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Biotransformation of 5´-O-β-D-galactosyl-floxuridine by immobilized β-galactosidase from *Kocuria rhizophila*

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ABSTRACT

5-fluorouracil-2’-deoxyriboside (FUdR), an antimetabolite known as floxuridine, is a halogenated nucleoside extensively used in the clinical treatment of colon carcinoma and hepatic metastases. This drug presents low bioavailability, thus requiring large doses and frequent administration, which leads to long-lasting and severe side effects in clinical practice. In order to overcome this problem, galactosylated nucleoside analogues were obtained using immobilized β-galactosidase in Ca-alginate with yields of 80% at only 7 h. Additionally, the obtained biocatalyst was stable for 6 months in storage conditions (4 °C) and could be reused at least 16 times without loss of its activity at 30 °C.

This work describes for the first time an efficient, eco-compatible and simple bioprocess for obtaining 5´-O-β-D-galactosyl-floxuridine using an immobilized biocatalyst.

1. Introduction

Nucleoside analogues are synthetic compounds that have been developed to mimic the physiological functions of their natural versions in order to interfere with the cellular metabolism and subsequently be incorporated into the DNA and RNA to inhibit cell division or/and viral replication [1]. These compounds have played an important role in anticancer and antiviral therapies for the last ten years. 5-fluorouracil-2’-deoxyriboside (FUdR), a halogenated pyrimidinic nucleoside, has been extensively used in the clinical treatment of colon carcinoma and hepatic metastases, and has also shown antitumoral activity in breast, head and neck cancers [2,3]. However, FUdR has some disadvantages such as low oral bioavailability and selectivity that lead to severe side effects in clinical practice [4]. It has been reported that the glycosylation of nucleosides masks their toxicity and improves their cell absorption [5]. For instance, Abraham et al. have shown that FUdR is a hundred times more toxic to bone marrow cells in Balb/c mice than 5´-O-β-D-galactosyl-5-fluorouridine (5-Gal-FUdR) [6]. Nucleoside drugs and their derivatives are mainly synthesized by chemical methods that involve arduous and tedious protection/deprotection steps causing unwanted accumulation of racemic mixtures that affect further purification; for these cases, biosynthesis is a promising alternative [7]. Biocatalysis has several advantages over chemical synthesis due to the general properties of enzymes, which are active under mild reaction conditions and display chemical enantio- and regioselectivity [1]. However, there are few publications on enzymatic glycosylation of nucleosides and in all these cases soluble enzymes are used, which hinders the product purification and does not allow its reuse [8,9].

Enzyme immobilization has received considerable attention in recent decades due to the fact that enzyme activity and stability can be improved by this technique [10]. Entrapment methods are widely used for enzyme immobilization; these techniques are based on the inclusion of the enzyme within a rigid network to prevent their release into the surrounding medium, while still allowing mass transfer [11]. The most commonly used supports for this methodology are agar, agarose, alginate, β-carrageenan, and polyacrylamide [12–16]. Alginate is a naturally occurring anionic polymer typically obtained from brown seaweed that has been extensively used in biotechnology, medicine and pharmaceutical industry due to its biocompatibility, low toxicity, and low cost [17].

The aim of this work was to biosynthesize, for the first time, 5-Gal-FUdR using a stabilized biocatalyst based on an immobilized β-galactosidase (3.2.1.23) from *Kocuria rhizophila* (Scheme 1).

2. Material and methods

2.1. Screening for β-galactosidase activity

Seventy bacterial strains from LIBioS collection were screened for

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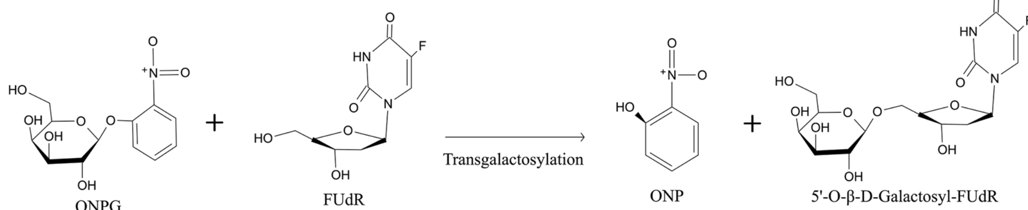

Scheme 1. Enzymatic galactosylation of FUdR.

extracellular β-galactosidase activity. Bacterial cultures were grown for 16 h at optimum conditions for each genus. β-galactosidase hydrolytic activity was determined by measuring of o-nitrophenol release from ortho-nitrophenyl-β-galactoside (ONPG) at 415 nm. The reaction conditions were ONPG 1 mM, NaCl 1 mM, and tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) 25 mM, pH 7 at 30 °C. One unit of enzyme activity (U) was defined as the quantity of enzyme that will liberate 1 μmol o-nitrophenol per min under assay conditions.

2.2. β-galactosidase purification

Kocuria rhizophila was grown in an optimized culture medium, until saturation, and the supernatant was ultrafiltered up to 100× volume concentration. The enzyme was characterized by electrophoresis. Native gradient gel electrophoresis according to the modified method of Laemmli was used to estimate the molecular weight of the protein by the detection of the enzyme activity in the gel [18].

2.3. Enzyme stabilization

According to Tanash et al. [19], different concentrations of β-galactosidase (50, 100, 200, 300, 600 μg/mL) solution were mixed with sodium alginate to final concentrations of 2%. The entrapment was carried out by dropping alginate solution in 0.1 M $CaCl_2$. Afterwards, the same assay was performed with different sodium alginate concentrations with the purpose of optimizing β-galactosidase retention in the matrix.

2.4. Optimization of FUdR galactosylation

Different reaction parameters such as pH (4, 5, 6, 7 and 8), temperature (20, 30, 45, 60 °C) and cation addition (Mg^{2+} and Na^{+}) were analyzed. Besides, the ONPG/FUdR molar ratio (1:1, 1:2 and 2:1 mM) was studied for FUdR galactosylation in 1 mL of reaction medium for 12 h. Finally, a kinetic of the reaction was evaluated at different times (1, 2, 3, 4, 5, 7 and 12 h).

2.5. Biocatalyst stability

Storage stability was tested at two temperatures (4 °C and 30 °C) and was defined as the relative activity of FUdR galactosylation between the first and the successive reactions using the same biocatalyst. Reusability of immobilized enzyme in Ca-alginate was evaluated using 5’-Gal-FUdR synthesis as standard reaction. The used beads were washed three times using buffer Tris-HCl pH 7 at the end of each cycle.

2.6. Analytical methods

FUdR galactosylation quantitative analysis was performed by HPLC (Gilson) at 254 nm (Detector UV/Vis 156, Gilson) with an Agilent Zorbax Eclipse XDB C-18 column (5 μm, 150 mm × 5 mm). The mobile phase consisted of a gradient elution (1.4 mL/min) with water/methanol (96/4, v/v) from 0 to 6.5 min, and then water/methanol (80/20, v/v) from 6.5 min was used. The retention times for 5-Gal-FUdR, FUdR and ONPG were 2.0, 5.4, and 12.0 min, respectively. The 5-Gal-FUdR was separated and purified by using a UHPLC equipped with UV–vis detector, automatic injector and fraction collector Thermo Scientific Dionex Ultimate 3000 Series (ThermoScientific®). Product identification was conducted by MS-HPLC under the above-mentioned conditions (5-Gal-FUdR; M+: 407) using a LCQ-DECAXP4 Thermo/Finnigan spectrometer with the electron spray ionization method (ESI) and one ion trap detector.

2.7. Statistical analysis

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

3. Results

3.1. Selection of strains with β-galactosidase activity

Seventy strains from several bacterial genera such as *Thermonospora, Brevibacterium, Chromobacterium, Citrobacter, Lactobacillus* and *Kocuria* were screened for β-galactosidase activity using batch fermentation. Among all evaluated strains, 14 strains that were able to hydrolyze ONPG were selected (Table 1). *Kocuria rhizophila* showed higher activity (0.15 U/mL) than all the other strains and was chosen for subsequent assays. The culture medium was optimized and β-galactosidase expression and activity were improved significantly, leading to an increase of 50% in enzyme activity.

3.2. β-galactosidase molecular weight

Enzyme characterization was carried out by electrophoretic techniques. By using a stained native electrophoresis, it could be confirmed that it was a lactase with demonstrated activity and their molecular weight was around 310 kDa (Fig. 1).

Table 1
Screening of strains for β-galactosidase activity.

Negative		Positive[a]	
Genus	Strains tested	Genus	Strains tested
Aeromonas	4	*Bacillus*	3
Arthrobacter	2	*Brevibacterium*	1
Bacillus	9	*Chromobacterium*	1
Enterobacter	3	*Citrobacter*	3
Enterococcus	1	*Kocuria*	1
Erwinia	3	*Lactobacillus*	2
Klebsiella	3	*Thermonospora*	1
Lactobacillus	7	*Xanthomonas*	2
Proteus	3		
Pseudomonas	3		
Serratia	2		
Streptomyces	15		
Xanthomonas	1		

[a] The positive response is expressed regarding ONPG hydrolysis.

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