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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.

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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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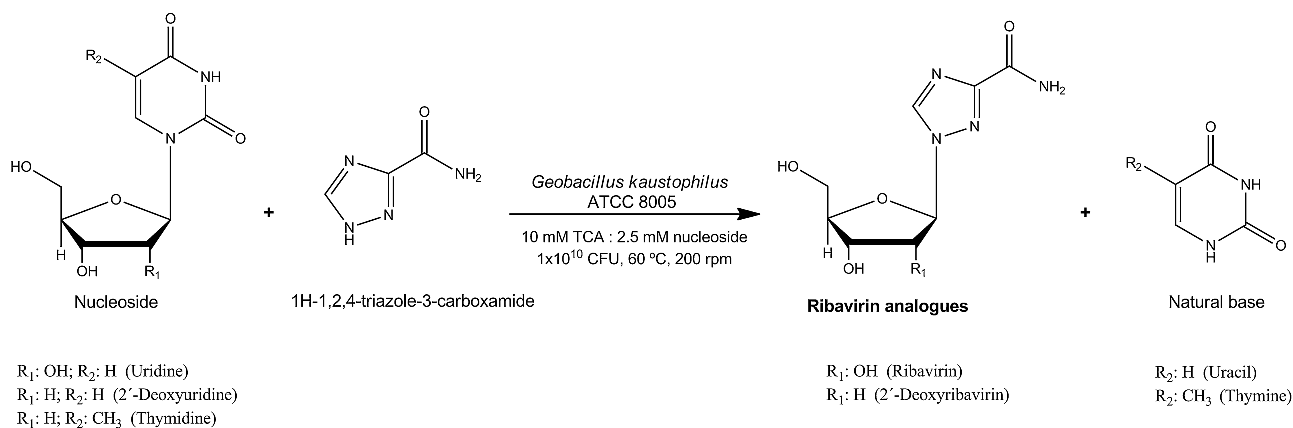

Scheme 1. Biosynthesis of ribavirin analogues using thermophilic microorganisms as biocatalyst. CFU: colony-forming units.

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.

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