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A bioreactor model system specifically designed for *Tetrahymena* growth and cholesterol removal from milk

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Abstract This work describes the configuration and operation of a bioreactor system especially designed for *Tetrahymena* cultivation and its use for milk improvement, particularly cholesterol elimination by the action of this cell. An advantage of the proposed method is the re-use of the growth medium; thus, the medium is used twice to provide two batches of *Tetrahymena* biomass without the need of further inoculation. This makes the procedure of producing the cell biomass faster and more economical. Cells are concentrated in the culture vessels by sedimentation at room temperature and then transferred to milk suspensions, where they can further grow for at least one generation with the benefit of reducing steeply cholesterol level. Milk treated according to this process is separated from the biomass by centrifugation. Under these conditions, less than 5% of the cells remain in the milk, and cholesterol elimination amounts to 75±10% of that initially present. No changes in sensorial properties of the milk, such as clotting or butyric odor, were observed as a result of this treatment. In addition, the bioreactor allows the aseptic recovery of the spent growth medium, which contains diverse enzymes of interest, and the cell pellets, to exploit particular lipids like phosphonolipids, abundant poly-unsaturated fatty acids and co-enzyme Q_8.

Keywords *Tetrahymena* · Bioreactor system · Milk treatment · Cholesterol removal

Introduction

Tetrahymena thermophila, a non-pathogenic ciliate, has been used thoroughly as a model cell system for morphogenesis, growth and nutritional studies (Wheatley et al. 1994). More recently, it has become relevant because of its potential for biotechnological applications, based on several outstanding features. These include the ability to produce and secrete valuable lysosomal enzymes (Kiy and Tiedtke 1991; Kiy et al. 1996). Some of these enzymes, such as proteases and phospholipases, have reached the production scale technology, using continuous high-density cultures, taking advantage of the exceptionally fast growth rate of this cell (Kiy and Tiedtke 1992a; Guberman et al. 1999). In addition, this protozoan has been shown to produce diisopropylfluorophosphatase that can be applied to environmental bioremediation (Landis et al. 1987). Moreover, these cells were also successfully used for bio-conversion of organic nitrates by the action of glutathione *S*-transferase (Ropenga and Lenfant 1987).

J. Florin-Christensen passed away during the revision of this manuscript. An obituary of his contributions to science will appear in this journal (Rasmussen and Tiedtke, in press).

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Further advantages of *Tetrahymena* are the abilities to capture and desaturate cholesterol into Δ^7-dehydrocholesterol (pro-vitamin D_3), and $\Delta^{7,22}$-*bis*-dehydrocholesterol (analogue of pro-vitamin D_2; Mallory and Conner 1970). It can also desaturate fatty acids taken up from the medium (Holz and Conner 1973). These peculiar properties have been used by our group to decrease cholesterol contents in foodstuffs like milk and egg with simultaneous enrichment in pro-vitamin D and unsaturated fatty acids (Valcarce et al. 2001, 2002). Moreover, we have observed that this ciliate is able to accumulate cholesterol in the form of intracellular steryl-esters and, in this way, eliminate cholesterol from foodstuffs by separating the cell biomass (unpublished results).

Several bioreactor configurations and medium compositions have been proposed for achieving high cell concentration and scale-up *Tetrahymena* cultures. They include classical stirred tanks, air lift reactors (Hellenbroich et al. 1999) and a perfused bioreactor (Kiy and Tiedtke 1992b). The latter represents a significant improvement with respect to maximal cell concentration and dry weight that can be obtained.

These methods apply to *Tetrahymena* cultivation but not to the foodstuff treatment. We have therefore designed and developed a simple bioreactor system that can simultaneously carry out cell cultivation and milk treatment, combined with spontaneous sedimentation for cell harvesting from the culture medium. The system allows the successive use of the same medium for two cell growth cycles, with economy in time, cost and labor.

In this work, we also investigated the effect of different inoculum sizes on the milk with the aim of optimising the process. Finally, we tested six methodologies for the separation of cell biomass from the treated milk.

As a result of the complete process, three types of products can be obtained: the improved milk that is reduced in cholesterol, the culture medium enriched in extracellular enzymes and finally abundant cell mass. The latter is source of compounds such as phosphonolipids (i.e glyceryl ether aminoethylphosphonate; Rosenthal and Pousada 1968), pro-vitamins D, poly-unsaturated fatty acids (PUFAs) and co-enzyme Q_8 (Holz and Conner 1973), all of them with biotechnological interest.

Materials and methods

Microorganisms

The ciliate *Tetrahymena thermophila mutant* MS-1, impaired in hydrolases secretion, was originated by conjugation of mutagenised *T. thermophila* CU-399 (Chx/Chx; cy sens., VI) with a vegetative C strain (Hünseler et al. 1987). The strain was supplied by Dr. A. Tiedtke of the University of Münster, Germany.

Materials

Tryptone was from Oxoid (Basingstone, UK). Yeast extract, glucose, iron citrate and solvents were purchased from Merck (Darmstadt, Germany). C-18 Ultrasphere (4.6× 250 mm) high-performance liquid chromatography (HPLC) columns were from Beckman (Palo Alto, CA). Lipid standards were obtained from Sigma (St. Louis, MO). Particles of ferromagnetic iron oxide (magnetite), finely ground to an average diameter of 5 μm, were kindly provided by the National Institute of Mineral Research (Buenos Aires, Argentina). Permanent Alnico magnetic bars (159×27 mm) were purchased from Sigma.

Cell cultivation

Tetrahymena thermophila MS-1 was maintained and grown in 250-ml Erlenmeyer flasks containing 50 ml of a tryptone–yeast extract–glucose (TYG) medium and incubated for 24 h with shaking at 32°C. Composition of the TYG medium consists of 1% tryptone, 0.1% yeast extract, 0.5% glucose and 0.003% iron citrate (pH 6.8). For growth in the bioreactor, the same medium was used. Commercial ultrahigh-temperature-processed (UHT) whole milk samples were used and diluted with sterile water (1:1 v/v), as *Tetrahymena* cells do not tolerate well the high osmolarity of undiluted milk (unpublished observations).

Bioreactor configuration

The configuration of the bioreactor model system designed is shown as a schematic diagram in Fig. 1. It consists of two vessels of 1 l each (A, B) with conical bottoms, suitable for cell cultivation and easy collection by sedimentation. The working volume is 0.8 l. Fresh medium is supplied to vessel A through a tube, and the same connection is used for inoculation. Culture vessels A and B are connected to each other at their upper ends by a system of tubes. These tubes are attached to a peristaltic pump that can propel the medium from vessel A to B and also serves as an outlet for the spent medium from vessel B. The tips of these tubes are hook shaped to avoid turbulence as the medium is removed. Vessel B is connected to a receiver (D) that saves the spent medium for latter enzyme recovery. The vessels are connected to a cylindrical container (C), located at a lower level, through tubes furnished with valves. Container C is used for the food treatment and has inlet/outlet separated connections for foodstuff addition and collection. An air pump with a filter provides sterile air required for mixing and aeration for the culture vessels and the food treatment container. The rate of air pumping was kept at 2 vvm. Vessels A, B and container C have vent tubes for CO_2 elimination. All the system runs under sterile conditions.

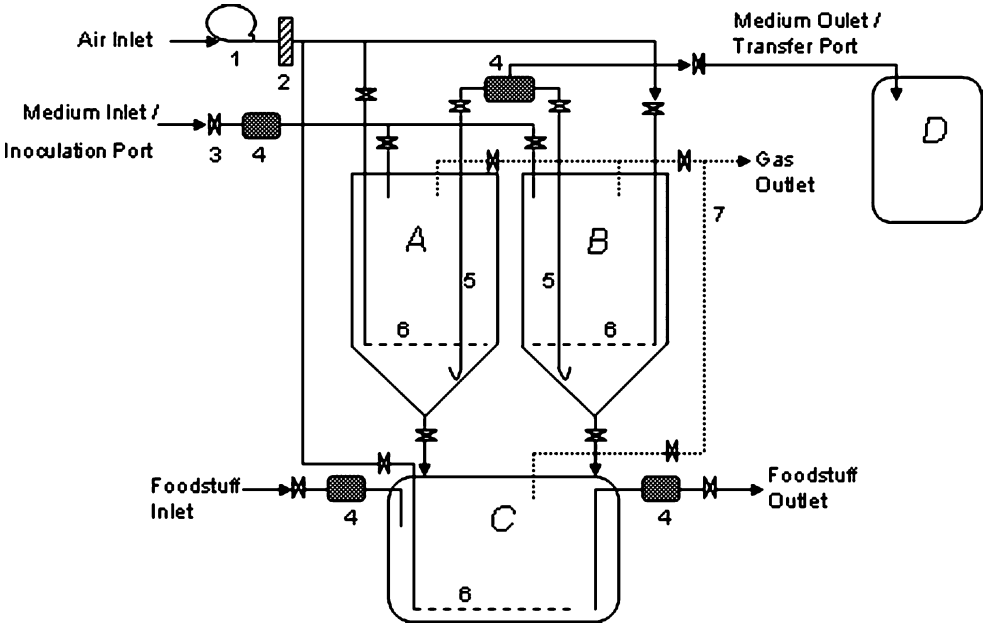

Fig. 1 Schematic diagram of the model bioreactor system for simultaneous cell culture and foodstuff treatment, with the following parts: *A* and *B*, culture vessels; *C*, foodstuff treatment container; *D*, spent medium receiver; *1*, air pump; *2*, sterile filter; *3*, valves; *4*, pump; *5*, transfer tube; *6*, air sparger; *7*, vent tube. Vessels A and B are used for *Tetrahymena* culture. Container C is used for foodstuffs treatment with the cells

Cell biomass separation techniques

Several methods were tested and compared for separation of cell biomass from the treated milk. These included the following: (1) sedimentation in the cold at 4°C, (2) cell killing by heating at 45°C and subsequent sedimentation at room temperature, (3) addition of 5% ethanol for 5 min and later sedimentation at room temperature or centrifugation at 1,000 *g* for 15 min, (4) centrifugation at room temperature at 1,000 *g* for 15 min, (5) centrifugation at 1,000 *g* for 15 min at 4°C and (6) separation with 100 μg/ml magnetite particles added to the treated milk with stirring at 100 rpm for 1 h. At the end of this period, the suspension was placed on a permanent magnetic field from 159×27 mm Alnico magnetic bars for 15 min to retain cells that ingested magnetic particles. In the latter case, the treated milk was decanted for separating from the cells.

Analytical methods

Cells counts

Cells were fixed by adding 10 volumes of 4% formaldehyde solution (in 0.9% NaCl solution) to 1 volume of sample. This treatment decreases cell motility and enables counting in a Neubauer chamber. For each sample, eight large squares were counted to estimate cell concentration.

Analysis of sterols

This was performed as described previously (Valcarce et al. 2001). After cell separation, an aliquot of 2 ml from the treated milk was saponified by addition of 1 volume of 2 M sodium hydroxide prepared in methanol/water (1:1 v/v) and heated at 60°C for 1 h. After cooling and mixing, 11.2 ml of chloroform/methanol (3:2 v/v) was added. Upon centrifugation, the sterols were extracted into the lower phase, concentrated under nitrogen and separated by HPLC. A C-18 Ultrasphere column was used with methanol/water (96:4 v/v) as mobile phase, operated at 41°C. The absorbance was monitored at 205 nm. For quantification, stigmasterol (100 μg/ml) was added to the milk aliquot, before saponification, and used as internal standard. This sterol separates neatly from cholesterol and its derivatives under the conditions employed. The identity of each compound was previously established by gas chromatography–mass spectrometry (Valcarce et al. 2000).

Results

Bioreactor operation

Tetrahymena cells grown in the TYG medium were aseptically transferred to vessel A and incubated in the same medium with vigorous sterile aeration (2 vvm) to ensure efficient mixing (Fig. 1). The initial cell concentration was 5×10^4 cells/ml, and after 24 h of growth, the cell concentration reached a value of 1×10^6 cells/ml (four to five doublings, approximately). At this time, the air flow was stopped, and immediate cells sedimentation started, also favoured by the conical bottom of the vessel. About 70% of the cells decanted spontaneously at room temperature after 30 min, forming a compact cell paste. Immediately after sedimentation, the supernatant was

aseptically transferred from vessel A to B aided by a peristaltic pump. This supernatant had a pH value close to 7.2, which is still optimal for cell growth. The volume transferred accounts for about 95% of the original volume. After this transfer, 40 ml of fresh TYG medium was added to replenish the initial working volume in vessel B (0.8 l). There was no need for a new inoculation in this vessel, as the remaining cell concentration was more than 3×10^5 cells/ml, and this amount is sufficient to initiate a new cycle of cell growth during the next 24 h. Thus, the inoculated culture medium is used twice, to generate two lots of *Tetrahymena* biomass.

Immediately after the removal of the spent medium, the cells from vessel A were transferred to container C, by opening the respective valve. Removal of the medium before transfer of the cell sediment to the milk avoids turbulence, thereby preventing an unnecessary passage of the culture medium to the foodstuff in container C. In the case of milk, a dilution of 1:1 (v/v) with distilled water was used in vessel C, owing to the low tolerance of *Tetrahymena* cells in non-diluted milk (unpublished results).

Cholesterol removal from milk reached 75±10 % in a period of 5 h (Table 2); beyond this period, milk became unstable and showed a tendency to coagulate. After 5 h the cells were separated from the treated milk (separation methods are discussed below).

Meanwhile, in vessel B, the second cycle of cell cultivation was conducted for 24 h until the culture reached again the concentration of 1×10^6 cells/ml. At this time, aeration in vessel B was stopped and cells allowed to sediment. The supernatant medium was removed and saved in container D for later enzyme recovery. At the end of the second cycle, the culture medium had a pH value around 7.4, with no change in cell viability.

The cell sediment was then again transferred to container C which has been emptied and filled with a new batch of milk. Upon incubation, the cell concentration in milk doubled.

At the end of the first cycle of cultivation using vessels A and B (48 h after the first inoculation), a new cycle is started in vessel A. This has been replenished with a fresh medium and inoculated to start a new cycle of operation. In this way, milk treatment can be run daily and uninterruptedly on the same bioreactor. In each case, after culturing the cells in milk, they are harvested from the foodstuff by centrifugation, producing on one hand improved milk and on the other cell biomass.

All the main features of the operation of the bioreactor system are summarised in Table 1. As a result of its operation, this bioreactor configuration can provide milk reduced in cholesterol (75% less), spent medium loaded with important enzymes and *Tetrahymena* biomass obtained after milk centrifugation, which has peculiar compounds of potential biotechnological interest.

Table 1 Operation data of the bioreactor model system

Main features	
Total volume of culture vessels	1 l
Working volume of the culture vessels	0.8 l
Total volume of foodstuff container	1 l
Working volume of the foodstuff container	0.8 l
Aeration rate	2 vvm
Cultivation temperature	32°C
pH range of the culture medium (TYG)	6.8–7.4
Initial cell concentration in TYG	5×10^4 cells/ml
Maximal cell concentration in TYG	1×10^6 cells/ml
Foodstuff treatment time	5 h
Cell harvest method in TYG	Sedimentation at 25°C
Cell elimination method from milk	Centrifugation at 25°C

Effect of inoculum size on milk improvement

We examined the efficiency of the milk treatment step in relation to inocula sizes, particularly with respect to cholesterol removal and subsequent cell separation from milk. As shown in Table 2, increasing the number of inoculated cells favoured subsequent cell sedimentation and separation. This effect may be attributed to aggregation of the cells as its concentration increases.

More dramatic has been the effect on cholesterol removal; in effect, increasing the concentration of cells in milk from 1.5 to 2.1 million per millilitre increased cholesterol removal from 42 to 75% during the usual 5-h incubation period. This reduction is evident in Fig. 2, where total sterols before and after milk treatment was compared (a and b, respectively). Hence, it was concluded that the inoculum size strongly influenced both cholesterol removal and the efficiency of cell separation from milk.

Table 2 Effect of inoculum size on cell elimination efficiency and cholesterol removal from milk

Initial cell population (cells/ml)	Final cell population[a] (cells/ml)	Cell elimination from treated milk[b] (%)	Cholesterol removal[c] (%)
0.7×10^6	1.3×10^6	87±2.5	20±6
1.5×10^6	2.8×10^6	93.5±4.5	42±9
2.1×10^6	3.7×10^6	96.5±3.8	75±10

Milk treated with different cell concentrations for a period of 5 h was evaluated for cell growth, cell elimination efficiency and cholesterol removal. Cell separation improved with increasing cell number, and cholesterol in milk was reduced accordingly.

[a] After incubation

[b] The separation technique was centrifugation at 25°C in all cases.

[c] Mean value ± SD (n=3). Initial cholesterol content in milk ranged from 125 to 160 mg/l.

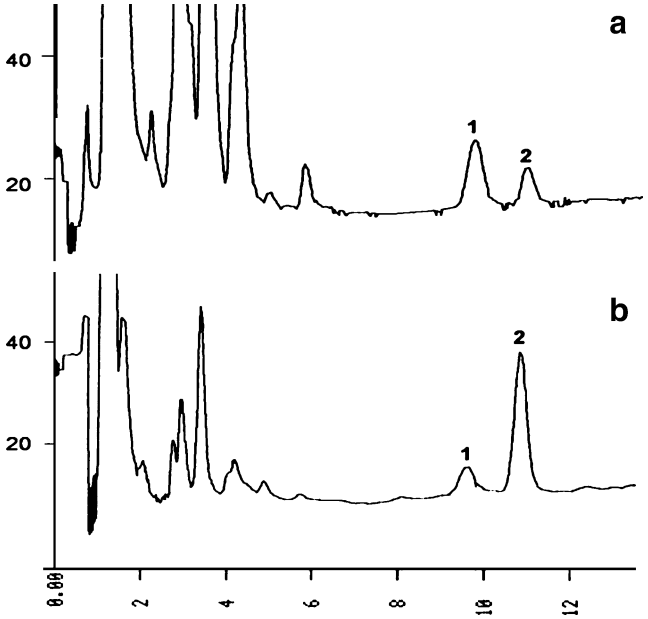

Fig. 2 Reduction of cholesterol level in milk by *Tetrahymena* treatment. Sterols were extracted and separated by HPLC using stigmasterol as internal standard. *Peak 1* corresponds to cholesterol and *peak 2* to stigmasterol. **a** Control milk without treatment. **b** Cholesterol removal in treated milk. The stigmasterol peak serves as reference to evaluate the efficiency of the extraction procedure and to calculate the concentration of cholesterol by area comparison. The difference in the size of the cholesterol peaks between **a** and **b** indicates the decrease of cholesterol in the treated milk

During the time of incubation in milk, cellular concentration increased, doubling approximately the initial value. Nevertheless, changes in sensorial properties such as clotting or butyric odor in milk were not detected.

Methodologies for separation of cells from milk

For the intact recovery of the treated milk, several cell separation techniques were compared. These methods are important, both for quality of the functional food and for achieving high yields of *Tetrahymena* biomass. Decreasing the temperature to 4°C for 1 h slowed down cell movement, but this procedure did not improve enough sedimentation to provide a separation method. Cell killing at 45°C induced a massive release of mucocysts which clotted the milk and prevented cell sedimentation. The addition of 5% ethanol for 5 min reduced cell motility but did not improve removal by centrifugation or spontaneous sedimentation. Magnetic separation with permanent magnets removed only 60% of the cells. This method was proposed to exploit the phagocytic activity of *Tetrahymena* and has been very efficient for the isolation of membrane proteins (Maicher and Tiedke 1999).

As shown in Table 3, centrifugation at 4°C provided a good separation of the cells (86.3%). However, if centrifugation is conducted at 25°C, the results were improved (97.3%). This may be due to a variation of the density of the cells with the temperature. Taken together, these results indicate that centrifugation at 25°C is the best method of those investigated for cell separation from milk.

Table 3 Cell separation from treated milk by centrifugation and magnetic separation

Separation method	Cell elimination (%)
Centrifugation at 25°C	97.3±2.4
Centrifugation at 4°C	86.3±4.6
Magnetic separation	60±2.6

Cell removal methods were compared using processed milk containing 3.5×10^6 cells/ml. Centrifugation at 25 and 4°C were both conducted at 1,000 *g* during 15 min. At 25°C, better cell separation was obtained.

Discussion

The bioreactor system designed here provides continuous operation to generate large densities of *Tetrahymena* and massive milk treatment with these cells. These operations can be run under aseptic conditions, and they can be easily subjected to automation with reduction in the labor cost.

The specific advantage of this bioreactor is that it allows the single re-use of the inoculated medium, as operation in both vessels can take place successively. Furthermore, the use of larger cell inocula at the beginning of the growth in the culture medium in vessel A shortens the time for achieving maximal reduction in the cholesterol content to only 4–5 h. This could allow the use of the system even twice a day.

Inoculum size has a remarkable effect on the removal of cholesterol from the milk. The mechanism by which this removal takes place likely involves the phagocytosis of fat globules. This provides prolonged and close interaction of the fat from milk with the endocytic membranes. Other endocytic paths may be involved such as pinocytosis. Finally, constant impact of milk globules on cell membranes can provide a mechanism for cholesterol transfer. In all cases investigated here, the larger the amount of cells present in the milk suspension, the higher are the levels of cholesterol removal.

Unexpectedly, larger cell concentration provided better pelleting. This may be due to improved cell aggregation at high concentration. In any case, this phenomenon favours also cholesterol elimination.

Although other industrial media have been proposed for high biomass yield (De Coninck et al. 2000) and high cell density cultures (Kiy and Tiedtke 1992a), we selected the TYG medium for *Tetrahymena* growth because of the requirement of a particle-free medium. In effect, for our purpose, a clear, large particle-free medium is essential to

avoid co-sedimentation of unwanted particles together with the cells. This condition is satisfied by a tryptone–yeast extract medium in which rapid sedimentation of the cells take place. In addition, cell sedimentation has been shown to be the least harming method for *Tetrahymena* harvesting (Hellenbroich et al. 1999).

According to our results, the cell concentration in milk can reach up to 3.7×10^6 cells/ml in a 5-h period, starting from an initial population of 2.1×10^6 cells/ml.

As the bioreactor is designed to manage large volumes of milk, it is evident that huge quantities of proteins and lipids will be generated. Among the proteins, several enzymes of interest are present. The lipids comprise phosphonolipids (i.e. glyceryl ether aminoethylphosphonate), which are resistant to enzymatic hydrolysis (Rosenthal and Pousada 1968; Florin-Christensen et al. 1986c) and abundant PUFAs among others compounds of interest.

The operation of the bioreactor also generates large amounts of spent medium that contains several extracellular acid hydrolases. Many of these have been characterised (Florin-Christensen et al. 1986a,b, 1989; Hunseler et al. 1988).

The emerging interest in biotechnological application calls for methods for massive and economical cell cultivation. The present bioreactor can address this issue, as it opens new possibilities to obtain and manage large *Tetrahymena* biomass and improve foodstuffs by cholesterol removal.

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