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Development of a nanostabilized biocatalyst using an extremophilic microorganism for ribavirin biosynthesis

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ABSTRACT

Ribavirin is a guanosine analogue commonly used as an antiviral compound for the treatment of Hepatitis C virus (HCV) infection. The biosynthesis of this compound using *Geobacillus kaustophilus* ATCC 8005 as biocatalyst is herein reported. This extremophilic microorganism has been successfully entrapped in an agarose matrix supplemented with bentonite, which was defined as bionanocomposite. This immobilized biocatalyst was stable for more than 580 h without activity loss, significantly improving operational stability and mechanical properties over the conventional agarose matrix.

Furthermore, a packed-bed bioreactor for bioprocess scale-up was designed, which was able to produce 370 mg L^{-1} of ribavirin. In conclusion, a smooth, inexpensive and environmentally friendly method to obtain ribavirin was developed in this study.

1. Introduction

Nucleosides are natural compounds which are essential precursors for nucleic acid biosynthesis. Nucleoside analogs have attracted considerable attention due to their broad spectrum of activity. These molecules can be obtained by synthetic or biological modifications of their natural counterparts and used in both antiviral and antitumor therapies [1,2].

Ribavirin is a guanosine analogue currently used as an antiviral compound for the treatment of Hepatitis C virus (HCV) infection in combination with pegylated-interferon α (PEG-IFN α) [3]. In the last years, this analog has become of great interest as a treatment option using telaprevir-based triple therapy in those patients not responding to conventional therapies [4,5]. Recently, ribavirin has been reported to exert antitumor activity in different types of cancer, including leukemia and lymphomas, making this compound relevant not only as an antiviral, but also as an antitumor agent [6]. Nowadays, biocatalytic methods are recognized as an alternative for nucleoside analogue biosynthesis [7]. These molecules can be obtained by a phosphorolytic reaction performed by nucleoside phosphorylases. These enzymes can be classified depending on their substrate specificity into purine NPs (PNPs; EC 2.4.2.1) or pyrimidine NPs (PyNPs; EC 2.4.2.2). NPs catalyze cleavage of N-glycosidic bonds of nucleosides to form a free base and its respective activated pentose moiety, which is then coupled to the desired modified base either by the same or a different NP to give a nucleoside analog [8]. These enzymes can be employed as biocatalysts in their isolated form or as whole cells. The use of whole cells provides one-pot reactions in a natural environment for enzymes and enable the efficient regeneration of cofactors (Scheme 1)[9].

Several ribavirin biosynthesis methods using different kinds of biocatalysts have been summarized by Luo et al., being 76% the best bioconversion value achieved after long reaction times (96 h) using *wild type* whole cells [10]. Although the *wild type* biocatalyst can be easily obtained and cultured, screening processes for detection of microorganisms with active enzymes are required to improve reaction productivity and decrease process costs.

Thermophilic microorganisms represent a novel source of highly active enzymes with attractive features for industrial bioprocesses due to their adaptability and stability under extreme conditions [11,12]. Thermoenzymes from these microorganisms allow to perform reactions at high temperatures, which result in lower medium viscosity, increased substrate diffusion coefficients and fewer microbial contamination risks [13,14]. However, to carry out these bioprocesses under preparative conditions, immobilization procedures are required to enable biocatalyst recovery and reusability. Entrapment techniques are the most widely used for whole-cell immobilization using hydrogels, thermogels and synthetic polymers as matrices. Thermogels are stable matrices having a wide field of application [15]. In particular, agarose is a linear polysaccharide composed of β-D-galactopyranose and anhydro-α-L-galactopyranose that polymerizes through a temperature change.

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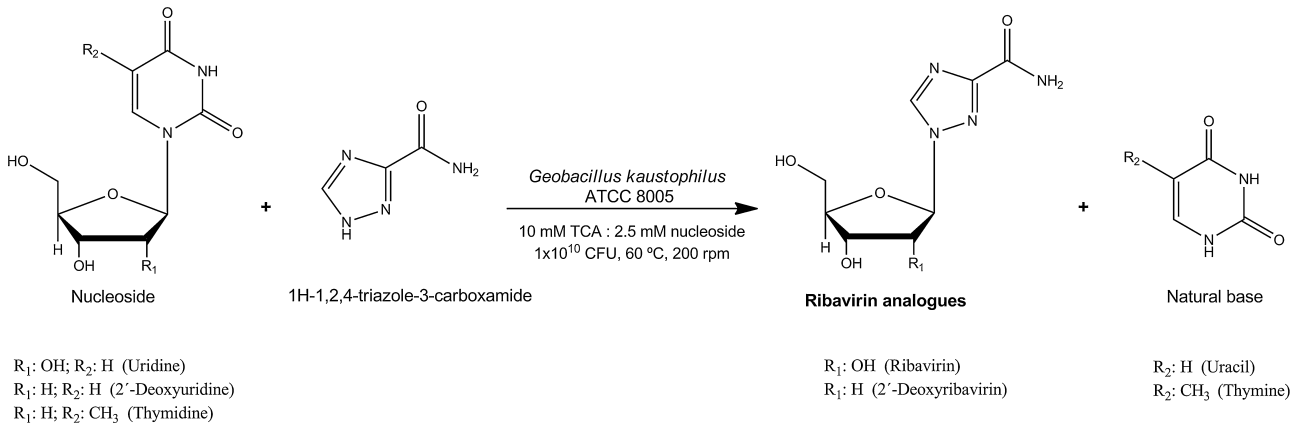

Scheme 1. Biosynthesis of ribavirin analogues using thermophilic microorganisms as biocatalyst. CFU: colony-forming units.

The strength and porous conformation of the matrix are dependent on monomer concentration [16]. Moreover, the addition of nanocomposites such as bentonite, a layered-structure clay which belongs to the smectite group, could improve the mechanical properties of this matrix. The biopolymer-clay bionanocomposites obtained by adding low amounts of bentonite to the agarose matrix are a new class of materials, based on the dispersion of hydrophilic nanoclay particles into the natural polymer matrix [17].

Although ribavirin biosynthesis using a stabilized *wild type* biocatalyst by immobilization in conventional agarose has been previously reported [18], in this work a more stable immobilized biocatalyst was obtained using bionanocomposites, which was able to operate at higher temperatures allowing a 2-fold increase in operational stability.

Therefore, we report here an optimized and environmentally friendly bioprocess for ribavirin biosynthesis using a novel nanostabilized extremophilic biocatalyst.

2. Materials and methods

2.1. Reagents and microorganisms

Nucleosides were purchased from Sigma Chem. Co. (Brazil). 1H-1,2,4-triazol-3-carboxamide (TCA) was purchased from Amfinecom (China). HPLC grade solvents were from Sintorgan S.A (Argentina). Culture medium components were purchased from Britania S.A. (Argentina). Microorganism strains belong to our own laboratory collection.

2.2. Growth conditions

Geobacillus strains were cultured at 55 °C and 200 rpm in media containing 10 g L^{-1} meat peptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl, and 4 g L^{-1} glucose (pH 7.0). *Thermomonospora* and *Streptomyces* strains were cultured at 50 °C and 200 rpm, in media containing 10 g L^{-1} malt extract, 4 g L^{-1} yeast extract and 4 g L^{-1} glucose (pH 7.0).

Microorganisms were harvested by centrifugation at 17,500 × *g* for 10 min, washed once with sodium phosphate buffer (30 mM, pH 7.0) and stored at 4 °C until use.

2.3. Screening

Ribavirin biosynthesis was assayed using 5×10^9 colony-forming units (CFU) in 1 mL sodium phosphate buffer (30 mM, pH 7.0) containing equimolar concentrations of uridine (Urd) and TCA (2.5 mM). Reactions were performed at 60 °C and 200 rpm in a period of 16 h.

2.4. Optimization of reaction parameters

2.4.1. Biocatalyst load

Different microorganism amounts (1×10^9, 5×10^9, 1×10^{10} and 5×10^{10} CFU) were assayed for Urd hydrolysis. Reactions were carried out at different reaction times (1, 3, 6 and 24 h) in 1 mL sodium phosphate buffer (30 mM, pH 7.0) containing 2.5 mM of Urd at 60 °C and 200 rpm.

2.4.2. Microbial growth phase

Ribavirin biosynthesis was performed at 60 °C using 1×10^{10} CFU at different stages of microorganism growth as exponential, stationary and death phase. Reactions were carried out for 6 h in 1 mL of sodium phosphate buffer (30 mM, pH 7.0) containing 2.5 mM Urd and TCA.

2.4.3. Optimal initial molar ratio

For substrate initial molar ratio analysis, reactions were performed at different TCA and Urd ratios as 1:1, 4:1 and 1:4 (where 1 = 2.5 mM and 4 = 10 mM) using 1×10^{10} CFU in 1 mL of sodium phosphate buffer (30 mM, pH 7.0) at 60 °C and 200 rpm for 6 h.

2.5. Biosynthesis of 2'-deoxyribavirin

Biosynthesis of 2'-deoxyribavirin was assayed using previously optimized reaction conditions (2.5 mM nucleoside donor, 10 mM TCA, 1×10^{10} CFU, 60 °C and 200 rpm). Ribose donors tested were thymidine (dThd) and 2'-deoxyuridine (dUrd).

2.6. Stabilization of G. kaustophilus using bionanocomposites

2.6.1. Biocatalyst immobilization

Geobacillus kaustophilus was stabilized by agarose immobilization as previously described [19] and the addition of nanocomposites was assayed to improve the biocatalyst mechanical properties. Tested matrices were agarose 3% (w/v) as control (Ag-Ctrl) and agarose 3% (w/v) with addition of 0.5% (w/v) bentonite (Ag-Bent). The ability of the immobilized biocatalyst to biosynthesize ribavirin was tested for 6 h using optimized conditions.

2.6.2. Immobilized biocatalyst characterization

Bed shape was quantified using the sphericity factor (SF), studying the bead diameter after polymerization. SF was calculated using the following equation:

$$SF = \frac{(d\max - d\min)}{(d\max + d\min)} \quad (1)$$

where $d$max is the largest diameter and $d$min is the smallest diameter, perpendicular to $d$max.

Moreover, gel beads having uniform diameters were placed in an excess of sodium phosphate buffer (30 mM, pH 7.0) at 60 °C for swelling ratio (SR) determination. Changes in bead volume during the course of swelling were monitored by measuring the weight using an analytical balance. SR was determined using the following equation:

$$SR{=}100 \times \frac{(Mt - Md)}{Md} \quad (2)$$

where Mt is the mass of swollen thermogel beads at a given time during swelling and Md is the dry mass of the thermogel. These parameters for matrix characterization were previously described by Cappa et al. [20].

Mechanical stability of immobilized biocatalysts was tested subjecting spheres to extreme agitation conditions (Multimatic 9N, Selecta). Shear stress was measured by mass loss, comparing initial and final weight of the spheres.

$$\text{Sphere mass loss}(\%) = 100 - \frac{(Mf \times 100)}{Mi} \quad (3)$$

where Mi is the initial weight and Mf is the final weight of the sphere.

Surface morphology and quantitative analysis of the chemical composition of Ag-Ctrl and Ag-Bent beads were assessed using a scanning electron microscope provided with energy dispersive X-ray analysis (SEM-EDS) employing a Philips SEM 505 microscope.

2.6.3. Operational stability

Reusability of the stabilized biocatalyst was assayed through successive ribavirin biosynthesis reactions. The catalyst reuse number was determined up to 50% of initial activity loss or matrix integrity loss. Each reuse was performed for 6 h in optimized conditions.

2.7. Scale-up

The packed bed reactor was designed using 2.5-fold the optimized biocatalyst load. Ribavirin biosynthesis was assayed in 10 mL of media containing 2.5 mM Urd and 10 mM TCA, at 60 °C. Constant flow (4 mL min^{-1}) was achieved using a peristaltic pump (Apema PC 26-20-F-D, Argentina).

2.8. Analytical methods

Ribavirin biosynthesis was quantitatively monitored by HPLC (Gilson) at 225 nm (Detector UV/Vis 156, Gilson) using a Phenomenex Luna® C-18 column (5 μm, 4.6 mm, 250 mm). An isocratic mobile phase (100% water) was used with a flow of 1.2 mL min^{-1}, using commercial ribavirin to determine retention time (Supplementary data).

Product identification was performed by MS-HPLC under the above mentioned conditions (Ribavirin; M^+: 245.1; 2'-deoxyribavirin: M^+: 229.1) using a LCQ-DECAXP4 thermo finnigan spectrometer with the electron spray ionization method (ESI) and one ion trap detector.

Table 1
Screening of thermophilic microorganisms for ribavirin biosynthesis.

Genera	Evaluatedstrains	Positivestrains	Ribavirinbiosynthesis[a]
Geobacillus	4	3	++
Streptomyces	5	4	+
Thermomonospora	4	2	+
Total	13	9	

[a] Conversion of 10–40% (+) and conversion higher than 40% (++).

2.9. Environmental parameters

Green chemical parameters of the aforementioned bioprocesses were calculated to demonstrate how bioreactor development increases the mass utilization efficiency.

Environment-factor (E-Factor) is a measurement of the industrial environmental impact. Carbon efficiency (C-Efficiency) and atom economy (A-Economy) are designed as parameters to evaluate the efficiency of synthetic reactions. All the abovementioned parameters were calculated as previously described [21].

3. Results and discussion

3.1. Screening

The ability of extremophiles to produce a wide variety of compounds of industrial interest has been demonstrated in previous reports [22]. Thus, several genera of thermophilic microorganisms were tested for ribavirin biosynthesis. Of the thirteen strains studied, nine were active, three of which belong to the genus *Geobacillus*, four to the genus *Streptomyces* and two to the genus *Thermomonospora*, being *G. kaustophilus* ATCC 8005 the one that proved to have the greatest biosynthetic capacity (Table 1).

This differential behavior can be explained by the different enzymatic selectivity, specificity or variability in the tertiary structure of the enzyme to accept non-natural bases [23].

3.2. Optimization of reaction parameters

3.2.1. Biocatalyst load

Urd hydrolysis was tested using different amounts of *G. kaustophilus* ATCC 8005 to prevent this step from becoming a limiting factor for ribavirin biosynthesis.

Although Urd hydrolysis was detected using 1×10^9 CFU, increasing the number of cells to 1×10^{10} CFU allowed to obtain better bioconversion values at shorter reaction times. When higher microorganisms loads (5×10^{10} CFU) were employed, a slight increase in hydrolysis was observed (Fig. 1). However, due to operational difficulties similar to those previously discussed in other biocatalytic systems [21], 1×10^{10} CFU was selected as the optimal biocatalyst amount for successive assays.

3.2.2. Microbial growth phase

Differential enzymatic expression is associated with nutritional cell requirements at different growth stages. In view of this fact, ribavirin biosynthesis was assayed using the selected microorganism at different growth phases [24].

When *G. kaustophilus* ATCC 8005 was evaluated at exponential (3 h) or death growth phase (16 h), low rates of ribavirin biosynthesis were detected. However, when these biocatalysts reached stationary phase (6–12 h), a significant improvement in reaction bioconversion values was detected, being this effect more marked at early stationary phase (6 h) (Fig. 2).

This behavior may be explained by NP involvement in purine and pyrimidine salvage pathways and their relation with nucleoside analogue biosynthesis. The activation of this recovery pathway

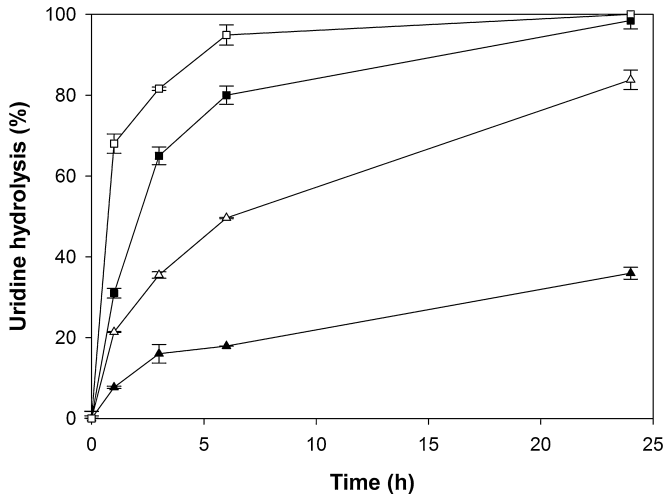

Fig. 1. Hydrolysis of uridine using different microorganism amounts. 5×10^{10} CFU (□), 1×10^{10} CFU (■), 5×10^{9} CFU (Δ), 1×10^{9} CFU (▲). CFU: colony-forming units. All reactions were performed three times, at 60 °C using 2.5 mM Urd, in 1 mL of sodium phosphate buffer (30 mM, pH 7.0). Hydrolysis rates were calculated as: (mmol uracil × 100)/(mmol Urd + mmol uracil).

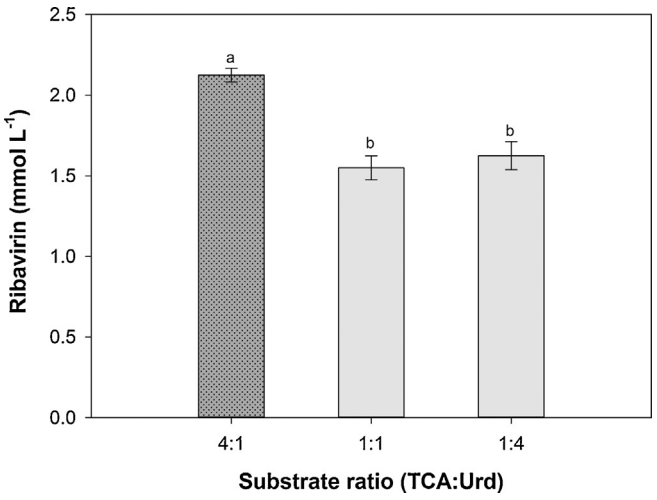

Fig. 3. Biosynthesis of ribavirin at different initial molar ratios. Substrate concentrations are designed as 4 = 10 mM and 1 = 2.5 mM. All reactions were performed three times, at 60 °C using 1×10^{10} CFU in 1 mL of sodium phosphate buffer (30 mM, pH 7.0). Each letter represents a group with significant differences (p-value <0.01). Bioconversion was calculated as: (mmol product/mmol limiting reagent) × 100.

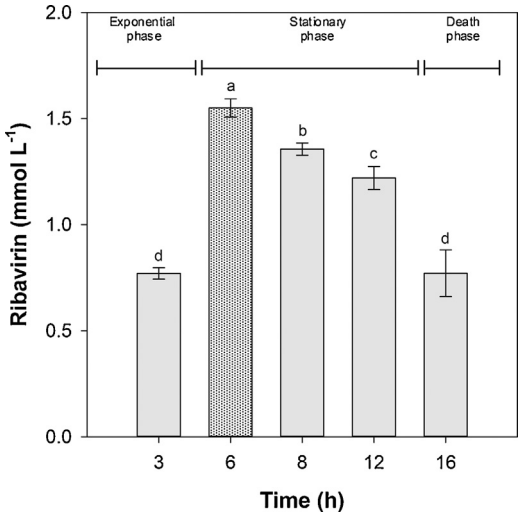

Fig. 2. Biosynthesis of ribavirin at different microorganism growth phases. All reactions were performed three times, at 60 °C using as substrates 2.5 mM TCA and Urd in 1 mL of sodium phosphate buffer (30 mM, pH 7.0). Each letter represents a group with significant differences (p-value <0.01). Bioconversion was calculated as: (mmol product/mmol limiting reagent) × 100.

at different microbial growth phases may be related to NP differential expression [25].

3.2.3. Optimal initial molar ratio

It has been widely reported that transglycosylation reactions are reversible and it has been shown that an excess of nucleoside ribose donor [21] or modified base [15] can shift the equilibrium towards the precursor or the desired product.

Bearing this in mind, reactions were performed using different substrate molar ratios. When an excess of TCA was tested, biosynthesis increased remarkably, obtaining 2.1 mM (84% bioconversion) of ribavirin (Fig. 3). However, no significant differences were observed when an excess of nucleoside donor or equimolar ratio were assayed. The uptake of TCA by NP could be hindered by the chemical and structural properties of the enzyme active site. An excess of the modified base would displace the reaction equilibrium towards product formation [26]. Therefore, a molar ratio of 4:1 (TCA:Urd) was selected as the best condition for subsequent reactions.

The effect of temperature in bioconversion values was tested using previously optimized conditions. Reaction temperatures of 30 and 45 °C were assayed for ribavirin biosynthesis. A marked decrease with respect to 60 °C was observed (data not shown). This behavior could be explained by an enzymatic optimal temperature near to culture conditions of the selected microorganism.

3.3. Biosynthesis of 2'-deoxyribavirin

The ribavirin analogue 2'-deoxyribavirin has shown improved antiviral activity against HCV compared to ribavirin [27]. Accordingly, the biosynthesis of this compound was tested with *G. kaustophilus* ATCC 8005 at the previously optimized reaction conditions using dUrd and dThd as ribose donors, achieving 2'-deoxyribavirin bioconversion values of 1.3 and 1.4 mM respectively. Therefore, dThd could be selected as susbtrate instead of dUrd for a potential scale-up, which will significantly reduce bioprocessing costs.

3.4. Stabilization of G. kaustophilus using bionanocomposites

3.4.1. Biocatalyst immobilization

It is well known that entrapment immobilization techniques improve the biocatalyst operational stability and simplify its recovery from the reaction media, favoring bioprocess scale-up. Microorganism stabilization by entrapment, particularly in agarose, has been successfully assayed by our group, and in some cases, a decrease in bioconversion values with respect to free cells was observed [26,28]. However, in the present work no significant activity loss was detected when *G. kaustophilus* ATCC 8005 was immobilized in agarose (Ag-Ctrl).

Furthermore, we have been able to stabilize this microorganism by entrapment using bionanocomposites (Ag-Bent). When the ability of this novel immobilized biocatalyst to produce ribavirin was assayed, a productivity of 0.27 mM h^{-1} was achieved, being this value slightly lower (about 10% less) compared to 0.31 mM h^{-1} of ribavirin obtained with Ag-Ctrl. However, it should be noted that such decrease is irrelevant in comparison with the significant operative stability achieved using nanostabilized biocatalysts (Section 3.4.3).

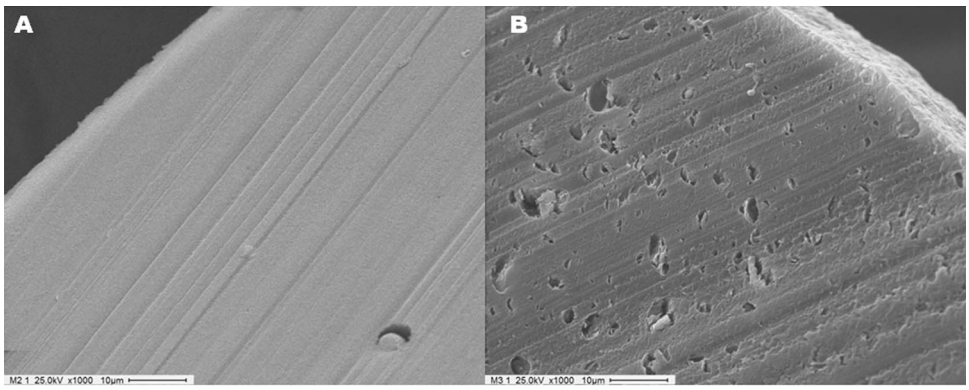

Fig. 4. Representative SEM images of fracture surfaces of (A) Ag-Ctrl and (B) Ag-Bent.

Table 2
Mechanical properties of immobilized biocatalysts.

	Ag-Ctrl	Ag-Bent
Sphericity Factor[a]	NV[b]	NV[b]
Swelling Ratio[a]	17.5	0.3
Shear Stress[a]	35%	<5%

[a] Mechanical properties were calculated as previously described in Section 2.6.2.
[b] NV: No variation was observed.

In this case, the slight reduction in productivity could be related to diffusional limitations due to the presence of the nanoclay, hindering substrate and product exchange between the immobilized microorganism and the reaction medium.

3.4.2. Immobilized biocatalyst characterization

To evaluate the mechanical properties of stabilized biocatalysts, several parameters such as sphericity factor (SF), swelling ratio (SR) and shear stress were assayed (Table 2). SF varies from zero to one to define a perfect or an elongated sphere, respectively [29]. Spherical and regular shaped beads were obtained by thermal gelation, being the average diameter 3 mm and the SF of about 0.005. Therefore, this entrapment technique allowed to obtain regular spheres and no significant differences were observed between spheres obtained using bionanocomposites or the control matrix.

SR is related to osmotic swelling, where water surrounding the sphere tends to get into the matrix [30]. Thus, modifications in the structure of the obtained biocatalysts were tested at 60 °C. Ag-Ctrl showed a marked increase in mass after incubation. However, no perceptible swelling was observed for Ag-Bent, showing the positive effect of bentonite addition on matrix stability.

The degradation of the matrix due to weathering processes was assayed for potential industrial application, by subjecting the spheres to extreme shear stress conditions. Biocatalyst disintegration was observed for Ag-Ctrl while Ag-Bent retained its integrity without significant mass loss. This behavior led us to select the Ag-Bent matrix as a better option for biocatalyst stabilization with the aim of applying it in further bioprocess scale-up.

Additionally, SEM images of fracture surfaces of stabilized biocatalysts are shown (Fig. 4). An increase in surface roughness without debris particles was observed in Ag-Bent, suggesting a strong bonding between agarose and bentonite [31]. This type of interaction could be related to the enhanced mechanical stability achieved. Moreover, the EDS spectra confirmed the presence of bentonite in the matrix by the identification of silicon and aluminum atoms belonging to these clays (data not shown).

3.4.3. Operational stability

Nanoparticle addition to conventional supports has proven to reinforce matrix stability and enhance its mechanical properties [31]. In this work, biocatalytic activity retention and matrix stability was tested for Ag-Ctrl and Ag-Bent in successive biosynthetic reactions.

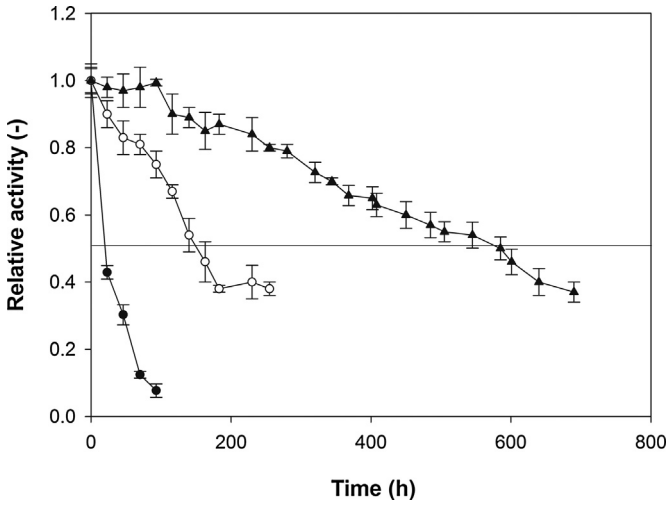

Fig. 5. Reusability of different biocatalysts: Free cells (●), Ag-Ctrl (○) and Ag-Bent (▲). Reactions were performed three times at 60 °C using 10 mM TCA, 2.5 mM Urd in sodium potassium buffer (30 mM, pH 7.0). Bioconversion was calculated as: (mmol product/mmol limiting reagent) × 100.

When the reusability of free cells was studied, a marked decrease in their biosynthetic activity was observed after 20 h, while immobilized cells in a conventional matrix (Ag-Ctrl) remained active for 150 h under operational conditions. In addition, the biocatalyst immobilized using bionanocomposites (Ag-Bent) retained its activity for more than 580 h, almost 4 fold compared to Ag-Ctrl. The marked decrease in operational stability shown by Ag-Ctrl may be explained as a result of matrix integrity loss and subsequent biocatalyst exposure to reaction media. Moreover, the improvements in the mechanical stability of the conventional matrix supplemented with bentonite allowed to maintain a steady microenvironment for the microorganisms, favoring retention of biocatalytic activity. This significant stability increase could favor subsequent bioprocess scale-up (Fig. 5).

In Table 3, the operational stability of different immobilized biocatalysts able to biosynthesize ribavirin was compared. A marked increase in operative hours without activity loss of the nanostabilized biocatalyst was observed. Therefore, the biocatalysts developed in this work could provide an inexpensive method for ribavirin biosynthesis, avoiding the use of recombinant enzymes or purification techniques.

Table 3
Biosynthesis of ribavirin using different immobilized whole cells.

Immobilized biocatalysts	Substrates	Temperature (°C)	Matrix	Operative hours(h)	References
G. kaustophilus	Urd/TCA	60	Bionanocomposite[b]	580	This study
E. coli[a]	Guo/TCA	60	Agar	40	[10]
E. coli	Urd/TCA	30	Agarose	270	[18]
E. coli	Urd/TCA	30	Polyacrylamide	81	[18]
A. hydrophila	Urd/TCA	60	Agarose	78	[32]

[a] Recombinant microorganism was used.
[b] Bionanocomposite (Ag-Bent).

3.5. Scale-up

For ribavirin biosynthesis scale-up, a packed-bed bioreactor was developed and, noteworthy ribavirin bioproduction was not affected with respect to micro-scale assays. The reaction was carried out at 60 °C and 370 mg L^{-1} of ribavirin were obtained. This kind of reactors are convenient because they reduce shear stress on the immobilized biocatalyst [18]. Moreover, it is remarkable that thermophilic enzymes are capable of retaining their activity under harsh processing conditions such as high temperatures, allowing the development of bioprocesses in which contamination risks are minimized and solubility of specific substrates is favored. Therefore, the described packed-bed bioreactor could be used in a continuous bioprocess for 580 h, with no activity loss.

Furthermore, green parameters were tested to study the environmental impact and the efficiency of the reaction. Generally, E-factor values of pharmaceutical compound synthesis are around 25-100. Using the bioreactor developed for ribavirin production, this value was lower than 4, suggesting mass utilization efficiency and a reduction in waste production. In addition, the C-Efficiency and A-Economy values were 66.7% and 68.5% respectively, which reflects a positive effect on atom recovery and process efficiency [33].

4. Conclusions

G. kaustophilus ATCC 8005 strain proved to be effective for ribavirin biosynthesis. After optimization of the reaction parameters, this microorganism was stabilized by entrapment in bionanocomposites. The addition of bentonite to the conventional agarose matrix significantly improved biocatalyst stability and reusability, which allowed to develop a continuous bioprocess using a packed-bed bioreactor producing 370 mg L^{-1} of the desired compound.

In this work, ribavirin, an anti-HCV drug, was biosynthesized using a thermophilic nanostabilized biocatalyst in a green, scalable bioprocess.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2015.08.006.

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