.

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