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Supporting Information available online

Bioproduction of Floxuridine Using Nanostabilized Biocatalysts

Bionanocomposites employing natural polysaccharides such as alginate and nanoclays, e.g., bentonite, are a promising alternative to developing stabilized biocatalysts used in the pharmaceutical industry due to their physicochemical properties, biocompatibility, and nontoxicity. Mechanical parameters such as swelling ratio, compressive strength, and fracture frequency were optimized, favoring scale-up. Moreover, storage stability and reusability of the biocatalysts were improved by more than 90 % compared with control conditions. In addition, immobilized lactic acid bacteria were used in the bioprocess scale-up to obtain floxuridine, showing a high productivity per gram of biocatalyst.

Keywords: Bioreactor, Biotransformations, Immobilization, Nanoparticles, Nanostabilized biocatalysts

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1 Introduction

Fluorinated organic molecules are known to perform a wide range of biological functions. Globally about 25 % of the drugs in the pharmaceutical industry contain at least one fluorine atom. The efficacy of fluorinated derivatives for the treatment of several cancer modalities is well-known [1]. Floxuridine or 5-fluoruracil-2'-deoxyriboside is used extensively as an antitumor drug in the clinical treatment of esophageal and colon cancer [2]. This modified nucleoside is mainly synthesized by chemical methods, however, its biocatalytic synthesis using microorganisms is a promising alternative [3]. Nevertheless, the application of microorganisms in soluble form is limited by their low stability [4]. Currently, different immobilization techniques have allowed to stabilize biocatalysts, facilitating their reusability, favoring their biocatalytic activity and bioprocess scale-up [5].

In the last decades, polymer hydrogels have attracted considerable scientific interest and important breakthroughs have been achieved as a result of the creation of nanocomposite hydrogels [6]. Among the inorganic nanoparticles, clay minerals such as bentonite have received more attention in the field of biopolymer nanocomposites due to their commercial availability [7], low cost, significant property enhancement, and relatively simple processability [8]. Furthermore, polysaccharides are the most promising alternatives to developing bionanocomposites because they are obtained from natural resources [6]. Among polysaccharides, sodium alginate is considered an efficient option because it is nontoxic, hydrophilic, biodegradable, and biocompatible [9]. Therefore, bionanocomposites exhibit improved mechanical, optical, and swelling/deswelling properties which could simultaneously overcome the limitations of conventional hydrogels [10]. Moreover, the use of bionanocomposites for microorganism stabilization to obtain pharmaceutical compounds is a promising option in the field of industrial biotechnology. In this context, large-scale production of chemotherapeutic drugs has acquired relevance in white biotechnology.

In the present work, a green bioprocess using stabilized *Lactobacillus animalis* ATCC 35046 in a bentonite-alginate-based bionanocomposite was developed. Scale-up biotransformation to obtain floxuridine using this biocatalytic system was studied to provide an improved alternative for the synthesis of antitumor compounds using a simple and environmentally friendly method.

2 Materials and Methods

2.1 Chemical Compounds

Nucleosides, bases, and chemicals were purchased from Sigma Chem. Co. (Brazil). Culture media compounds were obtained from Britania S.A. (Argentina). Sodium alginate was from Saporiti S.A.C.I.F.I.A (Argentina) and Patagonian bentonite was provided by Centro de Investigación y Desarrollo en Ciencias Aplicadas Dr. Jorge J. Ronco (Argentina). HPLC-grade solvents used in this study were from Sintorgan S.A. (Argentina).

2.2 Growth of *L. animalis*

L. animalis ATCC 35046 was grown until saturation in stationary phase, harvested by centrifugation for 10 min at 10 000 *g*,

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washed with 25 mM tris(hydroxymethyl) aminomethane-HCl (Tris-HCl) buffer pH 7, and stored at 4 °C until use. Media contained tryptone 15 g L^{-1}, soy peptone 5 g L^{-1}, and NaCl 5 g L^{-1}, pH 7.3.

2.3 Preparation of the Bionanocomposite Matrix

A 4 % w/v sodium alginate solution was prepared in physiological saline solution. Bentonite, a natural clay mineral, was added to a 4 % alginate solution under vigorous stirring for 16 h.

2.4 Microorganism Immobilization

L. animalis ATCC 35046 (1×10^{10} CFU) was immobilized by entrapment as previously described [11], using sodium alginate-bentonite (bionanocomposite) as support and $CaCl_2$ as cross-linking solution. Sodium alginate without bentonite was used as control of immobilization [12]. Moreover, a second control based on Sr-alginate gel beads was performed [13].

2.5 Optimization of Immobilization Conditions

Different immobilization parameters such as nanoclay concentrations (0–2 % w/v), $CaCl_2$ concentration (0.1–1 M), and exposure time (5–60 min) were analyzed. The immobilized biocatalyst activity was assayed by 5-fluoruracil-2′-deoxyriboside (5FUradRib) biosynthesis, using the methodology described by Britos et al. [12].

2.6 Characterization of Bionanocomposite Gel Beads

Fourier Transform Infrared Spectroscopy (FTIR) spectra were recorded with a Bruker Vertex 70 FT-IR infrared spectrometer with KBr optics and DTGS detector, spectral range 4000–400 cm^{-1}. Spectra were obtained by co-adding 80 scans collected at 4 cm^{-1} resolution.

Surface morphology and quantitative analysis of sample compositions were carried out employing a scanning electron microscope with energy dispersive X-ray detection (SEM-EDS) using a Philips SEM 505 microscope.

2.7 Physicochemical Properties

2.7.1 Mechanical Stability

The compressive strength of beads was determined using a Universal Testing Machine TC-500 II series (MegaTest). Young's modulus was calculated using the following equation:

$$\text{Young's modulus } (E) = \frac{F\,D_0}{A\,\Delta D} \tag{1}$$

where F[1)] is the force imposed on the gel beads, D_0 is the initial bead diameter, A is the gel bead area, and ΔD is the difference between the initial and final bead diameter.

2.7.2 Characterization of Swelling Behavior

Gel beads with a uniform diameter were placed in an excess of 25 mM Tris-HCl buffer pH 7 at room temperature. Changes in bead volume during the course of swelling were monitored by measuring their weight using an analytical balance. The swelling ratio (SR) was determined by using the following equation:

$$\text{Swelling ratio} = 100\times\frac{Mt - Md}{Md} \tag{2}$$

where Mt is the mass of swollen hydrogel at a given time during swelling and Md is the dry hydrogel mass. The mass was measured at specific times after removing the excess of water as described by Zhang et al. [14].

2.7.3 Fracture Frequency

The mechanical resistance of bionanocomposite gel beads was determined by a modification of the method described by Kathiravan et al. [15]. Beads were placed in a container with 25 mM Tris-HCl buffer under operative conditions (30 °C and 200 rpm for 40 h). Gel beads were filtered and counted. Resistance was expressed in terms of fracture frequency as given below:

$$\text{Fracture frequency} = 100\times\frac{N}{Nt} \tag{3}$$

where N is the number of fractured beads and N_t is the total number of gel beads.

2.8 Storage and Operational Stability

Storage stability of the immobilized biocatalysts was defined as the relative activity of 5FUradRib biosynthesis between the first reaction and successive batch cycles under optimized conditions. Two storage temperatures, 4 °C and 30 °C, were evaluated in 25 mM Tris-HCl buffer pH 7.

Reusability of immobilized whole cells in the bionanocomposite matrix was determined through 5FUradRib biosynthesis. *L. animalis* ATCC 35046 immobilized in Ca-alginate served as control system. The beads used for 5FUradRib biosynthesis were filtered after each 4-h cycle and washed three times with 25 mM Tris-HCl buffer pH 7.

1) List of symbols at the end of the paper.

2.9 Bioprocess Scale-Up

The batch bioprocess in a stirred tank with 15 mL of reaction medium was assayed to obtain 5FUradRib. Biosynthesis was carried out using 4.3 g of immobilized biocatalyst (7×10^{10} CFU), 6 mM dThd, and 2 mM 5FUra in 25 mM Tris-HCl buffer pH 7 at 30 °C with shaking at 200 rpm. Bioprocesses using *L. animalis* ATCC 35046 entrapped with Ca-alginate and Sr-alginate were performed as control reactions.

2.10 Green Chemistry Parameters

Green parameters such as environmental factor (E-factor), carbon efficiency (C-efficiency), and atom economy (A-economy) were evaluated by biosynthesis of halogenated analogues using the equations described by Sheldon [16].

2.11 Analytical Methods

Biosynthesis of 5FUradRib was qualitatively assessed by TLC Merck Silica gel 60 F254 in chloroform/methanol 80:20 v/v as mobile phase. Quantitative analysis was performed by HPLC (Gilson) at 254 nm with a Nucleodur 100 C18 column (5 µm, 250 mm × 4 mm). The isocratic mobile phase was water/methanol (94:6 v/v) and flow was 1.2 mL min^{-1}. Product identification was performed on a MS-HPLC LCQ-DECAXP4 Thermo Spectrometer with electron spray ionization methods (ESI). A Phenomenex C18 column (5 µm, 100 mm × 2 mm) and Xcalibur software version 1.3 (Thermo-Finnigan, USA) were employed. The mobile phase used for 5FUradRib (*t*: 14.0 min, M^+: 246.19) biosynthesis was 95:5 v/v water/methanol + 0.1 % acetic acid and the flow was 200 µL min^{-1}.

2.12 Statistical Analysis

All experiments were performed in triplicate. One-way analysis of variance (ANOVA) was performed to determine significant differences among variables. Differences with a probability value < 0.05 were considered significant and all data were reported as mean ± SD. Statgraphics Centurion XV software, version 15.1.02, was used.

3 Results and Discussion

3.1 Optimization of Immobilization Conditions

3.1.1 Nanoclay Concentration

To determine the best immobilization conditions for stabilizing *L. animalis* ATCC 35046 in the nanostructured matrix, different bentonite concentrations were evaluated through 5FUradRib biosynthesis. A significant decrease in 5FUradRib productivity was observed when bentonite concentrations higher than 0.1 % w/v were assayed using 0.1 M $CaCl_2$ (Fig. 1 a). This result could be related to the fact that bentonite occupied

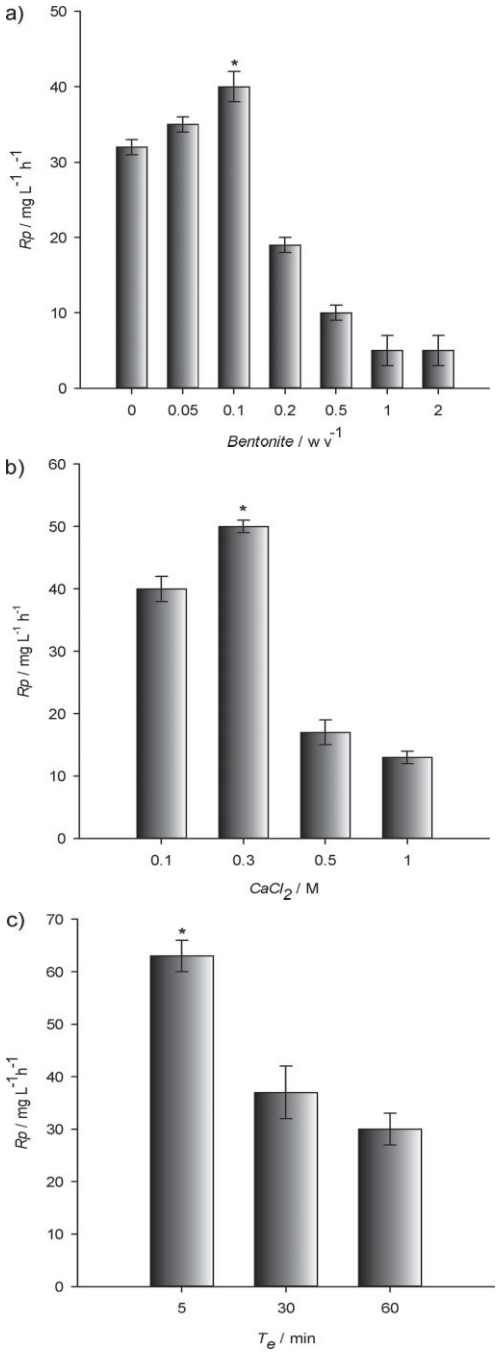

Figure 1. Optimization of immobilization conditions by *L. animalis* ATCC 35046. Floxuridine biosynthesis using 6 mM dThd and 2 mM 5FUra, Tris-HCl 25 mM, pH 7, 30 °C, and 200 rpm during 4 h was evaluated under different immobilization conditions. (a) Effect of increasing bentonite concentrations (0–2 % w/v). A significant difference was shown in the activity when 0.1 % w/v of bentonite was used (**p*-value < 0.05), cross-linking solution was 0.1 M $CaCl_2$ with 1 h exposure time. (b) Different concentrations of cross-linking solutions were assayed. A significant difference in floxuridine biosynthesis was observed when 0.3 M $CaCl_2$ solution was used with 1 h exposure (**p*-value < 0.01). (c) Effect of exposure times in the cross-linking solution was evaluated. A significant difference was shown at 5-min exposure (**p*-value < 0.05).

the interstitial space between the alginate layers. Interaction between functional groups of biopolymers and clay could generate a particular alginate-clay gel matrix which causes a reduction in substrate diffusion [14].

3.1.2 Cross-linking Solution

Cross-linking solution concentration is one of the main parameters to be standardized in this immobilization technique, therefore, different $CaCl_2$ concentrations were evaluated. In this case, the use of 0.3 M $CaCl_2$ allowed to significantly increase 5FUradRib productivity (Fig. 1 b). Moreover, the exposure time to the cross-linking solution was evaluated, achieving 5FUradRib productivity close to 60 mg $L^{-1}h^{-1}$ after 5 min of exposure (Fig. 1 c). This result not only allowed to improve biosynthesis productivity, but also had direct impact on operational feasibility by enabling more than a 12-fold total time reduction in the immobilization process. These results are consistent with a higher degree of cross-linking in the presence of an elevated cation concentration and longer exposure time, which causes a reduction in matrix permeability [17].

It is worth mentioning that cell release was assessed after seven days under the operating conditions. Optical density (600 nm) was lower than 0.1 which represents less than 1 % of the total immobilized cells.

3.2 Characterization of the Bionanocomposite Gel Beads

3.2.1 FTIR Spectroscopy

FTIR spectroscopy was applied to characterize the possible interaction between alginate and bentonite. A broad band between 3300 and 3750 cm^{-1} attributed to the hydroxyl stretching vibration of polysaccharides was observed. In addition, alginate-bentonite sample bands at 1700 and 1450 cm^{-1} assigned to carboxylate groups, as well as bands at 1060–1050 cm^{-1} associated with Si–O–Al bentonite tensions were detected. Below 1000 cm^{-1}, there was a band at 920 cm^{-1} corresponding to hydroxyl stretching vibration in the alginate sample, while this band shifted to 890 cm^{-1} in the bentonite sample (Fig. 2).

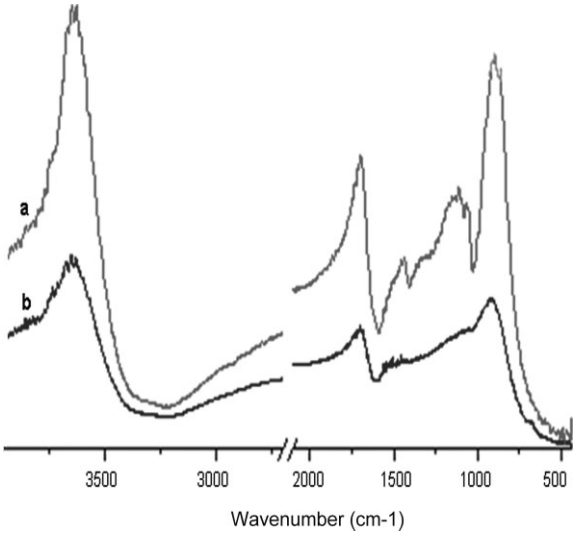

Figure 2. FTIR spectra of a bionanocomposite with 0.1 % of bentonite (a) and control matrix using Ca-alginate (b).

Although the negative charge of the carboxyl groups might have had electrostatic interaction with the positively charged sites existing at the bentonite edges, the presence of calcium ions could enhance and stabilize the interaction between alginate and clay [18]. The cross-linking process with the calcium ion caused a remarkable shift to higher wave numbers and a decrease in intensity of the carboxyl stretching vibration peak, indicating an ionic bond between the calcium ion and the carboxyl groups in alginate [19].

3.2.2 SEM Analysis

The analysis of the morphological characteristics of the bionanocomposite beads was performed by SEM. A homogeneous matrix was formed as a result of the interaction between bentonite and alginate (Fig. 3 a). This assay was also useful to determine that the methodology employed for bionanocomposite development was successful since no nanoclay aggregates were observed in the matrix. Additionally, the final microstructure of beads resulting from the internal gelation process of the alginate was modified in the presence of bentonite (Fig. 3 b).

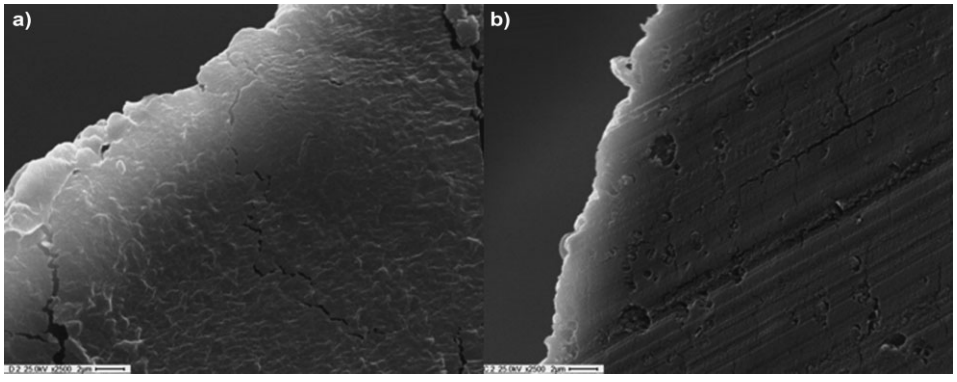

Figure 3. SEM analysis. Alginate-nanoclay bionanocomposite (a) and control matrix using Ca-alginate (b).

This change could be associated with the interaction of silica groups in bentonite with the carboxylic groups in alginate [20]. Further EDS assays showed the presence of calcium ions in the bentonite structure after bionanocomposite formation (see Supplementary Information).

3.3 Physicochemical Properties

Different parameters to evaluate the stability of bionanocomposite gel beads were determined and compared with immobilization control conditions.

3.3.1 Mechanical Stability

Youngs modulus or elastic modulus is the mathematical description of a material's tendency to be deformed elastically when a force is applied to it [21]. The effect of bentonite as alginate nanoreinforcement was evaluated (Tab. 1). The elastic modulus of the bionanocomposite beads was close to 60 kPa. In previous works, an improved immobilization method using Sr-alginate showed an elastic modulus higher than 20 kPa [22]. Therefore, the Young's modulus value obtained for the bionanocomposite beads was by 160 % better than that for the improved condition previously reported [22]. Nanosized clay can act as a reinforcing agent and improve mechanical properties as a result of changes in the matrix structure. The resulting competition between matrix-clay and clay-clay interactions are among the most important factors that determine the reinforcing effect of nanofillers [23]. Moreover, the formation of additional cross-links by inter-clay divalent cation (Ca^{+2}) interactions improved tensile and mechanical properties of bionanocomposites [24]. Each of these properties can increase biocatalyst stability for bioprocess scale-up.

Table 1. Physicochemical properties of alginate-bentonite bionanocomposite compared with immobilization control conditions.

Matrix	Young's modulus [kPa]	Swelling ratio [%]	Fracture frequency [%]
Bionanocomposite	63	23.7	< 5
Sr-alginate	24	2.8	< 5
Ca-alginate	17	15	< 5

3.3.2 Swelling Ratio

Osmotic swelling of the bead core is the principal cause of alginate polycation capsule breakage. In this study, a swelling ratio higher than 20 % was obtained (Tab. 1); however, it should be pointed out that the addition of bentonite, despite increasing the swelling capacity due to an increase in gel hydrophilicity, provides improved stability to breakdown [25]. This phenomenon is associated with higher mechanical bead strength. The optimum relationship between cation cross-linking concentration and exposure time promoted additional physicochemical stability of the matrix and improved substrate and product permeability due to film thickness [26].

3.3.3 Fracture Frequency

The fracture frequency of the bionanocomposite gel beads was not affected under the optimized immobilization conditions determined in this work compared with the controls using Ca-alginate or Sr-alginate (Tab. 1). Usually, the addition of nanocompounds to a polymeric matrix could result in its toughening and consequently in higher fragility. Immobilization parameters such as bentonite content, cross-linking solution and exposure time were optimized to avoid the negative effect of matrix toughening on fracture frequency. In our work, the presence of the bionanocomposite did not affect fracture frequency with respect to controls using Ca-alginate or Sr-alginate [27]. For *L. animalis* ATCC 35046 entrapment, 4 % w/v alginate, 0.1 % w/v bentonite, 0.3 M $CaCl_2$, and 5 min exposure time were used.

3.4 Storage and Operational Stability

Different storage temperatures were analyzed to ensure biocatalyst stability. When the biocatalyst was stored at 4 °C, biocatalytic activity was 10-fold more efficient than at 30 °C (data not shown). Moreover, the stability of *L. animalis* immobilized in the bionanocomposite was compared to that of Ca-alginate gel beads at 4 °C. The control condition remained active for 25 days while biocatalysts immobilized in the bionanocomposite for more than 55 days (Fig. 4 a).

Although there are a number of industrially effective reactions that can be catalyzed by immobilized biocatalysts, they are still too expensive when compared to chemical catalysts such as acids and bases [28]. Thus, higher biocatalyst reusability is beneficial from an economic viewpoint, since it is often an essential prerequisite for assessing the economic viability of the process and future scale-up of the developed biocatalyst. The ability of immobilized *L. animalis* to retain its biosynthetic activity during recycling was examined. The reusability test of stabilized *L. animalis* in the bionanocomposite retained more than 50 % of the initial activity after 20 successive batches. This biocatalyst had greater operational stability than that obtained using Ca^{2+} as cross-linking solution (Fig. 4 b). Ca-alginate lost its activity after four reuses, similarly to previous reports [29]. Therefore, the reusability of alginate-bentonite biocatalysts was twofold greater than previously reported values using $SrCl_2$ as cross-linking solution [22].

3.5 Bioprocess Scale-Up

Different laboratory-scale parameters of floxuridine biosynthesis were optimized in previous works using *L. animalis* as biocatalyst [12]. In the current work, floxuridine scaling-up biotransformation was evaluated. This process can be considered as first approximation to obtain an antitumor drug with enhanced yield using a microorganism immobilized in bionano-

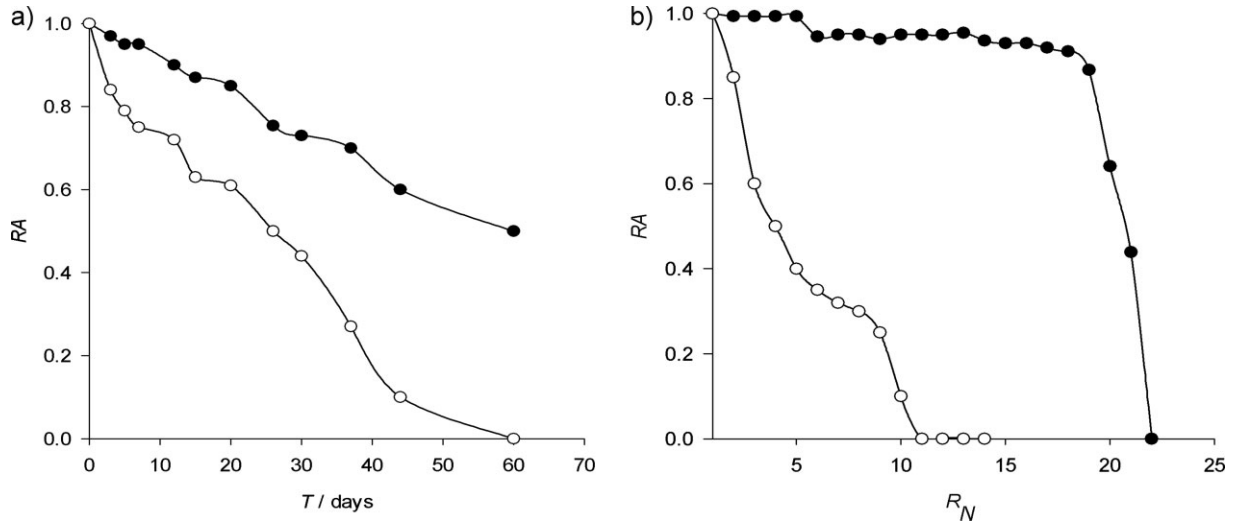

Figure 4. Evaluation of storage and operational stability. (a) Comparison of immobilized biocatalyst storage stability at 4 °C using bionanocomposite (●) and Ca-alginate (○) as matrices. (b) Biocatalyst reusability was evaluated through successive 5FUradRib biosynthesis (4 h) and the reusability number was determined.

composite. Bioprocess scale-up with immobilized microorganisms offers numerous advantages such as feasibility of continuous processing, flexibility in reactor design, cell stability, and lower costs of biocatalyst recovery and recycling, as well as the stabilization of several cell functions for biosynthesis applications [30].

Improved biocatalyst immobilization conditions using a bionanocomposite were applied to develop a bioprocess scale-up in order to obtain floxuridine by a batch operation mode. The floxuridine yield was greater than 120 mg L^{-1} at 4 h of reaction (Fig. 5). Batch configuration is most commonly employed due to easy operation and equipment flexibility [10]. Final catalytic productivity was 596 mg of floxuridine per gram of biocatalyst, until deactivation after 20 successive batches (Tab. 2). This bioprocess enables to reach a twofold higher productivity than that obtained under previously optimized conditions [22].

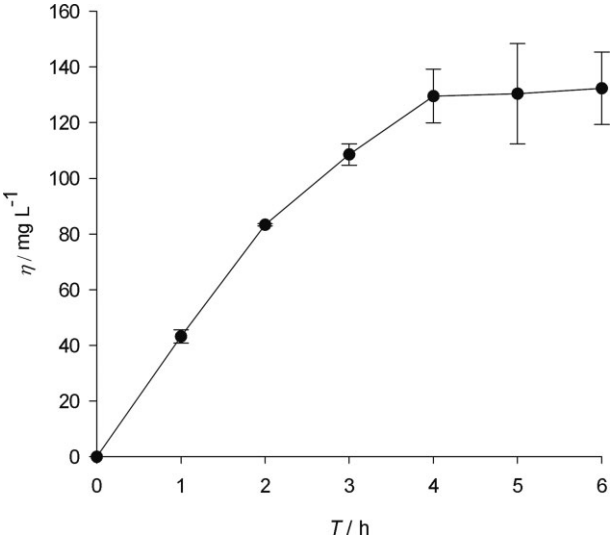

Figure 5. Scale-up bioprocess. Floxuridine yield in a batch operation mode using the biocatalytic system was evaluated at different times. Operational conditions: 7×10^{10} CFU, 6 mM dThd, and 2 mM 5FUra in 25 mM Tris-HCl buffer pH 7 at 30 °C with shaking at 200 rpm in 15 mL reaction volume. This assay was performed in duplicate.

3.6 Green Chemistry Parameters

The E-factor is a measurement of the environmental impact generated by chemical and pharmaceutical industries. E-factor values are around 25–100 for pharmacological compounds. A low E-factor indicates mass utilization efficiency and significant waste reduction [16]. In this work, E-factor values of 5FUradRib biotransformation using bionanocomposites as matrix were close to 7 (Tab. 2). Therefore, the support developed enabled to significantly increase the productivity and operational stability of the biocatalyst without affecting the environmental efficiency of the bioprocess. C-efficiency and A-economy were designed as parameters to evaluate

Table 2. Green bioprocess scale-up for floxuridine biosynthesis.

Matrix	Product	Operational stability [h]	Biosynthesis yield[a] [mg g^{-1} biocatalyst]	E-factor[b]
Bionanocomposite	5FUradRib	80	596	7.1
Sr-alginate	5FUradRib	40	347	5.4
Ca-alginate	5FUradRib	16	102	6.9

[a] Reaction conditions: 30 °C, 25 mM Tris-HCl buffer pH 7, considering a final volume of 1 L, 200 rpm, using 7×10^{10} CFU. [b] E-factor = total waste mass / product mass.

the efficiency of chemical synthesis. In all cases, C-efficiency values were 64 % and A-economy values were 66 %, both demonstrating the environmental efficiency of the bioprocess.

4 Conclusions

Alginate-bentonite is an improved mixed matrix using natural hydrogels and nanoclays. *L. animalis* ATCC 35046 immobilized in this bionanocomposite allowed to develop a novel biocatalytic system able to biosynthesize 5-fluoruracil-2′-deoxyriboside, a compound extensively used as an antitumor agent. This bioprocess scale-up may be employed in the pharmaceutical industry using a sustainable method.

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The authors have declared no conflict of interest.

Symbols used

A	[mm^2]	gel bead area
D_0	[mm]	initial bead diameter
ΔD	[mm]	difference between initial and final bead diameter
E	[kPa]	Young's modulus
F	[N]	force imposed on gel beads
Md	[g]	dry hydrogel mass
Mt	[g]	mass of swollen hydrogel
N	[–]	number of fractured beads
N_t	[–]	total number of gel beads
R_N	[–]	reuse number
RA	[–]	relative activity
Rp	[$mg\,L^{-1}h^{-1}$]	volumetric productivity
SR	[–]	swelling ratio
T	[h]	time
T_e	[min]	exposure time
η	[$mg\,L^{-1}$]	reaction yield

Abbreviations

CFU	colony-forming unit
5FUradRib	5-fluoruracil 2′-deoxyriboside
Tris-HCl	tris(hydroxymethyl) aminomethane-HCl

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