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One-pot biosynthesis of idoxuridine using nanostabilized lactic acid bacteria

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ABSTRACT

An efficient bioprocess using immobilized lactic acid bacteria to obtain 5-iodouracil-2′-deoxyriboside, known as idoxuridine, is reported. This nucleoside analogue is an antiviral agent used in the treatment of viral infections in the absence of efficient vaccines. Idoxuridine conversion was close to 90% in different nonconventional media.

A bionanocomposite (alginate-nanoclay) was employed as matrix to stabilize *Lactobacillus animalis* ATCC 35046 with improved stability in the presence of organic cosolvents. In this study, an eco-compatible and alternative bioprocess to obtain idoxuridine was developed for the first time. The scale-up biosynthesis of idoxuridine afforded a product yield close to 0.4 gr/L in the presence of 20% of DMSO.

1. Introduction

Nucleosides comprise a large family of natural and chemically modified analogues with great structural diversity and a broad spectrum of biological activities. These compounds are extensively used as antiviral and antitumor agents in clinical practice [1]. Halogenated pyrimidine nucleosides are among the most common nucleoside analogues used as pharmaceutical drugs [2].

Idoxuridine was the first effective antiviral nucleoside used in clinical trials [3]. Currently, idoxuridine is used as an effective agent against herpes simplex virus and different poxviruses such as vaccinia, variola, and cowpox [4]. Moreover, this compound is considered a chemotherapeutic drug for its radiosensitizing activity [5].

Analogue nucleosides are mainly obtained by chemical synthesis using multiple and complex reaction steps affecting the conversion yield and the sustainability of the process [6]. However, biocatalysis has appeared as an alternative to overcome these drawbacks. Biosynthesis has numerous advantages related to the general properties of enzymes as it takes place under mild conditions with high efficiency, and biocatalytic reactions are regio- and stereoselective avoiding undesirable racemic mixtures and simplifying downstream processing [7].

Some members of the lactic acid bacteria (LAB) group have an enzyme called 2′-*N*-deoxyribosyltransferase (EC 2.4.2.6), which catalyzes the transglycosylation between purine or pyrimidine bases and nucleosides in a one-step reaction, instead of conventional nucleoside phosphorylase enzymes that act in two successive steps [8]. In this context, the application of LAB is attractive for industrial biotechnology because it is a simple and effective alternative biocatalyst for an environmentally friendly process.

Nevertheless, the use of microorganisms in soluble form is limited by high production costs and low stability. Entrapment techniques using polymer hydrogels are the most widely used methodologies for whole cell immobilization [9]. These procedures allow the stabilization of microorganisms, facilitating their reuse, favoring their biocatalytic activity and bioprocess scale-up. Alginate is a natural polymer hydrogel used as a carrier matrix to encapsulate molecules of biological significance such as food products, enzymes, drugs and microbial, plant or animal cells [10]. The gelling process is induced by cations that interact with the carboxyl groups of mannuronic and guluronic acid of alginate chains and build a matrix around the cells [11]. Recently, polymer-nanoclay composite material was reported to stabilize microorganisms [12]. This bionanocomposite showed improved stability properties, extending its use to areas such as food preservation, textile printing agents and as burn dressing material. In this work, an efficient biocatalyst based on *Lactobacillus animalis* ATCC 35046 immobilized in an alginate-bentonite bionanocomposite was employed to obtain idoxuridine with improved bioconversion in nonconventional medium.

2. Materials and methods

2.1. Materials

Nucleosides and nucleobases were purchased from Sigma Chem. Co. (Brazil).

Culture media compounds were obtained from Britania S.A. (Argentina). Chemicals were acquired from Sigma Chem. Co. and

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Britania S.A. The HPLC grade solvents used were supplied by Sintorgan S.A. (Argentina). Sodium alginate was purchased from Saporiti S.A.C.I.F.I.A. (Argentina), and Patagonian bentonite (pore diameter 30–200 Å) was provided by Centro de Investigación y Desarrollo en Ciencias Aplicadas Dr. Jorge J. Ronco (Argentina).

2.2. Growth conditions

L. animalis ATCC 35046 was grown until stationary phase in MRS medium. Cells were harvested by centrifugation for 10 min at 10000*g*, they were then washed once with tris-(hydroxymethyl) aminomethane-HCl (Tris-Cl) buffer (25 mM, pH 7), and finally recentrifuged and stored at 4 °C until use.

2.3. Microorganism immobilization

First, 1×10^{10} CFU of *L. animalis* were harvested, centrifuged and washed. They were mixed with 1 mL of alginate-bentonite bionanocomposite, 4% and 0.1% (w/v) respectively. The mixture was then added dropwise to stirred 0.3 M $CaCl_2$ solution, with an exposure time of 5 min. Bionanocomposite gel beads were filtered and washed with physiological solution. Biological activity was evaluated through idoxuridine (5IUradRib) biosynthesis using dThd and 5-iodo-uracil (5IUra) in 25 mMTris-Cl at 30 °C and 200 rpm.

2.4. Optimization of biocatalytic activity for immobilized L. animalis

2.4.1. Optimum substrate molar ratio and microorganism loading

The response surface model was used to evaluate the interaction between different factors, each being simultaneously investigated at different levels. Based on Box-Behnken model, the three major factors, including reaction microorganism loading (1×10^9–1×10^{10} CFU) and a substrate molar ratio of 5IUra to dThd (6:6–2:2), were designed, and a 9-level-3-factor Box-Behnken for a total of fifteen experiments was developed. The response chosen was the product obtained by the immobilized *L. animalis*, and the factors were microorganism (C), 5IUra concentration (S_1) and dThd concentration (S_2). The second-order polynomial equation for the variables can be approximated to the following equation:

$$Y = a + a_1 S_1 + a_2 S_2 + a_{11} S_1^2 + a_{22} S_2^2 + a_{12} S_1 S_2 + b$$

Coefficients of the response function and their statistical significance were evaluated by Statgraphics Centurion XV (Stat Point Technologies Inc., Warrenton, VA, USA).

2.4.2. Optimum pH and temperature

Different pH values (5–9) and temperatures (30, 45 and 60 °C) were evaluated for 5IUradRib biosynthesis using *L. animalis* ATCC 35046 immobilized in bionanocomposite. Reactions were carried out using 1×10^{10} CFU, 6 mM dThd and 2 mM 5IUra in 25 mM Tris-Cl buffer and shaking at 200 rpm as standard reaction conditions. The reaction volume was 1 mL [14].

2.5. Biocatalytic activity in nonconventional media

The biosynthesis of 5IUradRib using immobilized *L. animalis* ATCC 35046, from dThd and 5IUra in 25 mM Tris-Cl at 30 °C and 200 rpm, was evaluated at 2 h employing 20% (v/v) of different cosolvents such as acetone, dimethylsulfoxide (DMSO), ethylene glycol, glycerol, and hexane.

2.6. Scale-up biosynthesis of idoxuridine in a stirred tank

Batch biotransformation was scaled-up in a 500 mL vessel. The bioreactor was equipped for the control of agitation (Rushton impellers) at 120 rpm stirring speed. Biosynthesis was carried out using 0.57 g/mL of biocatalyst (5×10^{11} CFU) in optimized reaction conditions: 2 mM dThd and 2 mM 5IUra in 25 mM Tris-Cl buffer pH 6 with DMSO 20% (v/v). The reactor was maintained at constant temperature (30 °C) using a heating bath. The total process volume was 50 mL.

2.7. Analytical methods

Quantitative analyses were performed by HPLC (Gilson) equipped with a UV detector (275 nm) using a Zorbax Eclipse XDB C18 column (5 μm, 125 mm, 5 mm). The isocratic mobile phase used was water/methanol (95:5, v/v) at room temperature and at a flow rate of 1.6 mL/min. Retention times of substrates and products for idoxuridine biosynthesis were as follows: thymine (2.9 min), 5-iodo-uracil (4.9 min), thymidine (7.6 min), and idoxuridine (16 min). Product identification was performed by MS-HPLC LCQ-DECAXP4 Thermo Spectrometer with Electron Spray Ionization methods (ESI). Phenomenex C-18 column (5 μm, 100 mm × 2 mm) and Xcalibur 1.3 software (Thermo Finnigan, USA) were used. The experimental conditions for idoxuridine (M^+:354.10) were: mobile phase (95:5, v/v) water/methanol + 0.1% acetic acid at a flow rate of 200 μL/min.

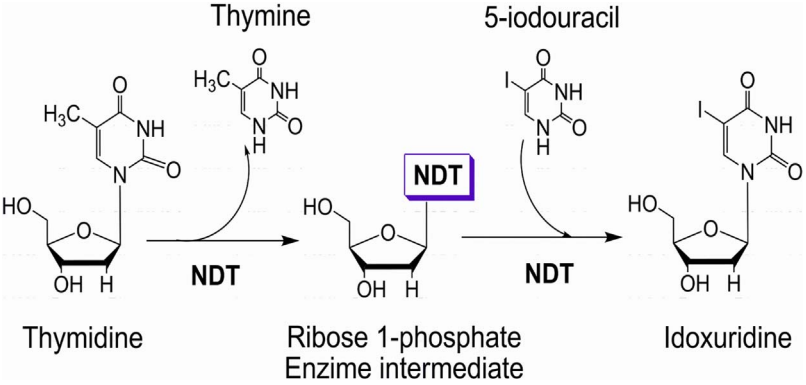
2.8. Statistical analysis

All experiments were carried out in triplicate. The 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. Biocatalytic activity of immobilized microorganism

L. animalis ATCC 35046 was selected by screening between different



Scheme 1. Biosynthesis of idoxuridine and catalytic mechanism of NDTs.

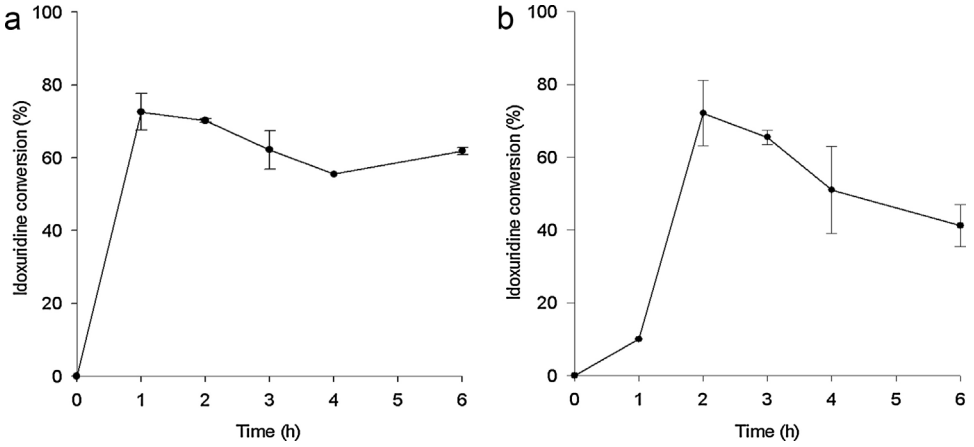


Fig. 1. Bioconversion of idoxuridine using free (a) or immobilized (b) *L. animalis* ATCC 35046. Reactions were carried out using 1×10^{10} CFU, 2 mM of 5IUra and 6 mM Thd in Tris-Cl buffer (25 mM, pH 7) at 30 °C and 200 rpm. Assays were performed by triplicate.

strains analyzed previously (data not shown) as the optimal microorganism to biosynthesize the product of interest (Scheme 1). This strain was able to produce idoxuridine with a yield close to 70% at only 1 h (Fig. 1a).

The selected microorganism was stabilized efficiently by immobilization in the bionanocomposite gel matrix using $CaCl_2$ as cross-linking solution. This biocatalytic system gave a 5IUradRib conversion yield similar to that reached with free microorganisms at 2 h (Fig. 1b). It was observed that the immobilized biocatalyst required longer time than free microorganisms to obtain the same result. This behavior is related to an increase in the diffusion restrictions of the matrix [15].

Additionally, biocatalyst stability was evaluated through a cell release assay. After seven days under operating conditions, *L. animalis* release was below 1%. The interphasic membrane of the bead can act as a barrier to retain microbial cells inside the bead [16]. Although alginate concentration above 2% improved cell retention, high alginate concentration can interfere with the diffusivity of the substrates through the beads [17].

3.2. Optimization of reaction parameters

The optimal conditions for best 5IUradRib performance were studied using the response surface methodology (RSM), which allows reducing the number of experiments without neglecting the interaction among the various parameters that affect the activity. The interaction effects between substrate concentration and microorganism load were studied using the Box–Behnken experimental design. RSM yielded the following regression equation:

$$P(mM) = 0.1625 + 0.05375 \times 5 \times 10^9 + 0.035625 \times S_1 - 0{,}02125 \times S_2 + 0{,}1025 \times (5 \times 10^9)^2 - 0.0375 \times 5 \times 10^9 \times S_1 + 0.07875 \times 5 \times 10^9 \times S_2 - 0.018125 \times S_1^2 + 0{,}018125 \times S_1 \times S_2 - 0.00375 \times S_2^2$$

where product (P) is the response, and the factor C (number of immobilized cells) was fixed at 5×10^9 CFU.

The RSM for immobilized microorganism activity as a function of base and nucleoside concentrations is shown in Fig. 2a. These results confirmed that the highest 2′-*N*-deoxyribosyltransferase activity occurred when a minimum concentration in an equimolar ratio was used. However, when substrate concentrations were greater than 5 mM, biocatalytic activity decreased completely. This behavior can be related to the mechanism of NDT. This enzyme has a specific catalytic site where the transglycosylation reaction takes place in one pot, and an excess of substrates could lead to an inhibitory process, especially when the base concentration increases [18]. It is important to note that under these optimized conditions, the immobilized biocatalyst was able to achieve a yield close to 72% at 1 h, improving the bioconversion time.

The significant factor affecting idoxuridine biosynthesis was microorganism concentration. The significance was identified from the Pareto chart as bars extending beyond the critical t-value, the vertical line on the chart (Fig. 2b). The increment of whole cells in the reaction medium is directly proportional to the amount of enzyme. The explanatory power of the model was assessed by the R^2 values. The R^2 value of 85.05% (0.85) showed that the model could account for 85% of the variability around its mean. ANOVA is used to test the variability in a given data set. The most significant effect was microorganism concentration with a p-value equal to 0.0093.

Additionally, the influence of pH and temperature on idoxuridine biosynthesis was evaluated. Values of pH below 7 resulted in improved idoxuridine productivities close to 100 mg/L h after 2 h of reaction (Fig. 3a). An additional benefit of these pH values was that hydrolysis of the nucleosides present in the reaction medium remained under 60% for over 4 h, while at the same time in neutral pH hydrolysis was above 80%, affecting the product stability (data not shown). At basic pH values the yield was below 10%, which can be related to the incorporation of 5IUra into the cell. Previous work revealed that alkaline pH values might interfere with an adequate internalization of the bases for subsequent use when pKa is close to the pH of the medium, in this case the pKa of 5IUra was 8.14 [13].

Idoxuridine productivity did not show significant differences between the evaluated temperatures. The immobilization process using the bionanocomposite developed maintained the stability of NDT activity even at temperatures above 55 °C (Fig. 3b).

3.3. Nonconventional media for idoxuridine biosynthesis

The addition of different cosolvents was evaluated in order to study idoxuridine conversion rate using the biocatalyst developed. It has been previously reported that nonconventional media increase the solubility of bases and nucleosides slightly soluble in water such as 5IUra [14]. The characteristics of this robust biocatalyst and its stability allowed the assessment of alternative reaction media using different organic solvents. Idoxuridine conversion was assayed employing hexane and DMSO, which gave a yield of 90% at 2 h, obtaining a conversion 1.4-fold higher than under the control conditions without cosolvent (Fig. 4). In terms of substrate polarity, the solubility of nonpolar drugs (log P greater than zero), such as 5IUra whose reported log P value is 0.13, increases in media with solvents that present positive log P values such as hexane. Moreover, this kind of cosolvent can exert an influence on the solubility of the drug, modify the concentrations of substrate and products in the vicinity of the biocatalyst and subsequently, the kinetics of biotransformation [19]. The presence of alcohols such as glycerol and ethylene glycol decreased the biosynthesis yield by 10% and 15%, respectively. On the other hand, the presence of aprotic polar solvents such as acetone or dimethyl sulfoxide (DMSO) had a positive effect on

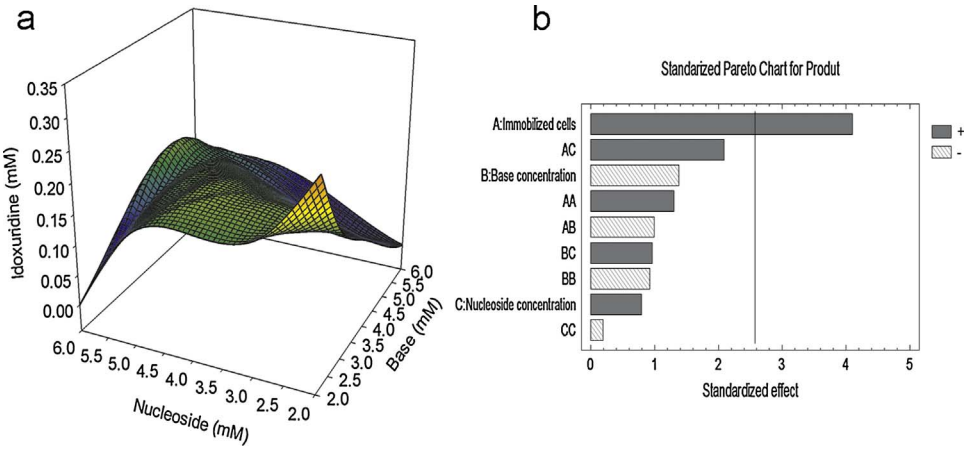


Fig. 2. Optimization of substrate concentrations for idoxuridine biosynthesis. (a) Response surface graph of interaction between different nucleoside and base concentrations using a fixed number of cells. (b) Pareto chart showing significant factors or combined factors affecting idoxuridine production (A: immobilized cells concentration, B: base concentration, C: nucleoside concentration).

the conversion yield. These solvents are used in pharmaceutical formulations to enhance the penetration of different topical drugs such as idoxuridine [20]. The use of nonconventional media could be an interesting alternative to improve the production of nucleoside analogues from bases and nucleosides with low solubility in aqueous phase.

4. Scale-up biosynthesis of idoxuridine

The objective to scale-up a process is to enlarge the production amounts with similar or higher productivity and product quality than that obtained at a microscale. The successful scale-up of biocatalytic processes requires a good understanding of the interactions between the biocatalyst and the chemical and physical environment in the reactor [21]. However, mass transfer is often found to limit the productivity of the bioreactor in heterogeneous systems [22]. The kinetic assay using a stirred tank in batch operation mode showed that the volumetric productivity of idoxuridine was 116 mg/L at 1 h. However, the maximum idoxuridine yield was 395 mg/L after 7 h of reaction under optimized conditions using DMSO as cosolvent and 30 °C. Temperatures above 30 °C may increase the costs of an industrial scale bioprocess, limiting its application. On the other hand, DMSO was selected for scale-up because it is used in pharmaceutical preparations and allowed obtaining a medium without interfaces at low stirring speed. When biosynthesis performance with and without cosolvent was compared after 6 h (Fig. 5), the presence of DMSO improved idoxuridine yield 2-fold. This behavior can be related to an increase in the permeability and kinetic diffusion of substrates and products.

The optimized biocatalysis conditions and stability of

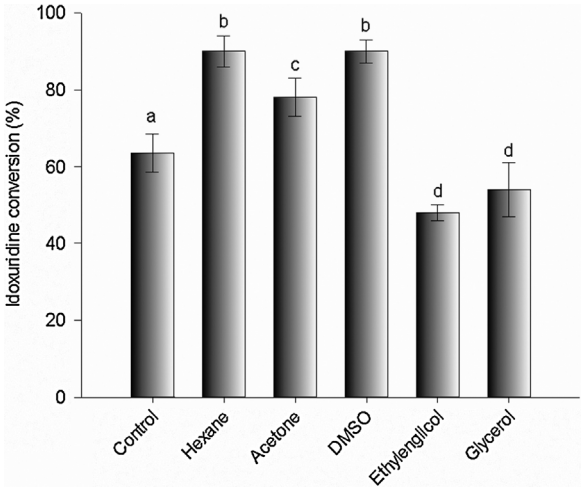


Fig. 4. Evaluation of co-solvents effect (20%, v v^{-1}) in reaction medium. Biosynthesis was carried out at 30 °C and 200 rpm in Tris-Cl buffer (25 mM, pH 6) using 1×10^{10} CFU, 2 mM dThd and 2 mM 5IUra. Each letter represent a group with significant differences (*$p < 0.001$). Assays were performed by triplicate.

nanostabilized microorganisms allowed overcoming the limitations associated with this configuration of reactor and operation mode.

5. Conclusion

In this report, a new one-pot bioprocess for idoxuridine production

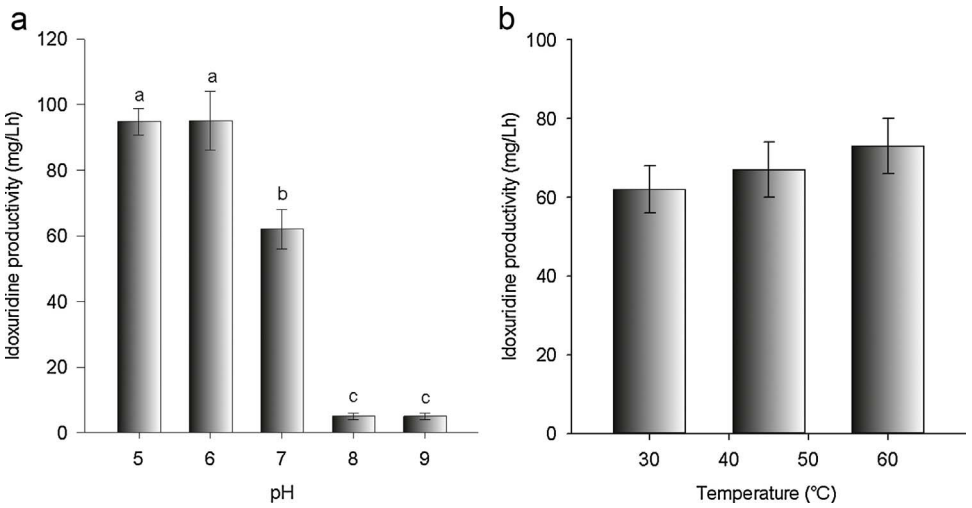


Fig. 3. Effect of pH (a) and temperature (b) on idoxuridine biosynthesis using immobilized *L. animalis*. Reaction was performed during 2 h using 1×10^{10} CFU, 2 mM dThd and 2 mM 5IUra in Tris-Cl buffer (25 mM, pH 7) at 200 rpm. Significant differences when pH was 5 and 6 (*$p < 0.0002$) were observed. Assays were performed by triplicate.

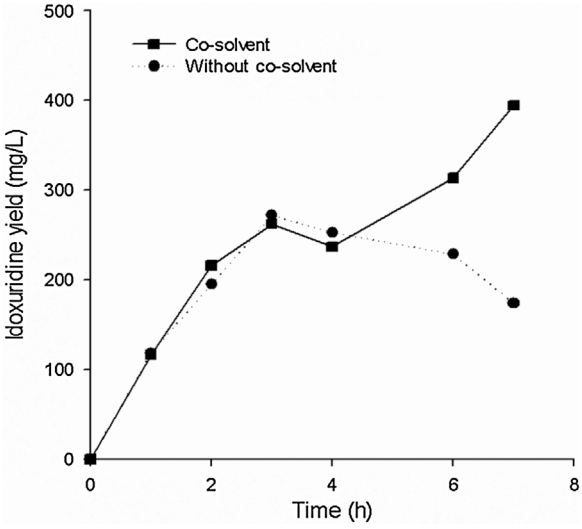


Fig. 5. Idoxuridine scale-up bioprocess using the biocatalytic system in a batch reactor. Biosynthesis was carried out using 0.57 g/mL of biocatalyst, 2 mM dThd and 2 mM 5IUra in Tris-Cl buffer (25 mM, pH 6) in presence of DMSO 20% (v/v) or without it. The reactor was maintained a constant temperature (30 °C) using a heating bath. Volume total of process was 50 mL.

has been described by direct transglycosylation using stabilized lactic acid bacteria in a bionanocomposite based on two natural and innocuous materials, alginate and nanoclay. This biocatalyst meets the requirements of high activity and stability in nonconventional media that allowed the development of a pilot scale-up with higher idoxuridine production. This result is a promising alternative for a future industrial application using a green methodology.

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