Green biosynthesis of floxuridine by immobilized microorganisms

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Abstract

This work describes an efficient, simple, and green bioprocess for obtaining 5-halogenated pyrimidine nucleosides from thymidine by transglycosylation using whole cells. Biosynthesis of 5-fluoro-2′-deoxyuridine (floxuridine) was achieved by free and immobilized Aeromonas salmonicida ATCC 27013 with an 80% and 65% conversion occurring in 1 h, respectively. The immobilized bio-catalyst was stable for more than 4 months in storage conditions (4 °C) and could be reused at least 30 times without loss of its activity. This microorganism was able to biosynthesize 2.0 mg L⁻¹ min⁻¹ (60%) of 5-chloro-2′-deoxyuridine in 3 h. These halogenated pyrimidine 2′-deoxynucleosides are used as antitumoral agents.

Introduction

Nucleosides have been considered of great interest because they have shown activity against various cancer cell lines in vitro and in vivo. Nucleosides and their analogues are implicated in the modulation of several signal transduction pathways causing growth inhibition, differentiation, apoptosis, and modulation of gene expression through different mechanisms of action (Wang et al., 2004; Rossi et al., 2009; Li et al., 2010). Therefore, nucleoside analogues can be used as powerful antitumoral agents. Halogenated derivatives are widely recognized today as an effective cancer treatment. The efficacy of fluorinated derivatives for the treatment of several cancer modalities is well known (Cantero et al., 2006; Bronckaers et al., 2008). Floxuridine or 5-fluoro-2′-deoxyuridine has shown activity in patients with colorectal, pancreatic, breast, head, and neck cancers (Liu et al., 2008). Many studies have demonstrated that 5-chloro-2′-deoxyuridine is useful in cancer treatment (Morris, 1993). Moreover, 5-fluoro-2′-deoxyuridine and 5-chloro-2′-deoxyuridine have been useful as substrates to design new prodrugs (Johar et al., 2005; Park et al., 2009).

Biocatalysis is frequently recognized as superior to conventional chemical methods in selective modification of polyfunctional substrates owing to high catalytic efficiency, inherent selectivity, and simple downstream processing. In addition, biotransformations take place under very mild conditions and offer environmentally clean technologies (Qian et al., 2008).

Transglycosylation is catalyzed by nucleoside phosphorylases. These enzymes catalyze reversible phosphorolytic cleavage of N-glycosidic bonds of nucleosides without addition of ATP, to form a free base and its respective activated pentose moiety, which is then coupled to the desired modified base to give a nucleoside analogue (only β-anomer; Bzowska et al., 2000).

Halogenation is usually applied to organic structures in order to confer or enhance antitumoral activity. Halogenation of nucleosides can be performed on the sugar
moiety as well as on the base moiety. Previous reports had described satisfactory halogenations on the sugar moiety mediated by fluorinase and chlorinase (O’Hagan et al., 2002; Eustaquio et al., 2008). However, these enzymes cannot introduce the halogen into the base moiety.

Biosynthesis of purine nucleoside analogues by transglycosylation has been extensively studied (Sinisterra et al., 2010). However, there have been few reports about obtaining pyrimidine nucleosides halogenated on the base moiety using whole cells. In all cases, conversion rates were <50% (Pal & Nair, 1997).

Microorganism immobilization is a good way to carry out the bioprocess under preparative conditions. Cell entrapment techniques are the most widely used for whole cell immobilization (Trelles et al., 2004). The main advantages of this methodology are high operational stability, easy upstream separation, and bioprocess scale-up feasibility.

The aim of this study was to obtain 5-halogenated 2'-deoxynucleosides with potential antitumoral activity using a smooth, cheap, and environmentally friendly methodology. We have been able to develop a bioprocess for 5-fluoro and 5-chloro-2'-deoxyuridine production using immobilized Aeromonas salmonicida ATCC 27013.

**Materials and methods**

**Materials**

Nucleosides and nucleobases were purchased from Sigma Chem. Co. (Brazil). Culture media compounds were obtained from Britania S.A. (Argentine). Chemicals were from Sigma Chem. Co. and Britania S.A. HPLC grade solvents used were from Sintorgan S.A. (Argentine). Most of the microorganisms were kindly supplied by the ‘Colección Española de Cultivos Tipo (CECT)’, Universidad de Valencia (Spain).

**Growth conditions**

Microorganisms were grown until stationary phase in LB medium (5 g L⁻¹ meat extract, 10 g L⁻¹ peptone, and 5 g L⁻¹ NaCl in deionized water adjusted to pH 7). Cells were harvested by centrifugation for 10 min at 17 500 g, were then washed once with potassium phosphate buffer (30 mM, pH 7), and finally recentrifuged and stored at 4 °C until use.

**Screening**

A taxonomic screening with bacterial strains was performed using the following genera: Aeromonas (10), Bacillus (8), Citrobacter (3), Chromobacterium (1), Enterobacter (6), Escherichia (7), Klebsiella (2), Micrococcus (3), Serratia (4), Proteus (7), and Xanthomonas (4). All microorganisms assayed were non pathogenic for humans.

The reaction to select the microorganisms was performed with 1 × 10¹⁰ CFU, 10 mM 5-fluorouracil and 2.5 mM thymidine or uridine in 1 mL of potassium phosphate buffer (30 mM, pH 7). Reactions were performed at 30 °C and 200 r.p.m. Samples were taken at 1, 3, 6, and 24 h and centrifuged at 17 500 g during 5 min.

**Optimization of 5-fluorinated 2'-deoxynucleoside biosynthesis**

**Effect of phosphate concentration, pH, and shaking speed**

Reactions were performed at 30 °C with 1 × 10¹⁰ CFU, 2.5 mM 5-fluorouracil and 10 mM thymidine at different phosphate concentration (20–40 mM), pH values (6–8), and shaking speed (100–300 r.p.m.).

**Effect of temperature**

Reactions were carried out with 1 × 10¹⁰ CFU, 2.5 mM 5-fluorouracil and 10 mM thymidine, potassium phosphate buffer (30 mM, pH 7) at two different temperatures (30 and 60 °C) and 200 r.p.m.

**Effect of sugar donors**

Different nucleosides were assayed at 30 °C and pH 7. Reaction mixtures contained 1 × 10¹⁰ CFU, 2.5 mM 5-fluorouracil and 10 mM uridine, thymidine, 2'-deoxyuridine, 2'-deoxycytidine or 2',3'-dideoxyuridine.

**Effect of initial molar ratio (base/nucleoside)**

Reactions were performed at different 5-fluorouracil and thymidine ratios (1 : 1, 4 : 1 and 1 : 4) at 30 °C, pH 7, and 200 r.p.m.

All assays were performed three times in 1 mL of reaction medium.

**Biosynthesis of other 5-halogenated 2'-deoxynucleosides**

Subsequent to system characterization with 5-fluorouracil, the incorporation of other halogenated pyrimidine bases was tested using 5-chlorouracil and 5-bromouracil. Reactions were performed in a 1 : 4 ratio (2.5 mM
halogenated base and 10 mM thymidine) in potassium phosphate buffer (30 mM, pH 7) at 30 °C.

**Microorganism immobilization**

**Entrapment in agar or agarose**

1 × 10¹⁰ CFU were mixed with 3 mL of 1, 2, and 3% (w/v) agar or agarose. The mixture was then added dropwise to stirred sunflower oil (20 mL) at 25 °C. The resulting gel beads were cooled, filtered, washed with hexane and then with physiological solution to obtain solvent-free beads.

**Entrapment in polyacrylamide**

1 × 10¹⁰ CFU were mixed with 3 mL of phosphate buffer (30 mM, pH 7) containing 15, 20, and 25% (w/v) acrylamide/bis-acrylamide, subsequently 50 μL of 10% (w/v) ammonium persulfate (APS) and 14 μL of N,N,N',N'-tetramethylethylenediamine (TEMED). The resulting gel was cut into small cubic pieces (1.0 × 1.0 × 0.2 cm).

**Analytical methods**

The biosynthesis of nucleoside analogues was qualitatively evaluated by TLC Merck Silica gel 60 F254 in chloroform/methanol (80 : 20, v/v) as mobile phase. The quantitative analysis was performed by HPLC (Gilson) equipped with a UV detector (254 nm) using a Nucleodur 100-5 C18 column (5 μm, 125 × 5 mm). The isocratic mobile phase used was water/methanol (95 : 5, v/v) at room temperature and at a flow rate of 1.2 mL min⁻¹.

Retention times of substrates and products were as follows:

- **Floxuridine biosynthesis:** (1) uracil (1.0 min), 5-fluorouracil (1.4 min), 2′-deoxyuridine (2.0 min), floxuridine (3.0 min); (2) cytosine (1.1 min), 5-fluorouracil (1.4 min), 2′-deoxyctydine (2.2 min), floxuridine (3.0 min); (3) 5-fluorouracil (1.4 min), thymine (2.6 min), floxuridine (3.0 min), thymidine (4.2 min).
- **5-fluorouridine biosynthesis:** uracil (1.0 min), 5-fluorouracil (1.4 min), uridine (1.8 min), 5-fluorouridine (2.8 min).
- **5-chloro-2′-deoxyuridine biosynthesis:** (1) uracil (1.0 min), 2′-deoxyuridine (2.0 min), 5-chlorouracil (4.8 min), 5-chloro-2′-deoxyuridine (6.0 min); (2) cytosine (1.1 min), 2′-deoxyctydine (2.2 min), 5-chlorouracil (4.8 min), 5-chloro-2′-deoxyuridine (6.0 min); (3) thymine (2.6 min), thymidine (4.2 min), 5-chlorouracil (4.8 min), 5-chloro-2′-deoxyuridine (6.0 min).

Product identification was performed by MS-HPLC (See Supporting Information, Data S1).

### Results and discussion

**Screening**

5-fluoro-2′-deoxyuridine biosynthesis from thymidine and 5-fluorouracil was used as reaction test for the screening (Fig. 1). Of all the strains tested (55), only *Aeromonas salmonicida*, *Aeromonas hydrophila*, *Enterobacter cloacae*, *Bacillus subtilis* and *Bacillus Stearothermophilus* were active. However, *A. salmonicida* ATCC 27013 and *A. hydrophila* ATCC 13136 were the ones showing the highest activity, the former exhibiting the best performance (Trelles *et al.*, 2011).

**Optimization of 5-fluorinated 2′-deoxynucleoside biosynthesis**

**Effect of phosphate concentration, pH, and shaking speed**

The importance of the presence of phosphate in the reaction for nucleoside phosphorolysis by pyrimidine nucleoside phosphorylase (PyNP) had been previously reported (Utagawa, 1999). Preliminary tests were performed to optimize different phosphate concentrations, pH values, and stirring speed. The results obtained were not significantly different (data not shown). Therefore, we continued using 30 mM potassium phosphate buffer at pH 7 and 200 r.p.m. as standard conditions.

**Effect of temperature**

PyNP enzyme (EC 2.4.2.2), which is responsible for transglycosylation reaction, remains active at 60 °C (Trelles *et al.*, 2005). Biosynthesis was performed at two temperatures (30 and 60 °C) using thymidine and 5-fluorouracil to evaluate the effect of other enzymes on substrates and products.

When the reaction was carried out at 60 °C, 1.5 mM of 5-fluoro-2′-deoxyuridine were obtained in 1 h in the presence of secondary products, which could be due to the effect of enzymes called dehalogenases that have been found in some mesophylic microorganisms, whose optimum temperature is between 45 and 60 °C (Liu *et al.*, 1994). When the reaction temperature was 30 °C, 2.0 mM of 5-fluoro-2′-deoxyuridine were gained in 1 h without secondary products, while at 3 h, the amount of 5-fluoro-2′-deoxyuridine was not significantly modified (2.1 mM; Fig. 2). The highest conversion for floxuridine biosynthesis was achieved at 30 °C.

**Effect of sugar donors**

Biosynthesis of 5-fluorouridine, 2′-deoxyuridine, and 2′,3′-dideoxyuridine counterpart by *A. salmonicida* ATCC 27013 was not significantly modified (2.1 mM; Fig. 2). The highest conversion for floxuridine biosynthesis was achieved at 30 °C.
27013 was evaluated using different nucleosides as sugar donors. These assays were performed at 30 °C and pH 7 with excess of thymidine, uridine, 2′-deoxyuridine, 2′,3′-dideoxyuridine and 2′-deoxycytidine to prevent the reaction from being limited by the production of ribose, 2′-deoxy- or 2′,3′-dideoxyribose-1-phosphate (depending on the nucleoside donor used).

*Aeromonas salmonicida* ATCC 27013 showed activity on uridine, thymidine, 2′-deoxyuridine and 2′-deoxycytidine. When 2′,3′-dideoxyuridine was assayed, no phosphorolytic activity was detected under the conditions tested. This microorganism was able to produce 1.0 mM (40%) of 5-fluorouridine when uridine was used. Biosynthesis of 5-fluorouridine with an excess of 5-fluorouracil, an excess of thymidine, or equal-molar quantities. Conversion was 38% in 1 h when 1 : 1 molar ratio was evaluated. Using 4 : 1 molar ratio (base / nucleoside), 5-fluoro-2′-deoxyuridine production was 52% after 1 h. When an excess of thymidine (1 : 4) was used, conversion was 80% (1 h) and productivity was 0.64-fold with respect to the reaction with modified base excess (Table 2).

### Table 1. Biosynthesis of 5-fluorinated nucleosides using different sugar donors. Reactions were carried out in 1 mL at 30 °C and pH 7. Reaction mixtures contained 1 × 10^10 CFU, 2.5 mM 5-fluorouracil, and 10 mM starting nucleoside.

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Reaction time* (h)</th>
<th>5-Fluorinated nucleosides (mM)</th>
<th>Conversion† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uridine</td>
<td>1</td>
<td>1.0</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.5</td>
<td>59</td>
</tr>
<tr>
<td>Thymidine</td>
<td>1</td>
<td>2.0</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.1</td>
<td>84</td>
</tr>
<tr>
<td>2′-deoxyuridine</td>
<td>1</td>
<td>2.0</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.1</td>
<td>84</td>
</tr>
<tr>
<td>2′-deoxycytidine</td>
<td>1</td>
<td>1.9</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.0</td>
<td>80</td>
</tr>
<tr>
<td>2′,3′-dideoxyuridine</td>
<td>1</td>
<td>0.0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.0</td>
<td>–</td>
</tr>
</tbody>
</table>

*The best times of reaction are shown.
†Conversion (%) = \( \frac{\text{mmol product}}{\text{mmol limiting reagent}} \times 100.\)

### Effect of initial molar ratio (base/2′-deoxynucleoside)

It has been widely reported that transglycosylation reactions are reversible (Pugmire & Ealich, 2002). For this reason, the initial rate of reaction was analyzed. It was shown that any excess of substrates improves transglycosylation. Trials were conducted to obtain 5-fluoro-2′-deoxyuridine with an excess of 5-fluorouracil, an excess of thymidine, or equal-molar quantities. Conversion was 38% in 1 h when 1 : 1 molar ratio was evaluated. Using 4 : 1 molar ratio (base / nucleoside), 5-fluoro-2′-deoxyuridine production was 52% after 1 h. When an excess of thymidine (1 : 4) was used, conversion was 80% (1 h) and productivity was 0.64-fold with respect to the reaction with modified base excess (Table 2).
Table 2. Biosynthesis of floxuridine using different initial molar ratios of substrates. Reactions were performed with 1 x 10^10 CFU at 30 °C, pH 7, and 200 r.p.m.

<table>
<thead>
<tr>
<th>Ratio* (5F/Thy)</th>
<th>Conversion† (%)</th>
<th>Productivity‡ (mg L⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 : 1</td>
<td>38</td>
<td>3.9</td>
</tr>
<tr>
<td>1 : 4</td>
<td>80</td>
<td>8.2</td>
</tr>
<tr>
<td>4 : 1</td>
<td>52</td>
<td>5.3</td>
</tr>
</tbody>
</table>

*5F = 5-fluorouracil and Thy = thymidine. 1 = 2.5 mM and 4 = 10 mM.
†Conversion is calculated relative to the limiting reagent concentration.
‡Productivity was evaluated at the best time of reaction (1 h).

Biosynthesis of other 5-halogenated 2′-deoxynucleosides

According to the conversions obtained for 5-fluoro-2′-deoxynucleoside biosynthesis, the specificity of *A. salmonicida* ATCC 27013 to accept other halogenated pyrimidine bases was evaluated.

Conversion was approximately 60% (3 h) in 5-chloro-2′-deoxynucleoside biosynthesis using 2′-deoxynucleoside, 2′-deoxyctydine, and thymidine as sugar donors (Table 3). Under the conditions tested, *A. salmonicida* ATCC 27013 accepted 5-chlorouracil but retained only residual activity (<10%) when 5-bromouracil was used.

Productivity of *A. salmonicida* was lower when 5-chlorouracil instead of 5-fluorouracil was assayed (5.4 and 8.2 mg L⁻¹ min⁻¹, respectively). Therefore, it can be postulated that steric hindrance because of the difference in atomic radii of halogens can probably reduce reaction conversion.

Microorganism immobilization

*Aeromonas salmonicida* ATCC 27013 was immobilized in agar, agarose, and polyacrylamide as previously optimized by Trelles and col. (Trelles et al., 2004). The minimum matrix percentage for preventing undesirable microorganism release into the reaction medium was assessed, being 3% and 25% the optimal percentage for agarose and polyacrylamide, respectively.

Immobilized microorganisms were assayed in floxuridine biosynthesis. Conversion values within 1 h of reaction were slightly lower than those obtained with free microorganisms (60% and 65% using polyacrylamide and agarose, respectively). It is well known that this difference is related to diffusion restrictions of these matrices.

Immobilization increases the biocatalyst stability. In this case, *A. salmonicida* ATCC 27013 was stable at 4 °C for more than 4 months without losing activity (about 90% retained activity). Besides, this immobilized biocatalyst could be used at least for 30 consecutive reactions (about 90% retained activity). Free microorganisms were stable at 4 °C for only 1 week and could not be reused for more than 10 times.

Agarose was selected to perform the preliminary test for bioprocess scale-up. These trials were conducted in a 10 mL batch reactor and results were similar to those obtained at microscale (1 mL).

Conclusion

In this report, an efficient one-pot bioprocess is described for the production of 5-fluoro- and 5-chloro-2′-deoxynucleoside by transglycosylation using immobilized *A. salmonicida* 27013 as biocatalysts.

This biocatalyst meets the requirements of high activity, stability, and short reaction times needed for low-cost production in a future preparative application.

Acknowledgements

This research was supported by Agencia Nacional de Promoción Científica y Tecnológica and Universidad Nacional de Quilmes. M.E.L. and J.A.T. are research members at CONICET; C.W.R. is a CONICET fellow, Argentina. Maria Luján Cuestas is also gratefully acknowledged for her kind participation in the scientific revision of this manuscript. We appreciated Valeria Cappa’s collaboration in some experimental works.

References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Data S1.** Identification of substrates and products by MS-HPLC.

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