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REVIEW ARTICLE

**Manuel Cánovas · José L. Iborra**

# Culture collections and biochemistry

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**Abstract** This review describes the relationships and links between culture collections, which act as sources of genomes, transcriptomes, proteome, and metabolomes, and fields of research biochemistry that demand their support and help. In addition, the invaluable but not always rewarded efforts of these organizations as a source and conservator of organism diversity is discussed. Biological waste-water treatment, ethanol as a non-finite source of energy, *Rhodococcus fascians* as the source of a citrus-juice debittering agent, the sporulation of filamentous fungi in liquid medium, and biotransformation with growing and resting cells are processes developed by the authors that demonstrate some of the applications of organisms from culture collections in the general field of biotechnology and related areas, including industrial biochemistry and biocatalytic synthesis.

**Keywords** Culture collections · Cell biocatalyst Reactor · Bioprocess · Source of genome

## Introduction

Microorganisms can be isolated from nature or obtained as pure cultures from permanent culture repositories. Industrially important microbial cultures are permanently preserved if they are of interest or useful. However, culture collections are much more than places for the deposit and supply of cultures. These resource centers are managed by mycologists, yeast and bacteria curators, and, nowadays, eukaryotic cell-line experts who provide advice on the specific culture of interest and its properties, identification (locality of origin, substrate, host, date of isolation/construction, name of person who isolated/constructed the resource, depositor, etc.) and preservation (methods applied, cultivation conditions, security and quality codes, characteristics, and restrictions). In addition, training and specialist services to industry on a contract consultant basis are available from the largest collections, a number of which also act as depositories for patent strains [22, 23, 32].

A growing awareness of the importance of biodiversity and its hidden genetic potential has resulted in both growing recognition of the value of culture collections of microorganisms and an increase in the number of such collections [22, 32]. Culture collections are long-term commitments, as explained in the guidelines of the World Federation for Culture Collections (WFCC) [23]. Culture collections are labour-intensive: accession, preservation and maintenance as well as the constant necessity to check the viability and to authenticate the biological material mean that good laboratory practice has to be guaranteed.

M. Cánovas · J. L. Iborra (✉)
Departamento de Bioquímica y Biología
Molecular B e Inmunología,
Universidad de Murcia,
Facultad de Química, Spain
E-mail: jliborra@um.es
Tel.: +34-968367398
Fax: +34-968364148

## Culture collections and biochemistry

The connection between culture collections and biochemistry and molecular biology is so obvious and important that one only needs to recall the definition of biochemistry: "[That] part of chemistry that studies the chemical composition and chemical reactions taking place within living things" [33]. For a biochemist, the study of either catalogue-known or research-screened microorganisms means that sooner or later he/she will be dealing with culture collection experts, since advice and/or a final deposit will be required. From an academic point of view, biochemistry students are taught cell biology (the structure and function of cellular components), enzymology, metabolism and its regulation, and most biochemistry textbooks cite examples from catalogued microorganisms (*Escherichia coli*, *Bacillus subtilis*, etc). Hence, culture collections have

always been the mainstay of the biochemist's teaching and research duties.

Culture collections, as sources for genome, transcriptome, proteome and metabolome, provide tools for biochemists and molecular biologists. In turn, biochemists and molecular biologists contribute knowledge, methodology, and techniques to an interdisciplinary approach known as biotechnology. Recent advances in biotechnology are being explored for their use in the treatment of metabolic disorders and genetic diseases. Furthermore, innovations in food production, environmental problem-solving technologies, and many other areas previously the domain of the chemical industry have been made by exploiting the metabolic characteristics of a wide variety of microorganisms. The relationship and the links between culture collections and biotechnology are discussed below.

## Biotechnology and industrial biochemistry

Biotechnology requires the existence of culture collections since the microorganisms maintained in them provide biocatalysts as well as sources of compounds with a wide variety of research and industrial applications. As a component of biotechnology, industrial biochemistry has been defined as "the science that studies the biochemical background/foundation of the biosystems used by bioindustry"[3]. In general, industrial biochemistry uses microorganisms either from culture collections or from screening projects and studies the stoichiometry and kinetics in closed and open environments (bioreactors), where bioprocesses are carried out in order to obtain products for the pharmaceutical, agricultural, and food industries. A very specific part of industrial biochemistry is that related to applied biocatalysis, also referred to as biocatalytic synthesis. This concerns "the use of biocatalysts such as enzymes, catalytic antibodies, artificial enzymes, organules and whole cells for the production of compounds" [3]. Its aims include how to stabilize biocatalysts, how to improve both their catalytic characteristics and the environments (open and closed bioreactors) in which they operate. A precise implementation and understanding of any biological process requires that culture collection experts must be consulted. Nowadays, the use of microorganisms for the production of everyday products has become routine in bioindustry. As early as 1929, J.B.S. Haldane was asked by a journalist for his opinion about pure chemists and their job, and he replied with the following joke: "Why trouble yourself to make compounds yourself when a bug will do it for you" [21]. Since then, many new techniques have been developed, such as cell culturing, reactor engineering, recombinant DNA, PCR, DNA shuffling, site-directed mutagenesis, metabolic engineering, protein engineering, and artificial enzyme synthesis. All these techniques and the resulting new-found knowledge have their applications in biotechnology, but always in connection with culture collections that supply the catalogued organisms. Research, then, begins and ends with such collections since the result, once modified and patented, is deposited in a culture collection. In fact, bioindustry, within a very short period of time, has pushed the scientific community to develop and apply new techniques to studying microorganisms, filamentous fungi, yeast, plant cells and mammalian cell lines, so as to be able to transform not only organic but also inorganic materials to yield products for the pharmaceutical, agricultural and food industries. Bioindustry requires culture collections as genome and proteome sources. More importantly, however, metabolic profiling—the metabolome era—is nowadays of critical importance for implementing new biotechnological processes and for developing new biocatalysts and techniques.

Biochemistry and molecular biology have focused their attention on four main areas: (1) signal transduction in cells; (2) protein engineering (structure and function, structure and activity, and structure and stability); (3) recombinant DNA technology, as a tool for studying and improving structure and function; and (4) metabolic engineering as a way to re-direct metabolic fluxes. Industrial biochemistry has used the resulting knowledge to optimize not only biocatalysts but also many other industrially oriented bioprocesses.

Some examples of bioprocesses that the authors have developed demonstrate the importance of culture collections in biochemistry and molecular biology research with applications to industrial biochemistry. These are described in the following sections.

## Some examples of bioprocesses

Parallel to the development of what has been referred to as classical biotechnology, i.e., the manufacture of food-enhancers (flavours and aromas) and food-products (dairy products, meat products, etc.), interest in other biotechnological processes, such as solvent production (acetone-butanol fermentation), antibiotics, and particularly bioremediation (waste-water treatment, aerobic and anaerobic organic matter degradation) has grown and many advances have been made.

### Biological waste-water treatment

Among waste-water treatment methods, anaerobic digestion involves transforming organic matter into substrates for cellular growth (10–15%), and a mixture of $CH_4$, $CO_2$ and $H_2S$ (85–90%), by a consortia of bacteria resembling an electron chain, in which organic matter acts as electron donor and acceptor. There are three different groups of bacteria (acidogenic, acetogenic and $H_2$-producing acetogenic and methanogenic) that consume a given substrate and produce substrates for the next group, the bottle-neck being methanogens due to their slow rate of growth [11]. Unfortunately, anaerobic digestion processes require long start-up times before operation is

efficient and stable because of the slow growth of acetate- and $CO_2$- as well as $H_2$-consuming methanogens. Fixed-film reactors to increase the biomass retention of the reactor were a significant advance [11, 12], and new bacteria were characterized and catalogued while the process was being evaluated. New high-rate anaerobic reactors and a novel method to accelerate the start-up of a down-flow percolating fixed-film anaerobic reactor were developed [2, 13] by growing both acidogenic and interspecies $H_2$-producing/$H_2$-consuming bacteria during two subsequent stages of reactor start-up [12, 27]. A model describing the stratified growth of the acidogenic and the methanogenic populations within the biofilm was generated, while the presence of the $F_{420}$ cofactor within methanogens was detected and used as a means of measuring methanogenic growth [7, 13]. From 1980 to 1990 a great number of new microorganisms and strains were referenced and catalogued; these were deposited in culture collections where they could be compared with previously characterized strains [38]. Part of our work was to maintain the cells by immobilizing them onto solid supports, either porous or non-porous material, and to compare microorganisms catalogued in the Spanish Type Culture Collection (CECT) with microorganisms observed in the reactor [7]. Table 1 lists both the type of cell-retention material and the reactor used in order to support cell growth and compound production.

## Ethanol as a non-finite source of energy

Due to their finite nature and continuing fluctuations in the price of fossil fuels (coal and petrol), interest in new and durable energy sources has increased. When we started our study of ethanol metabolism in *Zymomonas mobilis*, ethanol was a priority area of research within the European Community. Strain *Z. mobilis* ZM4 was kindly supplied by Prof. Baratti (INSA, Toulouse, France) and a flocculent strain obtained by our group was deposited in the CECT (CECT no. 560). *Z. mobilis* metabolizes glucose, fructose and sucrose by the Entner-Doudoroff pathway. This metabolism was studied using different types of reactors and different immobilization supports for cell retention. Long-term culture of our *Z. mobilis* strain in an immobilized cell reactor confirmed its genetic stability [14, 15, 26] (Table 1).

## *Rhodococcus fascians*, a citrus-juice debittering organism

The bitterness of certain citrus juices due to limonin is a major problem in the citrus industry worldwide and has significant economic impact. The naturally occurring form and precursor of limonin is limonoate A ring lactone [4]. After the juice is extracted, this limonoid moves into the liquid phase where it is chemically converted to limonin in a process accelerated by the acidic pH of the juices (4.0–5.0) and also by heat. Thus far, *Rhodococcus fascians* (CECT 3001) is the only organism that constitutively produces enzymes for the metabolism of limonoids [6], consuming limonin as carbon source for growth. Other bacteria require the presence of a limonoid inducer within the growth medium to produce cells capable of metabolizing limonoids. Despite the advantages of applying this microorganism as a possible citrus-juice-debittering biocatalyst, attempts to apply it in a commercially viable way were unsuccessful. We compared the behavior of *R. fascians* cells at the optimum pH for growth (pH 7.0) and at the usual pH of citrus juices (pH 4.0), at which cells have lower catalytic activity [5]. The limonin species used by *R. fascians* as possible cellular substrates at pH 4.0 were also tested [4]. In addition, as *R. fascians* is considered an aerobic microorganism, the cellular response to the extreme conditions imposed by the citrus juice (low pH) and the non-aerated conditions during its industrial processing were studied [6]. Moreover, with the purpose of finding a debittering biocatalytic system, the behavior of free and immobilized *R. fascians* cells within batch- and continuous reactors in the presence of synthetic citrus juices and under the conditions characteristic of processing citrus juices was monitored. Polyurethane foam was used as the solid support because of its high structural stability and biomass retention capability. It is also harmless to cells [6] and the product obtained is compatible with human use (Table 1).

**Table 1** Organisms and processes used for cell growth and production of compounds under different conditions and immobilized on different solid supports (see text for culture collections)

| Organism | Immobilization support | Product | Metabolism | Reactor type | Reference |
|---|---|---|---|---|---|
| Anaerobic Mixed cultures | Needle punched polyester | Methane | Anaerobic food chain from pyruvate | Downflow stationary fixed film | [7] |
| *Zymomonas mobilis* | Polyester/cellulose | Ethanol | Entner-Doudoroff | Packed bed | [15] |
| *Rhodococcus fascians* | Polyurethane | Fruit juice debittering | Limonoids | Continuous stirred tank | [5] |
| *Escherichia coli* strains | Various | L(–)-carnitine | Trimethylammonium compounds | High-density cell membrane | [9, 35] |
| *Proteus mirabilis* | Various | L(–)-carnitine | Trimethylammonium compounds | High-density cell membrane | [8] |
| *Penicillium chrysogenum* | Cellulose | Various | Biotransformations | Batch system | – |

## Filamentous fungi sporulation in submerged liquid medium

Filamentous fungi have two different types of reproduction: (1) sexual, which allows vegetative growth mycelium in liquid/solid media; and (2) asexual, which allows growth after spore germination [25, 40]. Sporulation methods of *Penicillium* sp. strains in submerged liquid media [40] represented a breakthrough in antibiotic production and biocatalyst spore production for the biotransformation of compounds such as steroids and certain aldehydes. Spore-latent status does not mean that no metabolic activity takes place. Therefore, in the field of biotransformations great effort is devoted to the use of spores of filamentous fungi for the production of compounds neither produced by single enzymes nor by other organisms [25]. The strains used for our studies were supplied by the CECT (CECT no. 2668) and by Antibióticos S.A. (León, Spain). The vegetative growth of filamentous fungi in liquid/solid media is affected by the environmental conditions and the composition of the growth medium. Morphological changes are likely to be observed as a result of changes in the environment, leading to a shift from sexual to asexual (sporulation) reproduction. By selecting liquid medium containing divalent cations such as $Ca^{2+}$ and $Mg^{2+}$ [40], the sporulation of several *Penicillium* sp. (Fig. 1) in submerged cultures was obtained (Cánovas and Iborra, unpublished data). Spores were used for the biotransformation of aldehydes to ketones.

## Biotransformations using growing and resting cells

This example is related to work currently being carried out by our group: optimization of the process for L(–)-carnitine synthesis from trimethylammonium compounds using wild-type and transformed *Escherichia coli* strains in metabolic and genetic engineering. L(–)-carnitine (R[–]3-hydroxy-4-trimethylaminobutyrate) transports long-chain fatty acids through the inner mitochondrial membrane [29], and several clinical applications for this process have been identified, leading to increased demand for this ccompound worldwide [31]. To satisfy this demand, chemical and biological production processes are being studied. Processes based on the chemical resolution of racemic carnitine produce D(+)-carnitine as a waste product, which can be converted into L(–)-carnitine by certain microorganisms (Fig. 2) [31]. Therefore, an otherwise worthless waste product can be used as substrate to produce a high-value compound [30]. *Escherichia coli* cells metabolize carnitine (through secondary trimethylammonium metabolism) but do not assimilate the carbon or nitrogen carnitine skeleton. Initial experiments have shown that resting or growing *E. coli* O44 K74 cells biotransform crotonobetaine into L(–)-carnitine (Fig. 2) [9, 39]. Furthermore, D(+)-carnitine can also be biotransformed under batch resting-cell conditions into L(–)-carnitine [16, 30]. In parallel, genetic studies were conducted to elucidate carnitine metabolism in *E. coli*. Two divergent structural operons, *caiTABCDE* and *fix*ABCX, are coexpressed during cell anaerobic growth in the presence of L(–)-carnitine or crotonobetaine [18, 19]. Molecular characterization of the *cai* operon has shown that high levels of carnitine racemase (bifunctional gene *caiD*) and L(–)-carnitine dehydratase (bifunctional gene *caiD*) as well as a cofactor (*caiE*) required for both enzyme activities are observed in a transformed strain of *E. coli*, strain K38 [17, 20].

In our work, *E. coli* K38 pT7-5KE32 was kindly provided by Prof. Kleber (University of Leipzig, Germany) and *E. coli* O44 K74 by DSM (DSM no. 8828) [17, 35]. *E. coli* K38 pT7–5KE32 (TS) contains the complete *cai*TABCDE and *fix* operons, and the plasmids pGP1-2, $Kan^r$ and pT7-5KE32 (a pBR322 derivative), $Amp^r$, which carries an insert with *caiD*, *caiE* and *caiF* from the *E. coli cai* operon; thus, this strain overexpresses carnitine racemase and L(–)-carnitine dehydratase [17]. The wild-type strain *E. coli* O44 K74 (WS) contains the complete *cai* and *fix* operons. The two-step pathway, including L(–)-carnitine dehydratase (L(–)-carnitine hydrolyase, protein CaiD) and a crotonobetaine reductase (protein CaiA) (Fig. 2) [17], is induced anaerobically in the presence of carnitine and/or crotonobetaine. This pathway is detectable not only in cells previously grown anaerobically but, to a lesser extent, also in cells grown under aerobiosis in the presence of the inducers [30, 36, 37]. To produce L(–)-carnitine, either the crotonobetaine reductase gene (*caiA*) must be knocked-out or the enzyme (protein CaiA) inhibited (Fig. 2).

Resting and growing cells of *E. coli* were used in batch reactors for substrate biotransformation. The term "resting cell" applies to cells that, after growth, are harvested and resuspended in the biotransformation medium, which contains D(+)-carnitine or crotonobetaine in 55 mM phosphate buffer, pH 7.5, in order to carry out biotransformation of either D(+)-carnitine or

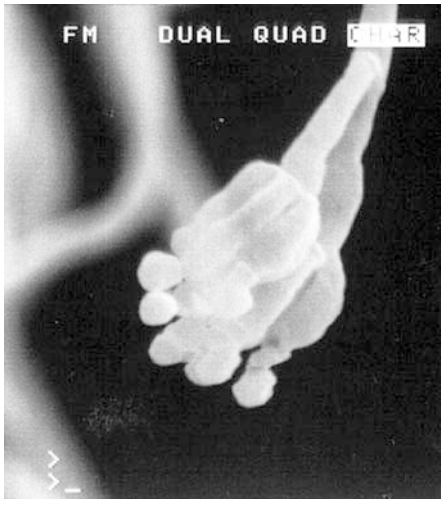


**Fig. 1** *Penicillium sp.* sporulation in submerged liquid media

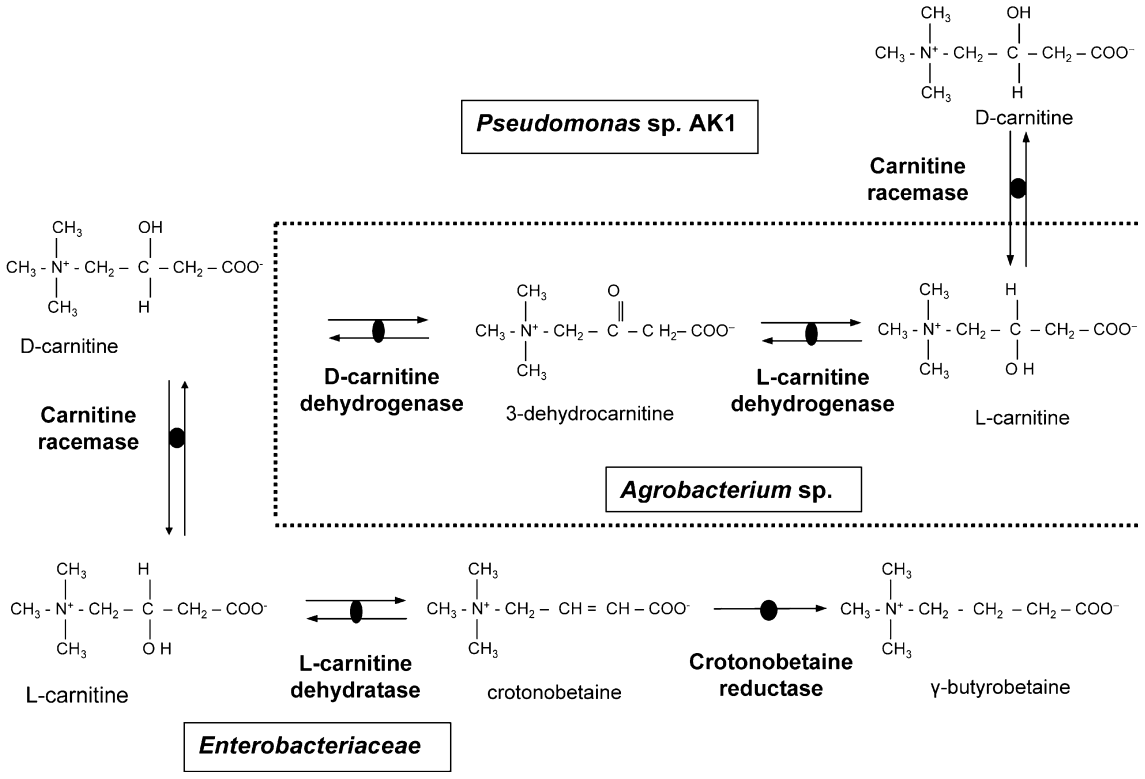


**Fig. 2** Metabolism of trimethylammonium compounds in different microorganisms. After [31]

crotonobetaine. During continuous processes, different cell immobilization procedures were utilized [34, 35, 36] (Table 1, Fig. 3). Membranes were also used to retain large components (e.g., cells), while allowing small molecules (i.e., the product) to pass through. Therefore, high-cell-density membrane reactors were run at different dilution rates, and strategies such as growth followed by resting periods to maintain continuous production of L(–)-carnitine were implemented so that stable operation was achieved [23, 36].

Furthermore, cell transport of trimethylammonium was studied under different conditions to determine the kinetics of the protein transporter [28] and its influence on the biotransformation of both substrates into L(–)-carnitine [1, 9]. The main research aim of our group is to study and optimize the biotransformation of D(+)-carnitine and crotonobetaine into L(–)-carnitine by obtaining a detailed understanding of carnitine metabolism and by overexpressing the relevant enzymes in *E.coli*. For this purpose, the activities L(–)-carnitine dehydratase and crotonobetaine reductase of *E. coli* are being studied under different growth conditions in batch and high-cell-density membrane reactors.

## Influence of electron acceptors: oxygen and fumarate

Resting cell experiments showed a positive influence of electron acceptors, such as oxygen and fumarate, on the D(+)-carnitine racemization process carried out by WS (Fig. 2) [16, 30]. Higher growth rates and biomass concentrations of TS were achieved with increased oxygen concentration. Furthermore, during aerobiosis TS carried out trimethylammonium metabolism in which crotonobetaine reductase was not detected (Fig. 2). Thus, 15–30% oxygen saturation was fixed as the optimum concentration since L(–)-carnitine was obtained without concomitant γ-butyrobetaine production (Fig. 2). With respect to WS, batch growth studies during aerobiosis showed that there was little metabolism of trimethylammonium, unlike during anaerobiosis, since low levels of L(–)-carnitine dehydratase and D(+)-carnitine racemase were detected after growth in the presence of crotonobetaine or D(+)-carnitine. Based on these results, the biotransformation process was improved by the addition of fumarate [16, 35]. However, the role of fumarate in trimethylammonium metabolism by *E. coli* growing under anaerobiosis is not totally understood; the effect might be at the energy-producing or the anaplerotic pathway levels. Moreover, for both strains, crotonobetaine was shown to be the best substrate for L(–)-carnitine production due to the expression of enzyme activity or to the fact that membrane transporters for the substrate and product have been reported to be the same [10], implying that there are different transport affinities.

## Overexpression of CaiD

The biotransformation of D(+)-carnitine by TS under optimal reactor conditions (15–30% oxygen saturation, 41 °C, and a minimal medium), resulted in the production of 0.15 g L(–)-carnitine/l/h and a conversion of 32%.

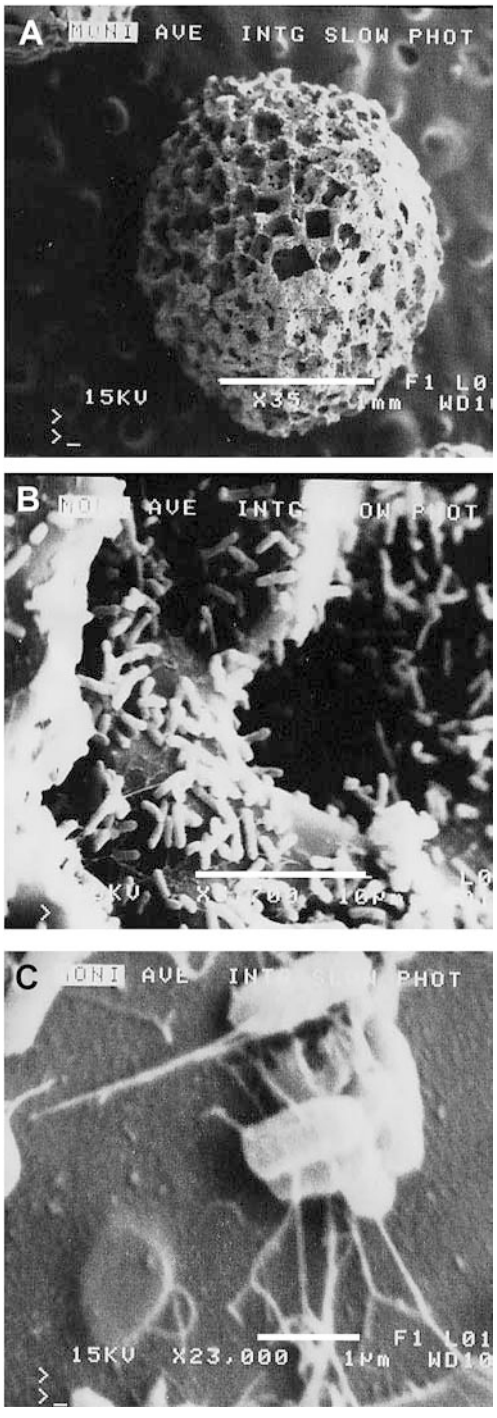


**Fig. 3** *Escherichia coli* O44 K74 growth and biotransformation of trimethylammonium compounds on synterized glass for cell immobilization at **A** ×35 (*bar* 1 mm), **B** ×3,700 (*bar* 10 μm), and **C** ×23,000 (*bar* 1 μm). Experiments were carried out at 37 °C using a complex medium supplemented with 50 mM fumarate under anaerobiosis

Moreover, no plasmid loss was observed during batch reactor experiments [17]. Experiments showed that overexpression of CaiD by TS results in a racemase activity capable of transforming D(+)-carnitine into L(–)-carnitine, although with $\gamma$-butyrobetaine production.

### High-cell-density reactors

In recombinant-protein production, not only is a high level of gene expression important but also a high-cell-density reactor, meaning that cell-recycling membrane reactors offer good possibilities for improving productivity and final product concentrations as well as the genetic stability of strains [36]. Moreover, for transformed strains in continuous reactors, conditions must be established in which plasmid copy number, transcription, and translation efficiency are optimally balanced to maximize productivity. Furthermore, strategies need to be found to understand cell physiology in process conditions, using the transformed cells in the chosen reactor and subjecting them to production conditions in order to ascertain their dynamic responses. Results showed that TS is segregationally and structurally unstable in continuous high-cell-density membrane reactors under the conditions tested. Nevertheless, since medium composition strongly affects structural instability, new studies in which the the growth rate is decreased by controlling the medium composition are currently underway.

WS cells were also cultured anaerobically at 37 °C in a continuous reactor in which they were retained by means of different microfiltration membranes (assembled in flat-sheet modules) of 0.1-μm pore size and subjected to recycling. It was concluded that the continuous production of L(–)-carnitine from crotonobetaine by WS in a cell-recycle reactor could reach values as high as 6.5 g/l/h [1, 9], with L(–)-carnitine conversions of 35–46%. In order to assess the stability and potential productivity of the reactor, we carried out perturbation studies, changing the dilution rate and crotonobetaine concentration, and followed the effects on strains of *E. coli* WS and *Proteus* sp. (kindly provided by Sigma-Tau, Roma, Italy) [8, 34]. Biotransformation resulted in nearly the same L(–)-carnitine yield as obtained in the previous operation, though with nearly double the productivity (11.5 g L(–)-carnitine/l/h). Thus, WS was shown to be suitable for process operation and even capable of being subjected to dynamic stress, which may be useful for control and optimization strategies [1]. Moreover, L(–)-carnitine dehydratase activity/biomass ratio decreased. This fact is important and might imply cellular control of the biotransformation catalysis reaction, which, to our knowledge, has not previously been detected in trimethylammonium metabolism studies in *E. coli*. Furthermore, the reactor was remarkably robust throughout the experimental run. Currently, our work is directed at finding the link between central carbon or primary metabolism and secondary carnitine metabolism of *E. coli,* in order to be able to redirect the metabolic fluxes. Cell metabolic engineering tools will then be applied for process optimization.

## Conclusions

Maintaining culture collections requires commitment, hard work and continuous research (not always with

the best means), although the effort involved is not always acknowledged by the scientific community. Culture collections have helped not only in the development of food-production-related processes, but also in the simple non-sterile production of solvents, organic acids, biomass, etc., and the complex sterile production of pharmaceutical and fine chemicals. The important contribution of culture collections to biochemistry in the areas of enzymology, molecular biology, genetic engineering, and bioprocess engineering in the form of help and advice, as well as the transmission of know-how, should be recognized and acknowledged. This review has provided some examples to show the strong links between culture collections and biochemistry and biotechnology in order to highlight their importance in our changing and evolving world.

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