Introducing the Concept of Biocatalysis in the Classroom: The Conversion of Cholesterol to Provitamin D₃

Abstract

Biocatalysis is a fundamental concept in biotechnology. The topic integrates knowledge of several disciplines; therefore, it was included in the course “design and optimization of biological systems” which is offered in the biochemistry curriculum. We selected the ciliate tetrahymena as an example of a eukaryotic system with potential for the biotransformation of sterol metabolites of industrial interest; in particular, we focused on the conversion of cholesterol to provitamin D₃. The students work with wild type and recombinant strains and learn how sterol pathways could be modified to obtain diverse sterol moieties. During the course the students identify and measure the concentration of sterols. They also search for related genes by bioinformatic analysis. Additionally, the students compare biotransformation rates, growing the ciliate in plate and in a bioreactor. Finally, they use fluorescence microscopy to localize an enzyme involved in biotransformation. The last day each team makes an oral presentation, explaining the results obtained and responds to a series of key questions posed by the teachers, which determine the final mark. In our experience, this course enables undergraduate students to become acquainted with the principles of biocatalysis as well as with standard and modern techniques, through a simple and robust laboratory exercise, using a biological system for the conversion of valuable pharmaceutical moieties. © 2016 by The International Union of Biochemistry and Molecular Biology, 45(2):105–114, 2017.

Keywords: biotechnology education; biocatalysis; undergraduate; tetrahymena thermophila; cholesterol; provitamin D3

Introduction

Biocatalysis is a fundamental concept in biotechnology. It is defined as the use of biological systems (whole cells, cellular extracts, or isolated enzymes) to catalyze the regio- and/or stereo-specific conversion of (bio)chemicals. The field of industrial biocatalysis, including biotransformation and bioconversion, has grown rapidly in the last decade, driven by the increasing demand for enantiomerically pure compounds in the pharmaceutical industry, the necessity to promote sustainable development and environmentally friendly technologies and the opportunity to develop purely chemical strategies in the synthesis of complex glycoproteins, oligosaccharides, and lipids [1]. For these reasons, biocatalytic processes are now widely applied for pharmaceutical, agrochemical, chemical, nutritional, and bioremediation purposes, among others [2].

Steroidal hormones have been one of the earliest and most successful products obtained by biotransformation. Since the introduction of a mucor type fungi in the 1950s for the hydroxylation of steroids [3], microbial biotransformations became an important strategy for the production of novel steroidal drugs, as well as for the efficient production of steroid active pharmaceutical ingredients (APIs) and key intermediates. Steroids, along with amino acids, carbohydrates, alcohols, peptides, and fat derivatives, are the main type of compounds produced using biotransformation processes [4]. More than 300 approved sterols and steroid drugs are known to date. They represent one of the largest sectors in the food and pharmaceutical industry with world markets of more than US$10 billion and the production exceeding 1,000,000 tons per year [5, 6].
In addition to their use as pharmaceuticals, sterols, including steroids, are a vast group of terpenoid—lips indispensible for an array of physiological processes. In most eukaryotic organisms, they are essential in modulating membrane fluidity, controlling permeability barrier properties and signaling processes, serving as precursors of oxysterols, steroid hormones, and vitamin D in mammals [7], brassinosteroids in plants and fungi [8], ecdysteroids in arthropods [9], and dafachronic acids in nematodes [10].

In the course "design and optimization of biological systems" the ciliate *Tetrahymena thermophila* was used as an example of a biocatalyst system employed for the biotransformation of metabolites of industrial interest. This ciliate, along with Paramecium, are the major ciliate model systems [11]. We focused on sterol metabolism, specifically in the conversion of cholesterol to provitamin 

# Background and Goals

*Tetrahymena* spp are fresh-water nonpathogenic protozoa widely used as models of study in basic and applied science as well as in education [12]. The use of *Tetrahymena* as a biological system for biotransformation has several advantages: among the highlights are: rapid growth (doubling time of 1.4 h), which enables high cell densities (up to 5.0 × 10^6 cells mL^-1) in a short time, easy culture in axenic conditions in complex and cheap media and in bioreactors of simple design [13] and a convenient size (20–50 μm) for an easy follow up with conventional microscopes (at 40×). Additionally, *T. thermophila* is extensively studied and developed for providing new techniques and procedures that can be applied in laboratories of medium complexity, aimed both at gene silencing and over-expression of recombinant proteins [14].

The metabolism of sterols in *T. thermophila* and *T. pyrifor-
mis* has been intensely investigated in the late 60s [15]. *Tetra-
hymena* does not produce nor require sterols for growth; in their absence, this protozoan synthesizes triterpenoid alcohols, mainly tetrahymanol (a sterol surrogate similar to the hopa-
noids found in prokaryotes) (Fig. 1A). In contrast, when cells are grown in the presence of sterols, tetrahymanol biosynthesis is suppressed and sterols are preferentially incorporated into the cells, with or without modification, depending on the sterol supplied. To date, four modifications have been reported: desaturations at positions C5(6), C7(8), and C22(23) and the removal of the ethyl group at position C24 from 29-carbon steroids, leading to the accumulation of provitamin D analogs [16], (Fig. 1B). The transformation of cholesterol into the C7(8) desaturated derivative cholesta-5,7-dien-3β-ol (provitamin D₃, Fig. 1C) has attracted particular attention, because of the pharmaceutical and food-related applications, as this enzyme activity may be used to decrease cholesterol content in foodstuffs with simultaneous enrichment in provitamin D₃ in a single step [17, 18]. This method is also proposed for the synthesis of provitamin D₃ from cholesterol, as an alternative to the more complex and highly contaminating chemical method, in use since 1960 with few improvements [19].

The advantages of the organism, together with its capacity of sterol biotransformations, make this laboratory exercise ideal for undergraduate students that select a biotechnology specialization. They analyze the bioconversion of cholesterol in wild type and recombinant strains, compare different growth systems, from microplates to bioreactors, and use various analytical techniques, such as HPLC for the measurement of sterols, PCR for the identification of strains and fluorescence microscopy for intracellular localization of substrate and enzymes involved in the biotransformation.

To our knowledge this is the first course for college students, conducted with this advantageous organism, with the aim to teach different aspects of biotransformation and biocatalysis integrating other disciplines and previous knowledge.

# Materials and Methods

## Course Outline

This hands-on activity is planned for undergraduate students in their last semester of the BSc degree in Biochemistry (5 years and a half in Argentina) at the Faculty of Pharmacy and Biochemistry of the University of Buenos Aires in Buenos Aires, Argentina. Some topics covered during the elective course “Design and optimization of biological systems” are: screening and isolation of microorganisms of industrial interest, strain improvement by breeding methods and systems for cloning and expression, regulation and coordination of microbial metabolism, biotransformations of industrial interest and applications related to the production of foodstuffs, pharmaceuticals, environmental biotechnology and biofuels. The students enlisted have attended previous courses in biology, genetics, biochemistry, physics, maths, microbiology, and analytical chemistry.

The course has been assigned 32 hr in a quarter of a semester; therefore it was organized in 5 weeks, and scheduled for 6 hr attendance once a week. Eventually, during
another day of the week, selected groups of students have to
go to the lab to inoculate cells, take samples or perform sterol
quantiﬁcation by RP-HPLC (~2 extra hours for each group).
Regularly, between 15 and 20 participants enroll in the
course and are organized in groups of three to four members.
The timeline of the course is summarized in Table I.

**Strains, Growth Conditions, and**
**Biotransformation Assays**

*T. thermophila* strain CU428 mpr1-1/mprr1-1 (mp-s, VII),
designated the wild type (WT) in this work, was acquired
from the Tetrahymena Stock Center, located at Cornell Uni-
versity. *T. thermophila* DES7 KO (sterol C7 desaturase
knockout) is a mutant with a deletion in the *DES*
7 gene,
expressing paromomycin resistance under the control of an
*MTT* promoter, which is induced by CdCl2. *T. thermophila*
DES7 GFP is a C-terminally eGFP tagged version of the ste-
rol C7 desaturase. Construction of the strain has been pre-
viously described [20].

All *Tetrahymena* strains were grown in 250-mL Erlen-
meyer ﬂasks containing 30 mL SPP medium of the follow-
ing composition (wt/vol): 1% proteose-peptone (Oxoid,-
United Kingdom), 0.1% yeast extract (Merck, Germany),
0.2% glucose (Merck, Germany), and 0.003% iron citrate
(Sigma–Aldrich). For growth of DES7 KO strain, paromomy-
cin at 120 μg mL⁻¹ ﬁnal concentration was added from a
200 mg mL⁻¹ stock solution in water, together with 1
μg mL⁻¹ of CdCl2, which was prepared as a 0.5 mg mL⁻¹
stock solution in water [21]. In bioconversion assays, medi-
um was supplemented with cholesterol at a ﬁnal concen-
tration of 20 μg mL⁻¹, which was added from 5-mg mL⁻¹
stock solutions in ethanol. Cultures were inoculated with a
1:10 dilution of a 24-hr culture. Cultivation was carried out
in a rotary shaker (150 rpm) at 30°C. For cell density deter-
mination, cells were treated with 1% trichloroacetic acid
(TCA) and counted in a Neubauer chamber (between 30
and 100 cells, in duplicates).

**Bioreactor System**

Growth and biotransformation was carried out in a 1 LStandard Benchtop Bioreactor (Applikon Biotechnology
B.V. Delft, Netherlands) equipped with a digital myControl
controller unit. The temperature was kept constant at 30°C.
A Rushton impeller was used for agitation. The stirrer
speed was limited to 600 rpm to avoid cell damage. The
dissolved oxygen tension was controlled at 40% by aeration
and agitation rate. During cultivation the pH was main-
tained at 7.0 using 4 M acetic acid.

The identification and quantification of sterols, as well
as the DNA manipulation procedures and the microscopy
analysis are described in the Supporting Information S1.
Results

Through the course, the students performed three main activities. The results shown here are examples of the activities carried out in the 3 years in which the course was conducted.

Biotransformation in Bioreactor versus Erlenmeyer Flasks

**Goal**

In this activity students compare product yields obtained in cultures at constant pH and saturation oxygen tension rate (bioreactor) versus those obtained in typically non-controlled conditions (Erlenmeyer flasks).

For undergraduate students, the setting up and operation of an automated bioreactor system is a challenging experience. Because these operations and process objectives match those of a real-world fermentation facility, design, calibration, system assembly, and aseptic sampling give professors and students the chance to deal with good manufacturing practices and process hazard analysis in biotechnology. These topics are discussed during preparation, sterilization, and inoculation of the bioreactors.

Bioreactor-based bioprocess and biotransformation additionally gives students the real picture behind the practical activities, this is, the advantages of a myriad of possibilities and potential of process control they have with bioreactors compared to a process performed in Erlenmeyer flasks or sterile multiwell plates.

Students learn that bioreactors are culture devices that enable configuration and control of growth parameters, such as dissolved oxygen tension and pH, among others. Their effect on product yield becomes evident from this experiment.

In this activity the students prepare and sterilize media, inoculate and run the cultures and measure growth and sterol(s) biotransformation using a wild type strain of *T. thermophila* in two different culture systems: 1 L bioreactor and 250 mL Erlenmeyer flasks.

In the case of the bioreactor a batch culture was set up in SPP medium supplemented with cholesterol, as explained in Materials and Methods. During the time course of the experiment the students had to modify the agitation speed and the aeration rate in order to keep the dissolved oxygen tension above a chosen value (40%). The bioreactor was inoculated with cells from a late log phase culture, grown at a cell density of $1.0 \times 10^4$ cells mL$^{-1}$. Culture samples at 0, 24, and 48 hr were removed aseptically from the bioreactor for the measurement of cell density and sterols, including cholesterol and the biotransformation products cholesta-5,7-dien-3β-ol (7-dehydrocholesterol, provitamin D3), cholesta-5,22-diene-3β-ol (22-dehydrocholesterol) and cholesta-5,7,22-triene-3β-ol (7,22-bis-dehydrocholesterol). Additionally, the students used the Windows Remote Desktop/RD Client software for Windows, Android and IOS to monitor and control the cultivation remotely, Fig. 2A.

The arrows in Fig. 2B show the increase in the aeration (VVM) and agitation rate (RPM) made by the students in order to keep the dissolved oxygen tension above 40%. The overall calculated duplication time of *T. thermophila* was 2.6 hr and the concentration of cells reached $9.6 \times 10^5$ cells mL$^{-1}$. Figure 2C shows a time course in the bioreactor: growth, cholesterol conversion to unsaturated sterol products formed at various intervals of the batch culture. At 24-hr culture, 76% of the cholesterol initially present was converted to unsaturated sterols, at 48 hr the conversion reached 82% and at 72 hr, the conversion was maximal (87%).

The biotransformation yield obtained in flasks (incubated at the fixed rate of 180 RPM, without pH regulation) was lower than in the bioreactor: only 55% of the cholesterol initially present was recovered in the form of desaturated sterols at

| Day | Objective | Activity |
|-----|-----------|----------|
| 1   | Biotransformation of cholesterol in bioreactor. Genomic identification of blind strains. | Bioreactor preparation and inoculation of wild type *Tetrahymena* strain PCR |
| 2   | Metabolic identification of blind strains. Biotransformation of cholesterol in bioreactor. | Inoculation of *Tetrahymena* blind strains in microplates/HPLC setup Sampling/HPLC setup |
| 3   | Metabolic identification of blind strains and biotransformation of cholesterol in bioreactor. | Sampling/Sterol extraction and identification by HPLC |
| 4   | Intracellular localization of substrates and enzymes. | Fluorescent microscopy |
| 5   | Assessments. | Oral presentation/Exam |

TABLE 1 Course timeline
72-hr culture, in spite that all other conditions, including medium composition, temperature incubation and initial cell density, were identical. From these results, students learn on the importance of controlling and adjusting cell culture conditions, especially those that impact significantly in aerobic organisms grown in complex media: the dissolved oxygen rate (by agitation and aeration) and pH.

Strain Identification

**Goal**
In this activity students learn to search for desired properties in strains using physiological and genetic properties. For the purpose, they perform the identification of biotransformation products by HPLC and the amplification of putative gene-fragments by PCR. They also learn to design recombinant strains, so as to modify metabolic pathways and obtain chimera compounds.

One of the main variables in a biotransformation process is the election of the biocatalysis agent, either an enzyme or a whole-cell. Detailed information about enzymatic activities and microbial biocatalysts can be found in several databases and sources, such as BREnda, ExPASy-ENZYME, culture cell collections, literature, and others. The first exercise consists in becoming familiar with these databases, searching for different enzymatic activities as proteases, lipases, hydroxylases and desaturases. See Supporting Information S2.

Some techniques currently used to identify enzyme activities include: high performance liquid chromatography (HPLC), mass spectrometry (MS), capillary electrophoresis (CE), all assays that give spectroscopy signals, PCR and gene sequencing [22]. The platform is also used to design new activities or improve the existing ones for creating value-added products, developing biocatalysts and to speed up technological advances, provided that the screening of strains from the environment and the generation of new recombinant strains by recombinant DNA technology remain the major strategies [23].

During our lab exercise, each group of students receives a wild type or a recombinant strain (with its sterol metabolism impaired by a specific gene knocked out, named A or B). To identify the blind strain they have to...
perform the cholesterol biotransformation assay in a microplate scale and analyze the sterol profile by HPLC. Further, they must confirm the identity of the assigned strain by the presence or absence of the gene/s responsible for the enzymatic activity. For this purpose, they search for gene fragments belonging to the wild type or knockout locus, using selected primers and PCR amplification on previously purified genomic DNA obtained from all the strains.

Figure 3 shows the sterol profile obtained by HPLC of two different strains. In the strain A all the desaturated-derivatives were present in the chromatogram, thereby identifying this strain as the wild type (WT). By contrast, in the chromatogram corresponding to strain B, only cholesterol (the substrate) and 22-dehydrocholesterol (the product) were present. This result indicates that the strain lacks C7(8)-desaturase activity, thus corresponding to a Des7KO strain.

PCR amplification of specific fragments in the Des7 gene analyzed on agarose gels confirmed that strain A correspond to the wild type, and strain B to a Des7KO. Figure 4 shows a gel in which the amplification product of the wild type locus is only detected in genomic DNA from strain A whereas the fragment corresponding to the knockout locus is detected only in strain B.

Intracellular Localization of Substrates and Enzymes

Goal

In this activity students learn about the typical features of this organism at the microscopic level and the use of fluorescence staining techniques for tracking substrates and enzymes localization.

In a biotransformation process where the enzymatic activity is intracellular and substrates are mainly hydrophobic compounds, it is important to gather information on the efficiency of the uptake of the substrate from the culture media and the correct localization of substrate and expressed enzymes.

The larger size of ciliates compared to yeasts and bacteria offer a unique advantage in the field of microscopy, allowing the study of the uptake of substrates and also its intracellular distribution with simple equipment. As a matter of fact, several biological processes, such as microtubule cytoskeleton organization, membrane trafficking, various types of movement, and cellular remodeling during sexual reproduction (conjugation) were extensively studied in the ciliates [24].

With the aim to track the pathway of cholesterol uptake, and localize at least one enzyme involved in the biotransformation process to desaturated-derivatives, fluorescence
microscopy was used by the students. For tracking the uptake of cholesterol, fluorescent bodipy-cholesterol was added to the culture medium and samples were taken at several intervals.

It is important to note that *T. thermophila* has at least two well characterized pathways for substrate uptake, the endocytic at parasomal sacs and phagocytosis [25]. To analyze which uptake system is used for cholesterol, the students add cells of *E. coli* that express a red fluorescent protein, DsRed-Express2 in the culture medium, as a label for phagocytosis [26]. The appearance of red-stained particles in the ciliate (a well-known bacteriovorus organism) would confirm the use of this pathway. Figure 5A shows co-localization between fluorescent *E. coli* and bodipy-cholesterol, suggesting that cholesterol is taken up via phagocytosis. These results are in accordance with previous experiments carried out in the ciliate Paramecium [27].

To localize the enzyme sterol C7-desaturase, responsible for the conversion of cholesterol to 7-dehydrocholesterol in *Tetrahymena*, the students use a recombinant strain that expresses the enzyme C-terminally linked to the eGFP protein (Des7p:eGFP). The tagged gene was inserted in its endogenous locus to avoid potential miss-expression artifacts (Fig. 4A). Because of high autofluorescence background in wild type cells, we increased the selectivity of the signal using an anti-GFP antibody and indirect immunofluorescence microscopy for detection. As shown in Fig. 5B, the Des7p:eGFP strain showed a strong signal around the nucleus, in a pattern that is consistent with nuclear envelope localization, and a more diffuse one in the cytoplasm. This pattern is consistent with localization of the enzyme in the endoplasmic reticulum (ER) of these cells [28].

**Assessment**

During the 3 years that the course was carried out (2013–2015), different types of evaluations were held at the end of the course, to assess the knowledge acquired by the students. They included: written examinations (during the 3 years), group written reports (2013 and 2014) and group oral presentations (only 2015). The written exam consisted of five questions (each question 2 points), two related to the experimental assays/results (four points in total) and three to the complementary bibliography of the course (six points in total). Some examples on the type of written questions and average marks are available as Supporting Information S3.

In the year 2013 (15 students) the average of the points obtained of the experimental assays/results were 2.6/4.0 (65%) while in 2014 (20 students) was 2.9/4.0 (72%) and in 2015 (18 students) 3.2/4.0 (80%).

The final written report or the oral presentation, also at the end of the course, was presented by each group including all the data generated, experimental descriptions and conclusions. We noticed that the oral presentations were a better way of evaluation than the joint written reports, because teachers can judge each student individually. In addition, many doubts and concerns were answered by their peers and teachers during the presentations.

Finally, to get a feedback from the course, the student opinions were evaluated by an anonymous on-line survey at the virtual campus of the University. Students were asked to answer several questions about the lectures, practical course, bibliography, virtual campus and teachers. The questions were ranked from 1 (very bad/unsatisfactory/inadequate) to 10 (very good/satisfactory/adequate), as described in Table II. In addition it was asked to students any suggestion to improve the course. See Supporting Information S4.

**Discussion**

The use of *Tetrahymena* in the class room has been advocated from various aspects. From the biological point of view, the organism has the following advantages: *Tetrahymena* is a...
large unicellular non-pathogenic eukaryote, easy to grow in cheap defined medium, at high cell densities. It swims waving its cilia and phagocytes food particles through the oral apparatus. Cell (ultra)structure, physiology, biochemistry, genetics, and molecular biology have been extensively investigated in the organism, and a considerable set of molecular tools and techniques are available to make the system easily accessible. These features favor not only its use in research but also in education.

Additionally, a number of publications are available for the use of this organism as a teaching resource. An excellent chapter written by Joshua Smith et al. [29] outlines the use of the ciliate in teaching and summarizes a number of laboratory courses in K-12 classrooms and undergraduate and graduate college. In that chapter different types of modules can be found, from a complex one, such as “Protein Interaction through Immunoprecipitation and Mass Spectrometry” to more simple ones, such as “Effects of Cigarette Smoke in *Tetrahymena*.”

Another important collaborative research initiative in education is the creation of the Ciliate Genomics Consortium (faculty.jsd.claremont.edu/ewiley/), which motivates undergraduate students to investigate gene function in *T. thermophila*.

Several biotransformation processes performed by this ciliate have been described in the scientific literature, including the biodegradation of the pollutants pentachlorofenol [30], arsenic [31], toluene, xylene [32], and the bioconversion of isosorbide dinitrate into isosorbide mononitrate [33]. Nevertheless, to our knowledge, a laboratory course on the subject has not been presented to date.

Various reports and publications have shown the advantages to teach science, technology, engineering and mathematics (STEM) topics to undergraduate [34, 35], because it provides students with greater capacities to face a highly competitive labor market, helps them acquire tools to solve problems efficiently and also provides them ideas to generate new paths. This leads not only to the benefit of the person, but also the whole society [36].

However university education in STEM subjects is a complicated goal because it is difficult to motivate the students. In this regard, various institutions around the world call for improvements on this situation [37, 38]. To try to cover this deficiency, a good strategy is to engage students in the same lines of research as their teachers; in this way the students are introduced into state-of-the-art science but facing also real problems and learning how to solve them together.
information and communications technologies (ICTs) have control parameters in the bioreactor. The correct uses of cell phone applications for remotely monitoring and were used for motivation of students, such as the use of more empathetic to solve them. Besides, new technologies way they take the problem as their own challenge and are blind strains was meant precisely for that purpose. In this encounter in our own research. The identification of the call their interest was to share with them the problems we obtained without a clear purpose [41, 42]. Another way to more than when they work with examples of experiments real problems involves students in STEM subjects much help the student in the future. Furthermore, working with microscopy, were used to cover diverse areas that couldduce the concept of biocatalysis to students, performing a biological system for the conversion of a simple substrate (cholesterol) into a valuable pharmaceutical product (provitamin D₃) and the genetic and engineering strategies that can be used for the optimization of the process.

In conclusion, this course has been designed to introduce the concept of biocatalysis to students, performing a simple and robust laboratory exercise, using a biological model for the optimization of the process.

**The cholesterol bioconversion course presented here deals with these difficulties. The laboratory exercise was based on a research on sterol metabolism in *Tetrahymena* which is going on for over 15 years in a group of the Department of Biotechnology of the Faculty of Pharmacy and Biochemistry, University of Buenos Aires [20, 39, 40]. For that reason, all the goals detailed in the three main activities were systematically carried out and there is plenty of expertise in the techniques. Fermentation, HPLC analysis, bioinformatics, molecular biology techniques, and microscopy, were used to cover diverse areas that could help the student in the future. Furthermore, working with real problems involves students in STEM subjects much more than when they work with examples of experiments obtained without a clear purpose [41, 42]. Another way to call their interest was to share with them the problems we encounter in our own research. The identification of the blind strains was meant precisely for that purpose. In this way they take the problem as their own challenge and are more empathetic to solve them. Besides, new technologies were used for motivation of students, such as the use of cell phones applications for remotely monitoring and control parameters in the bioreactor. The correct uses of information and communications technologies (ICTs) have shown a positive correlation with the learning approaches, educational gains, and other forms of engagement [43, 44]. Students also make use of deeper approaches of learning, like higher order thinking and integrative learning, and they reported higher advantages in education, practical capability, and personal and social development [45]. In our case, we realize that many students, especially those with shy personalities, increased their participation and commitment through the use of distance learning programs, thereby improving their teaching.

**In conclusion, this course has been designed to introduce the concept of biocatalysis to students, performing a simple and robust laboratory exercise, using a biological model for the conversion of a simple substrate (cholesterol) into a valuable pharmaceutical product (provitamin D₃) and the genetic and engineering strategies that can be used for the optimization of the process.**

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