RESEARCH LETTER

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 biocatalyst 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 1×10^{10} 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 1×10^{10} 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 1×10^{10} 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 1×10^{10} 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

1×10^{10} 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

1×10^{10} 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 F_{254} 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′-deoxycytidine (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′-deoxycytidine (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

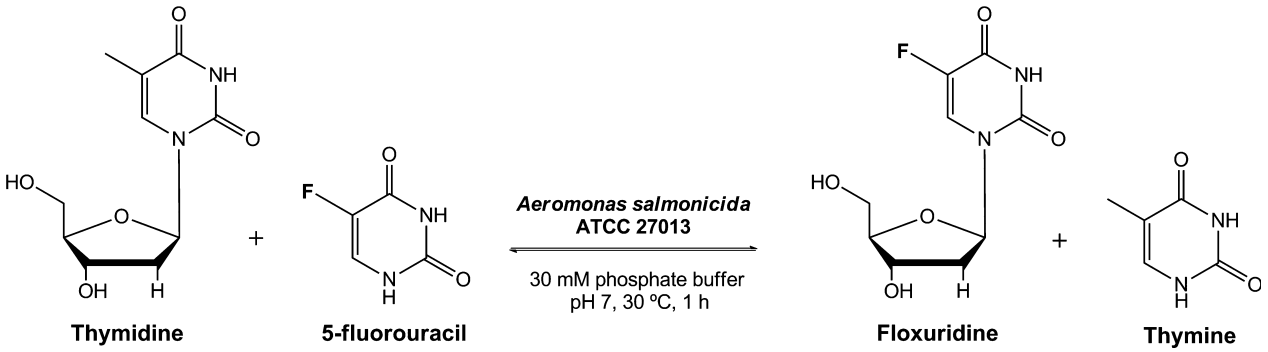

Fig. 1. Biosynthesis of floxuridine by microbial transglycosylation.

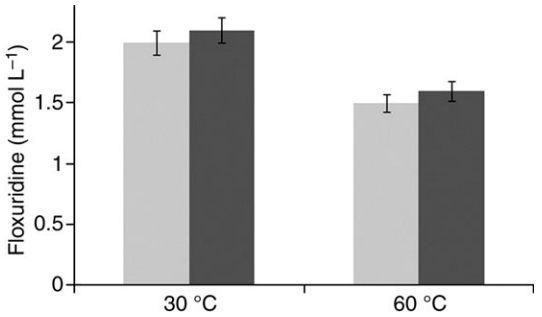

Fig. 2. Biosynthesis of floxuridine by *Aeromonas salmonicida* ATCC 27013 at different temperatures. Reactions were performed with 1×10^{10} CFU, 2.5 mM 5-fluorouracil and 10 mM thymidine in 1 mL of phosphate buffer (30 mM, pH 7) at 200 r.p.m. The best times were 1 h (white) and 3 h (gray).

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-fluoro-2′-deoxyuridine was 2.0 mM (80%) in 1 h when thymidine and 2′-deoxyuridine were evaluated as sugar donors and 1.9 mM (76%) when 2′-deoxycytidine was used (Table 1). Owing to the fact that higher conversion was obtained when thymidine and 2′-deoxyuridine were used, it was decided to continue working with thymidine (PyNP's natural substrate) because it reduces the costs of a subsequent scale-up of this bioprocess.

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

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

*The best times of reaction are shown.

†$\text{Conversion}\,(\%) = \frac{\text{mmol product}}{\text{mmol limiting reagent}} * 100.$

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 1×10^{10} CFU at 30 °C, pH 7, and 200 r.p.m.

Ratio* (5F/Thy)	Conversion† (%)	Productivity‡ (mg L^{-1} min^{-1})
1 : 1	38	3.9
1 : 4	80	8.2
4 : 1	52	5.3

*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′-deoxyuridine 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′-deoxyuridine biosynthesis using 2′-deoxyuridine, 2′-deoxycytidine, 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.

Table 3. Biosynthesis of 5-chloro-2′-deoxyuridine 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-chlorouracil, and 10 mM starting nucleoside

Nucleoside	Reaction time* (h)	5-Chloro-2′-deoxyuridine (mM)	Conversion† (%)
Thymidine	1	1.3	52
	3	1.4	56
2′-deoxyuridine	1	1.3	52
	3	1.5	60
2′-deoxycytidine	1	1.3	52
	3	1.4	56

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

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′-deoxyuridine 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.

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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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