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A facile whole-cell biocatalytic approach to regioselective synthesis of monoacylated 1-β-D-arabinofuranosylcytosine: Influence of organic solvents

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ABSTRACT

The lyophilized *Pseudomonas fluorescens* cell was an efficient alternative catalyst to enzymes for highly regioselective acylation of a polar nucleoside, 1-β-D-arabinofuranosylcytosine (ara-C). The cells showed an evident solvent dependence in the reaction. Among the tested solvents except for acetonitrile–pyridine, catalytic activity of the cells clearly increased with increasing the polarity of the organic solvents used. Among all the tested solvents both pure and binary, the best results were observed in isopropyl ether–pyridine system, in which the catalyst also showed good thermal and operational stabilities. For the biocataylsis in isopropyl ether–pyridine, the optimal isopropyl ether concentration, water content, acyl donor/ara-C ratio, biocatalyst dosage and reaction temperature were 30% (v/v), 4%, 45, 50 mg/mL and 30 °C, respectively, under which the initial rate, yield and 5′-regioselectivity were 2.93 mM/h, 77.1% and 97.3%, respectively. The bacterial cells exhibited comparable 5′-regioselectivity to the expensive immobilized enzyme, which could also have environmental and cost advantages.

1. Introduction

Nucleoside analogs are compounds of high significance in medicinal chemistry with antitumor, antiviral and immunosuppressive effects (Bergman et al., 2004; Li et al., 2010a; Wagner et al., 2000). Due to a fairly close resemblance in structure, they can be used as competitors of natural nucleosides, be inserted into a growing DNA/RNA strand by the polymerases after phosphorylation and thus disturb the normal DNA/RNA replication (Wagner et al., 2000). However, some nucleoside analogs with high hydrophilicity, such as 1-β-D-arabinofuranosylcytosine (ara-C), have major clinical shortfalls in the treatment of solid tumors, since they cannot easily transfer across the cell membrane by passive diffusion and might undergo a rapid enzymatic deactivation in plasma. To improve the clinical efficiency of those analogs, lipophilic modification has gained much attention as a promising strategy, which is also valuable for new nucleoside drug/prodrug discovery and development (Bergman et al., 2004; Li et al., 2010a).

Selectively modification of only one hydroxyl group of nucleosides is considered as an arduous task to chemists, due to the multiple hydroxyl groups of similar reactivity (Li et al., 2010a,b). During the past decade enormous efforts have been made in the development of synthetic methodologies for lipophilic derivatives of nucleosides (Li et al., 2006a,b, 2010a,b; Wechter et al., 1975). Among these synthetic tools available to chemists, application of enzymes in organic chemistry has become one of the most attractive alternatives to the conventional chemical methods for its high regioselectivity, and environmental friendliness (Klibanov, 2001). In contrast, regioselectively introducing acyl group to sugar moiety of nucleosides by chemical method was an arduous task needing time-consuming protection/deprotection steps and bulky acylating regents (Li et al., 2010a; Morís and Gotor, 1993).

The main hurdle to the industrial application of an enzymatic approach in the synthesis of nucleoside esters are the high cost of the procedure, low thermo- and operational stability of free enzymes in organic solvents. To cope with the limitations, solvent engineering strategies were developed to improve the stability of the enzymes. Our previous research reported the successful application of ionic liquid-containing systems and binary organic solvent mixtures to synthesis of monoesters of nucleosides (Li et al., 2006a, 2010b). However, the use of ionic liquids made handing (filtrating, etc.) more difficult and constrained mass transfer. Other methods focused on the screening of non-expensive lipases (Yang et al., 2010) and searching for effective immobilization carrier for free enzymes (Mitchell and Perez-Ramirez, 2011). The latter, however, added to the complexity and cost of the enzymatic procedures.

Compared to the typically used enzymes, whole-cell biocatalysts offer several benefits to organic synthesis (Schmid et al., 2001; Klibanov, 2001; de Carvalho, 2011). One is that whole-cell biocatalysis eliminate the need for enzyme purification and

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immobilization which account for a large part in the enzyme cost. Another is that whole-cell biocatalyst provide a natural environment for enzyme location, which protects the enzymes from a rapid deactivation in non-aqueous solvents (Duetz et al., 2001). Whole-cell biocatalysis has recently attracted interest in some areas of organic synthesis in nonaqueous media, including asymmetric reduction of ketones in neat substrates (Jakoblinnert et al., 2011), transesterification of oils for biodiesel production (Fukuda et al., 2008), etc. However, to our knowledge, the use of whole-cell catalysts in regioselective acylation of nucleosides has so far remained unexplored.

For whole cells biocatalysis, one of the most important challenges is to find effective strains that not only exhibit moderate to good catalytic activities, but are stable in toxic organic solvents (Schmid et al., 2001; Torres et al., 2011). In addition, whole-cell catalysis being investigated previously in nonaqueous media mainly involved the hydrophobic compounds, which could be well dissolved in apolar solvents and easily transfer across the cell membrane (Ni and Chen, 2004; Schmid et al., 2001). The difficulty or even inability to dissolve the hydrophilic substrates in common organic solvents with low cytotoxicity was also the main limitation in the application of whole-cell biocatalysis to the acylation of polar substrates in organic media.

In continuation of our research on the synthesis of nucleoside derivatives with potential bioactivities (Li et al., 2006a,b, 2010a,b), we reported here the application of a whole-cell biocatalyst from *Pseudomonas fluorescens* to acylation of a typical hydrophilic nucleoside ara-C, a traditional valuable agent for therapy of acute leukemias. Vinyl propionate (VP), an irreversible acyl donor, was adopted in the biocatalytic reaction. The aim of this study was to check the catalytic properties, stability and the feasibility of the reuse of bacterial whole-cell biocatalyst suspended as a lyophilized powder in organic solvent, which might be of interest in industrial biotechnology. The influence of some influential factors, such as the types of organic solvents, water content and substrate ratio, were also investigated in detail.

2. Methods

2.1. Biological and chemical materials

P. fluorescens AS1.823 was supplied by CGMCC (China General Microbiological Culture Collection Center, China). 1-β-D-arabinofuranosylcytosine and VP were purchased from Sigma (USA). Refined soybean oil was purchased locally. All other chemicals were from commercial sources and were of the highest purity available.

2.2. Cell culture conditions and whole cell biocatalyst preparation

The precultivation was performed in the medium which contained 1% glucose, 1% beef extract, 1% peptone, 0.5% K_2HPO_4 and 0.5% NaCl at 30 °C for 24 h. Then 2% seed culture was inoculated to the culture medium which contained (g/L) $(NH_4)_2SO_4$ 5.0, K_2HPO_4 1.0, $MgSO_4$ $7H_2O$ 0.2, soybean oil 5.0 and yeast extracts 1.0. To obtain whole cell biocatalyst, the cultivation was carried out in 500 mL flasks containing 100 mL culture media on a rotary shaker at 30 °C and 180 rpm. The bacteria cells were harvested by centrifugation to remove the supernatant, washed twice with distilled water, freeze-dried at −30 °C for 24 h and then stored at 4 °C.

2.3. General procedure for whole-cell mediated acylation of ara-C with VP

In a typical reaction, 2 mL pure or binary organic solvents containing 20 mM ara-C, 300 mM VP, 4% water and 50 mg/mL freeze-dried cells were incubated by shaking (140 rpm) at a fixed temperature for 72 h. Aliquots were withdrawn at specified time intervals from the reaction mixture, and then diluted 100 times with water–methanol prior to HPLC analysis. To structurally characterize the product of the whole cell-catalyzed acylation of ara-C with VP, the reaction was scaled up (0.08 mmol of ara-C and 3.6 mol VP). Upon completion of the reaction, the reaction mixture was centrifuged to remove the biocatalyst and evaporated under vacuum. The residue was separated and purified through flash column chromatography using acetic acetate/methanol (1:5, v/v) as the mobile phase. After crystallization from ethanol, two products were obtained as white powder. All reported data are averages of experiments performed at least in duplicate.

2.4. Polarity parameter $E_T(30)$ determination for pure and binary solvents

1.5 mg of Reichardt's dye was dissolved in 100 mL of a pure solvent or a two-solvent mixture. Then, the maximal UV–Vis absorption band of this dye was recorded on a UNIICOTM WFZUV-2102 PC spectrophotometer (UNICO instruments Co. Ltd., China). The polarity of the reaction medium was estimated using the empirical parameter $E_T(30)$ which was calculated from the equation: $E_T(30)$ (kcal/mol) = $h\nu N = h(c/\lambda)N$ (h: the Planck's constant; ν: the light frequency; c: the velocity of light; λ: the maximal absorption band of the dye; N: the Avogadro's number).

2.5. Determination of activation energy

The reactions were performed at different temperatures (varying from 20 to 45 °C). Biocatalysts (50 mg/mL) were added to 2 mL of 30% (v/v) isopropyl ether–pyridine (containing 0.04 mmol ara-C, 1.8 mmol VP and 80 μL water) for the whole cells or 28% (v/v) hexane–pyridine (containing 0.04 mmol ara-C and 0.4 mmol vinyl propionate) for Novozyme 435 (Li et al., 2006b). The mixture was then incubated in a water-bath shaker at 140 rpm. The apparent activation energy (E_a) of the whole cell-catalyzed acylation of ara-C with VP was calculated according to the linear regression analysis of the Arrhenius plot.

2.6. Determination of the thermal stability of the biocatalyst

In order to assess the thermal stability of the enzyme, 100 mg aliquots of dried cells were added into separate screw-capped vials containing 2 mL of the selected medium {30% (v/v) hexane–pyridine or 30% (v/v) isopropyl ether–pyridine} and the mixture was incubated for 6 h at various temperatures from 30 to 60 °C. Then the biocatalyst was recovered by filtration from the solvent mixtures and added to a fresh volume of the same reaction medium containing 0.04 mmol ara-C and 0.9 mmol VP. The assay reaction was then incubated at 140 rpm and 30 °C and the catalytic activity of the cells was measured by HPLC quantitation of the product ester. The relative activity was expressed as the ratio of the retained activity after incubation to the original activity of the cell biocatalyst in the same reaction system.

2.7. Determination of the operational stability of the biocatalyst

To assess the operational stability of the whole cells, the re-use of the biocatalyst was investigated over 4 reaction cycles. Initially 60 mg aliquots of the dried cells were added into separate screw-capped vials each containing 1 mL 30% (v/v) isopropyl ether–pyridine, ara-C (0.02 mmol), VP (0.9 mmol) and water (40 μL). The reaction was then repeated over 4 cycles (12 h per batch) at 30 °C and 140 rpm. Between cycles the cells were filtered from