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Regioselective synthesis of cytarabine monopropionate by using a fungal whole-cell biocatalyst in nonaqueous medium

Mei-yan Yang[a], Hui Wu[b], Zhi-hong Lu[b], Xiao-feng Li[a,b*], Fu-rao Lai, and Guang-lei Zhao[a**]

[a] State Key Laboratory of Pulp and Paper Engineering, South China University of Technology, Guangzhou, 510641, China.

[b] College of Light Industry and Food Sciences, South China University of Technology, Guangzhou, 510641, China

Correspondence:

* Xiao-feng Li, Ph.D.

College of Light Industry and Food Sciences, South China University of Technology, Wushan Road 381, Guangzhou, 510641, China.

Tel: +86-20-2223-6819. Fax: +86-20-8711-2853.

E-mail: xflibio@gmail.com，zhaoguanglei@gmail.com

Abstract

The utilization of a dehydrated fungal biocatalyst of *Aspergillus oryzae* cells was successfully performed to achieve efficient aylation modification of a polar nucleoside cytarabine (ara-C). Organic solvents showed evident influence on the reaction catalyzed by the *A. oryzae* whole-cells. Except for hexane-pyridine, the catalytic activity and regioslectivity of the whole-cells clearly increased with increasing the polarity of the hydrophobic organic solvents used. The effects of some crucial factors on the reaction were further examined. The best reaction medium, hydrophobic solvent concentration, vinyl propionate/ara-C ratio, reaction temperature and shaking speed were confirmed as isopropyl ether (IPE)-pyridine, 30% (v/v), 90, 30˚C and 140-180 rpm, respectively. The cell biocatalyst also showed good thermal stabilities in both IPE-pyridine and hexane-pyridine systems. In addition, the desired 3′-O-propional derivative of ara-C was synthesized with the yields of 88.3% and regioselectivity (>70%). The resulting biocatalytic system appears to be an effective alternative, and can thus be employed for application in highly regioselective modification of nucleoside analogues.

Keywords: Fungal whole-cell; nucleoside; acylation; solvent engineering; regioselectivity

Regioselective acylation of only one out of several hydroxyl groups of polyhydroxyl compounds is a useful reaction for modification of the drug candidates bearing sugar residues to improve their clinical efficiency, which is also valuable for new drug/prodrug discovery and development.[1, 2] For an example, some nucleoside analogues with high hydrophilicity, such as aracytosine (ara-C), have major clinical shortfalls in the treatment of solid tumors, since they can not easily transfer across the cell membrane by passive diffusion and might undergo a rapid enzymatic deactivation in plasma. Regioselective acylation of the nucleoside analogues could significantly improve their antitumor, antiviral and immunosuppressive effects. And even just a single acylation of those compounds many led to promising candidate compounds, such as the 3'-0-acyl-ara-cytidinesor the antibiotic puromycin.[3, 4] In addition, regioselective acylation of nucleosides represents a way of introducing protecting groups commonly used in drug discrovery.[3] However, it still remains as a significant challenge in classic organic chemistry.[1, 2, 5]

During the past decade enormous efforts have been made in the development of synthetic methodologies for selective preparation of lipophilic derivatives of nucleoside analogues.[6-8] Among these synthetic tools available to chemists, the use of enzymes has become one of the most attractive alternatives to the conventional chemical methods for its high regioselectivity, and environmental friendliness.[9] However, in terms of efficiency and cost-effectiveness, the need for isolated enzymes is disadvantageous.

Biocatalytic reactions can also be performed by use of whole-cell biocatalysts. Compared to the isolated enzymes, whole-cell biocatalysts offer several benefits to organic synthesis.[9, 10] This type of biocatalysts provide a natural environment for enzyme location, which protects the cell-bound enzymes from a rapid deactivation in

non-aqueous solvents.[11] In addition, the use of microbial biocatalysts can eliminate the need for enzyme purification and immobilization which account for a large part in the enzyme cost. Until recently the majority of whole-cell mediated bio-processes were carried out in aqueous media for hydrophobic compounds.[12] Only a few researches have been involved in non-aqueous biocatalysis with whole-cells, including asymmetric reduction of ketones in neat substrates,[13] transesterification of oils for biodiesel production, etc.[7]

The finding of microbial strains that are not only tolerant to organic solvents but possesses good catalytic performance plays an important role in whole-cell biocatalysis. A wide range of microbial cells have been explored for their capability in catalysis, including both bacterial and fungal strains. Among them, filamentous fungi have been attracted much attention. They have been used for fermentative food processing since ancient times, and thus are considered to be quite safe for use in pharmaceutical industry. Recently, several strains of filamentous fungi have been exploited as whole-cell biocatalysts in asymmetric reduction,[8] kinetic hydrolysis of (R,S)-benzyl glycidyl ether,[14] etc, in which the cells exhibited moderate to good catalytic activity and high selectivities. In continuation of our research on the "green" synthesis of nucleoside derivatives with potential antitumor and antiviral activities,[3, 4, 15] we reported here the application of a whole-cell biocatalyst from *Aspergillus oryzae* in acylation of the hydrophilic nucleoside ara-C, a traditional clinical agent for therapy of acute leukemias. The influence of some influential factors, such as the types of organic solvents, substrate ratio and shaking speed, were also investigated in

details. In addition, the catalytic properties and stability of the fungi whole-cell biocatalyst in organic solvents were investigated (Scheme 1). [16, 17]

(Insert Scheme 1 here)

Table 1 showed the effects of a series of solvents both pure and binary on the catalytic behaviors of *A.oryzae* cells in acylation of ara-C.[18] No product was detected when reactions were carried out in DMSO and DMF which have high polarities [in terms of $E_T(30)$]. In pyridine, the microbial cells exhibited certain catalytic activity, which may be due to its lower polarity than DMSO or DMF. Thus a "co-solvent" strategy was proposed by using the mixture of the polar solvent pyridine and another hydrophobic solvent as reaction medium. Table 1, entries 3-8 showed that the organic solvent mixtures had an evident effect on the whole-cell catalytic performances. Except for hexane-pyridine, the catalytic activity and regioselectivity of the cells clearly increased with increasing the polarity of the hydrophobic organic solvents used, suggesting that the polarity of the solvents was a crucial factor affecting the whole-cell catalyzed reaction and might be used to approximately predict the solvent effects. Furthermore, two kind organic solvents with a quite similar polarity (hexane-pyridine and IPE-pyridine) gave significant differences in product yield and regioselectivity, indicating that the catalytic behaviors of the *A.oryzae* cells in organic solvents were not only polarity-dependent. Hence, IPE-pyridine mixture was chosen as the most suitable reaction solvent due to the best reaction results achieved.

(Insert Table 1 here)

To get a better insight into the fungus whole-cell mediated acylation of ara-C in

IPE-pyridine system, the effects of several key variables were further optimized. Considering that varying the volume ratio of IPE to pyridine enabled the reaction media to controllable $E_T(30)$ values, the influence of IPE content on the reaction was investigated. As Fig.1a showed, when changing the IPE content from 10% (v/v) up to 30% (v/v), the initial rate, yield and 3′-regioselectivity of the reaction went up markedly. Further increased in the content of hydrophobic IPE, however, caused visible insolubility of ara-C in the reaction media. Thus, when the IPE content was increased from 30% (v/v) to 40% (v/v), both the yield and 3′-regioselectivity of the reaction decreased.

Fig.1b confirmed that the initial rate and yield of the whole-cell catalyzed reaction can be greatly boosted by increasing the VP/ara-C ratio from 10 to a high level of 50 (mol/mol), beyond which the initial rate and yield were improved slowly, and then hardly changed when the ratio was above 90 (mol/mol). Excessive supply of vinyl acyl donor in the acylation reactions was considered to be necessary for vinyl ester-involved biotransformation due to the rapid enzymatic hydrolysis of vinyl esters, a side reaction along with the enzymatic acylation of the nucleoside. As illustrated in Fig.1c, the cells exhibited catalytic activity in a wide range of temperature (from 20°C to 50°C). The highest initial rate and yield of the reaction were achieved at 30℃. Temperature may be not only closely related to the thermodynamic equilibrium of the reactions but also involved in the deactivation of biocatalyst. It can be found that the increase of the temperature above 30℃ resulted in significant decrease of the 3'-regioselectivity from 71.3% to 16.1%. This phenomenon suggested that higher

temperature influenced the confirmation of the enzymes active center, and thus the recognition site of the *A.oryzae* whole-cells on sugar moity of ara-C changed from 3'-OH to 5'-OH.

In biocatalysis, substrates have to diffuse to the active center of enzymes from reaction media by solutions of the salvation, and the products also need diffuse to the reaction media from the active center of enzymes. These processes were related to the mass transfer resistance, viscosity of the media and the solution of substrates and products in the reaction media and so on. Previous researches showed that the catalytic efficiency of whole-cell biocatalysts was 1-2 classes lower than free enzymes[19] due to the low permeability of the whole-cell catalysts. Hence, the shaking speed of the reaction system was particularly important to facilitate the mass transfer in the reaction. Fig.1d showed that the initial rate of the reaction increased as raising the shaking speed at the shaking speed of <180 rpm, confirming the existence of mass transfer resistance in the reaction system. However, the reaction rate decreased when the shaking speed was above 180 rpm, which was mainly due to that the drastic shaking made the whole-cell catalysts adhere to the wall of reaction flasks and thus reduced the efficient cell concentration. Changes in shaking speeds showed little influence on 3'-regioselectivity of the reaction. For analysis of the statistically significant difference between the effect of these key variables and biocatalytic reaction, the Duncan's post-test at 0.05 level was done. Results showed that the change of IPE concentration had no significant effect on the reaction, while changes of VP/ara-C and shaking speed resulted in significant differences of both initial

reaction rate and yield of the reaction. Variation of temperature, however, only led to significant difference in the initial reaction rate of the reaction.

(Insert Fig.1 here)

From both a practical and a theoretical viewpoint, it was of considerable importance to understand the stability of the whole-cell biocatalyst in the reaction media.[20] As showed in Fig.2, the *A. oryzae* whole-cells only had a little loss of catalytic activity after incubated in 30% (v/v) IPE-pyridine solvent under 30 ℃-35 ℃. Further improving of the incubation temperature led to the decrease of their relative activities. When the incubation temperature was as high as 60 ℃, the relative activity was decreased to 37.8%. Fig.2 also illustrated the deactivation profile of the whole-cells of *A.oryzae* by incubating the cells in different co-solvent media with similar $E_T(30)$ values at various temperatures. The relative activity after incubation in 30% (v/v) hexane-pyridine was almost the same like in 30% (v/v) IPE-pyridine. Furthermore, the Duncan's post-test showed that there was no statistically significant differences between them at $P<0.05$. It demonstrated that the whole-cell biocatalyst had similar thermal stability in IPE-pyridine to that in hexane-pyridine.

(Insert Fig.2 here)

In conclusions, a fungus whole-cell biocatalytic system was developed for 3′-regioselective synthesis of ara-C propionate. The catalytic behaviors of the whole-cell biocatalyst from *A.oryzae* showed a clear solvent dependence within the range of organic solvents tested. Binary mixture was shown to be more suitable for the fungus whole-cell catalyzed acylation of hydrophilic substrate than the pure polar

solvents. The 3'-regioselectivity and thermal stabilities observed in organic solvents renders the whole-cell biocatalyst a promising candidate for non-aqueous production of bioactive nucleoside esters. In addition, the fungus cell biocatalysts can be produced in abundance via cultivation and avoid tedious purification of free enzymes, thus enabling the whole-cell catalyst being superior to the free enzymes in cost and handling. It is hoped that this new whole-cell biotechnological strategy may benefit some related pharmaceutical processes, making them greener and more cost-effective. Extension of the whole-cell biocatalytic technology to modification of other bioactive drugs is currently under development and will be reported in due course.

Acknowledgements

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16. Analytical methods: The reaction mixture was analyzed by RP-HPLC on a 4.6mm × 250 mm (5μm)

Zorbax SB-C18 column (Agilent Technologies Co. Ltd. , Massachusetts, USA) using a Waters 600E pump and a Waters 2996UV/photodiode array detector (Waters Corp., Massachusetts, USA) at 276 nm. A mixture of ammonium acetate buffer (0.01 M, pH 4.27) and methanol (80/20, v/v) was used as mobile phase with the flow rate of 0.9 ml/min. The retention times for ara-C, 3′-O-propionyl ara-C and 5′-O-propionyl ara-C were 3.14, 11.89 and 13.81 min, respectively. Regioselectivity was defined as the ratio of the HPLC peak area corresponding to the indicated product to that of all the products formed upon a certain reaction time. The yield of ara-C to its 5′-O-ester was defined as the ratio: [moles of 5′-regioisomer in the acylation reaction]/[initial moles of ara-C]. The average error for this determination was less than 1.0%. The biomass of the strain was calculated in term of dry cell weight.

17. ^{13}C NMR of Ara-C: ^{13}C NMR δ 164.9 (C-4) , 143.4 (C-6), 92.5 (C-5) , 86.5 (C-1′) , 85.3 (C-4′), 76.3 (C-3′) ,74.9 (C-2′) , 61.2 (C-5′); FT-IR (KBr, cm-1) v 3214-3342 (OH,NH), 2922 (CH), 1649 (C=C), 1117/1051 (C-O-C)., M.δ.C. ^{13}C NMR of 5′-O-propionyl ara-C: ^{13}C NMR(DMSO-d6, 166.10 (C4-N), 155.86 (C=O 2), 143.40(C-6), 93.11(C-5), 86.34 (C-1′), 82.20(C-4′), 77. 25 (C-3′), 74.81(C-2′), 64.20 (C-5′), 27.22 (CH3) , 9.39 (CH3) ESI-MS ((C12H17N3O6, 299.30): m/z(%)=298.90(100) ([M-H]+)., M.δ.C. ^{13}C NMR of 3′-O-propionyl ara-C：：^{13}C NMR(DMSO-d6, 166.10 (C4-N), 155.85(C=O 2), 143.18 (C-6), 93.11 (C-5), 86.34 (C-1′), 83.15 (C-4′), 79. 20 (C-3′), 72.75(C-2′), 61.66(C-5′), 27.20(CH2), 9.40 (CH3)；ESI-MS (C12H17N3O6, 299.30): m/z(%)= 298.90(100) ([M-H]+).

18. *Aspergillus oryzae* 3.5232 (Collection No. 3.5232) was supplied by CGMCC (China General Microbiological Culture Collection Center, China). 1-β-D-arabinofuranosylcytosine and vinyl propionate (VP) was purchased from Sigma (USA). All other chemicals were from commercial sources and were of the highest purity available. General procedure of whole-cell acylation of ara-C：2 ml organic solvents containing 20 mM ara-C, 600 mM VP, 4% water and 50 mg/ml freeze-dried Aspergillus oryzae 3.5232 (Collection No.3.5232) were incubated by shaking (140 rpm) at a fixed temperature for 24 hours. 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. Upon completion of the reaction, the reaction mixture was centrifuged to remove the cell masses, isolated, and purified by half-preparation HPLC with a semi-preparation column. The acquisition was concentrated to

about 1 mL by vacuum rotary evaporation. After crystallization under 4℃, two products were obtained and then determined by 13C NMR (Bruker AVANCE Digital 400MHz Nuclear Magnetic Resonance Spectrometer, Bruker Co., Germany) at 100 MHz. The ESI-MS spectra of the product were recorded on a Thermo LCQ DECA XP Plus ESI Mass Spectrometer with a spray voltage of 4.5 kV (Thermo Finnigan, USA). All the experiment was repeated twice.

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20. 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) IPE-pyridine] and the mixture was incubated for 6 hours at various temperatures from 30 to 60 ℃. Then the biocatalyst was recovered by filtration from the solvent mixtures and added to a fresh volume of the same reaction medium containing 20 mM ara-C and 600 mM VP. The assay reaction was then incubated at 140 rpm under 30 ℃. The relative activity was expressed as the ratio of the residual activity after incubation to the original activity of the cell biocatalyst in the same reaction system.

Figure Caption

Scheme 1 Acylation of ara-C with VP catalyzed by whole-cell（1：Ara-C；2：VP；3：Vinyl alcohol (byproduct of the reaction); 4: Aldehyde; 5: 3'-O-propionyl ara-C; 6: 5'-O-propionyl ara-C）

Fig.1 (a) Effect of the IPE content on acylation of ara-C catalyzed by *A.oryzae* cells (reaction conditions: 50 mg/mL biomass, 20 mM ara-C, 600 mM VP, 4% water, 1 mL solvent medium, 30 ℃,140 rpm and 24 h). (b) Effects of VP/ara-C ratio (reaction conditions: 50 mg/mL biomass, 20 mmol/L ara-C, 4% water, 1 mL solvent medium, 30 ℃,140 rpm and 24 h). (c) Effect of reaction temperature (reaction conditions: 50 mg/mL biomass, 20 mmol/L ara-C, 1800 mmol/L VP, 4% water, 1 mL 30%IPE-pyridine, 140 rpm and 24 h). (d) Effects of shaking speed (reaction conditions: 50 mg/mL biomass, 20 mmol/L ara-C, 1800 mmol/L VP, 4% water, 1 mL 30% IPE-pyridine, 30 ℃and 24 h).

Fig.2 The thermal stabilities of *A.oryzae* whole-cells in 30% IPE-pyridine and 30% hexane-pyridine (The reaction conditions: 50 mg/mL biomass, 20 mmol/L ara-C, 1800 mmol/L VP, 4% water, 2 mL 30%IPE-pyridine, 30 ℃, 140 rpm and 24 h)

Tables

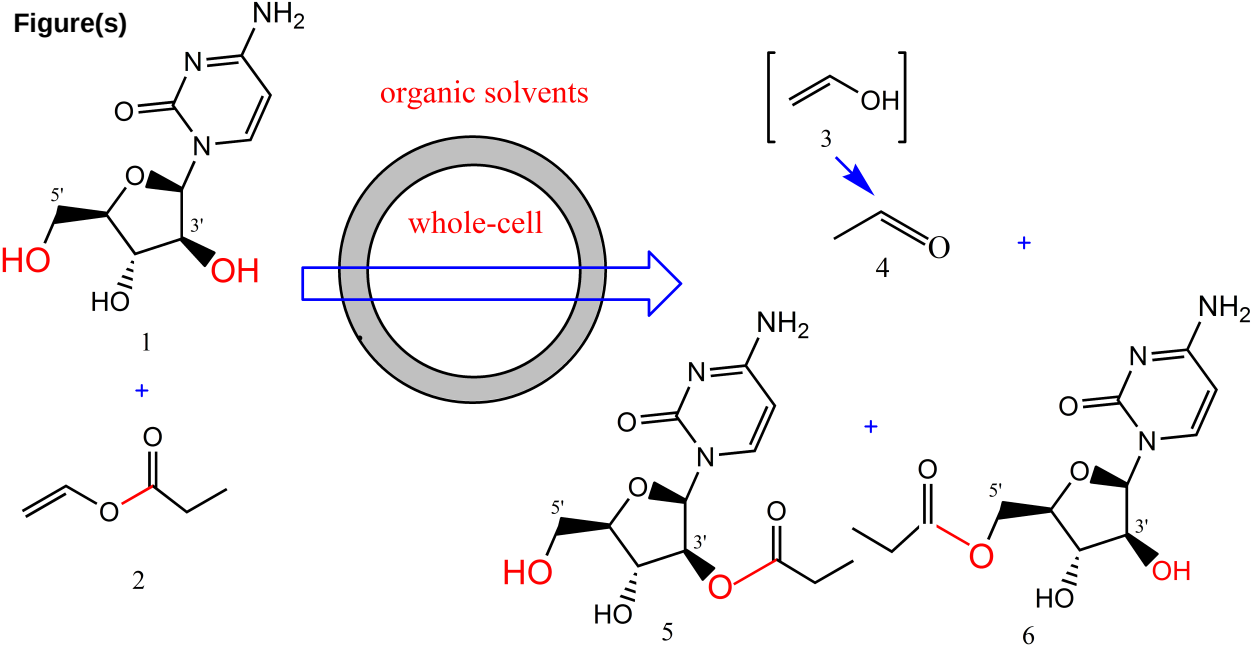
Table 1 Acylation of ara-C with VP in various pure and binary organic solvents catalyzed by whole-cells of *A.oryzae* [a]

Entry	Solvent [b]	$E_T(30)$ (kcal/mol)	V_0 (mmol/L·h)	Y (%)	3'-Regioselectivity (%)
1	DMSO	45.10	0	0	0
2	DMF	43.50	0	0	0
3	Pyridine	40.20	0.52 A	16.05 A	64.16 A
4	*t*-Butanol-pyridine	42.01	0.56 A	21.36 B	63.97 A
5	*t*-Pentyl alcohol-pyridine	41.88	0.88 B	26.82 C	64.98 A
6	THF-pyridine	40.17	1.27 C	40.60 D	68.83 B
7	IPE-pyridine	40.06	3.06 D	57.37 F	69.08 B
8	Hexane-pyridine	40.05	3.30 E	44.92 E	63.53 A

[a] The reaction conditions: 50 mg/mL biomass, 20 mM ara-C, 600 mM VP, 4% water, 1 mL solvent medium, 30 ℃,140 rpm and 24 h; different capital letters denote significant differences at Duncan's post-test between different solvent systems.

[b] 75% pyridine was added in the binary solvents

Figure(s)



Scheme 1

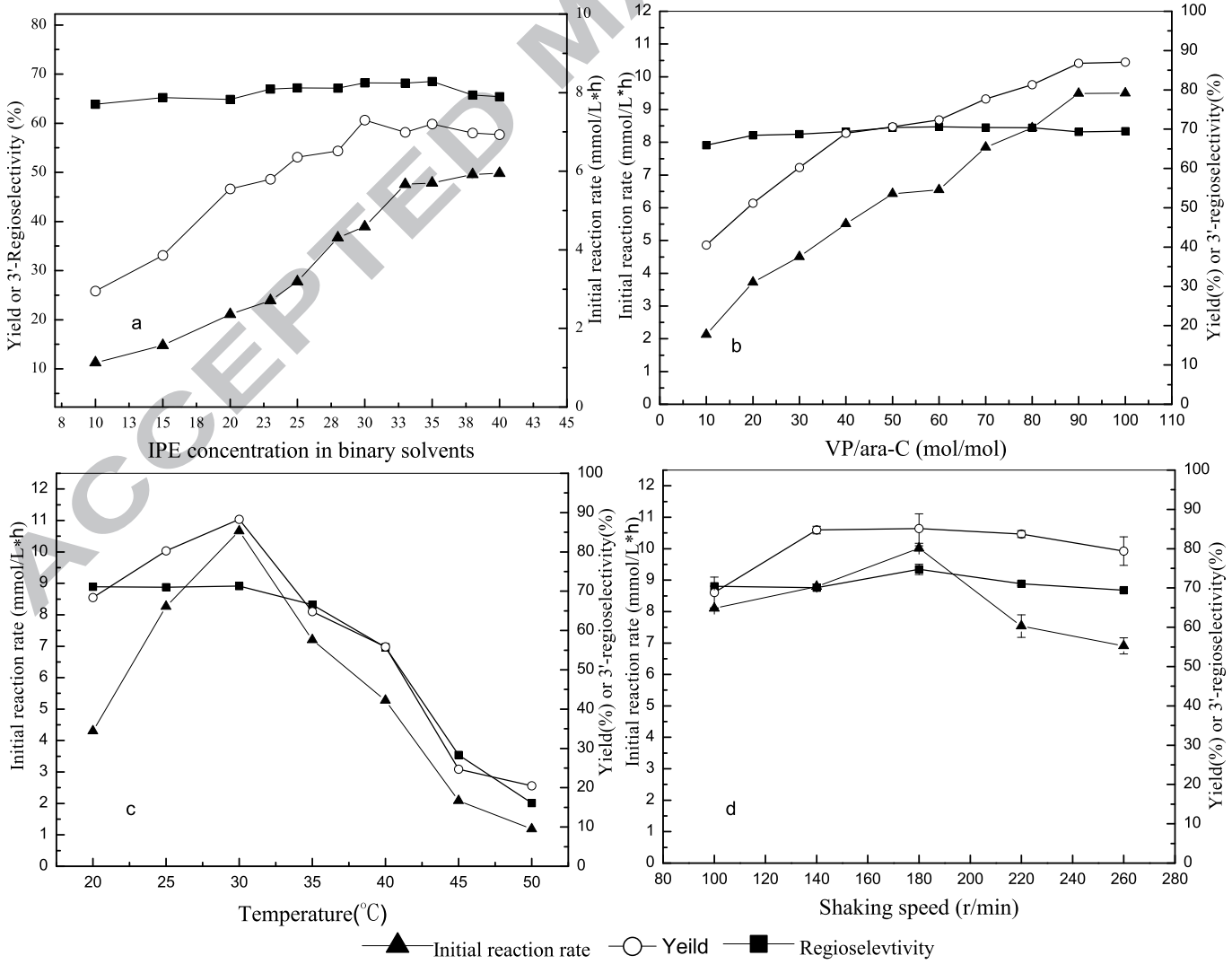


Fig.1

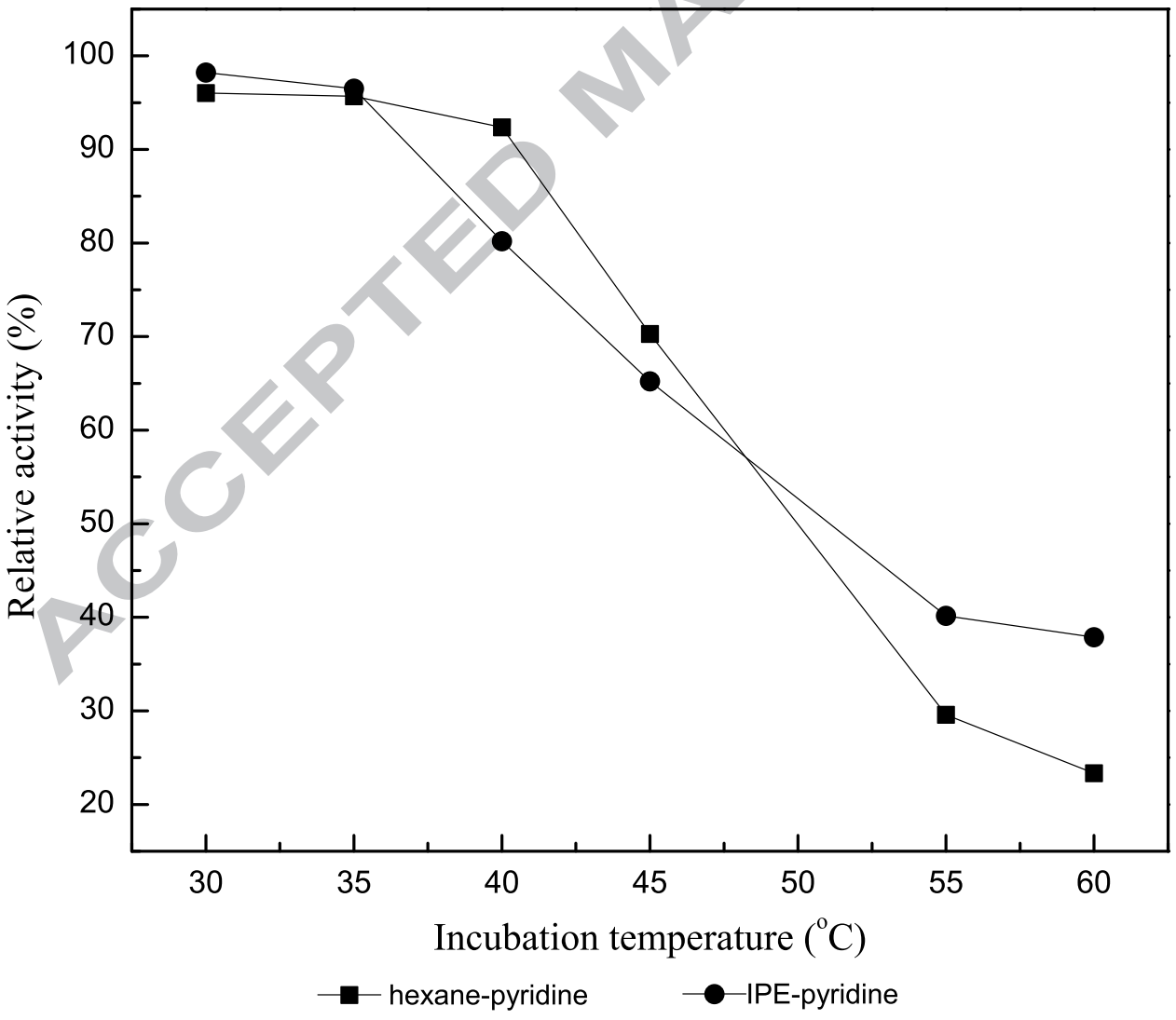
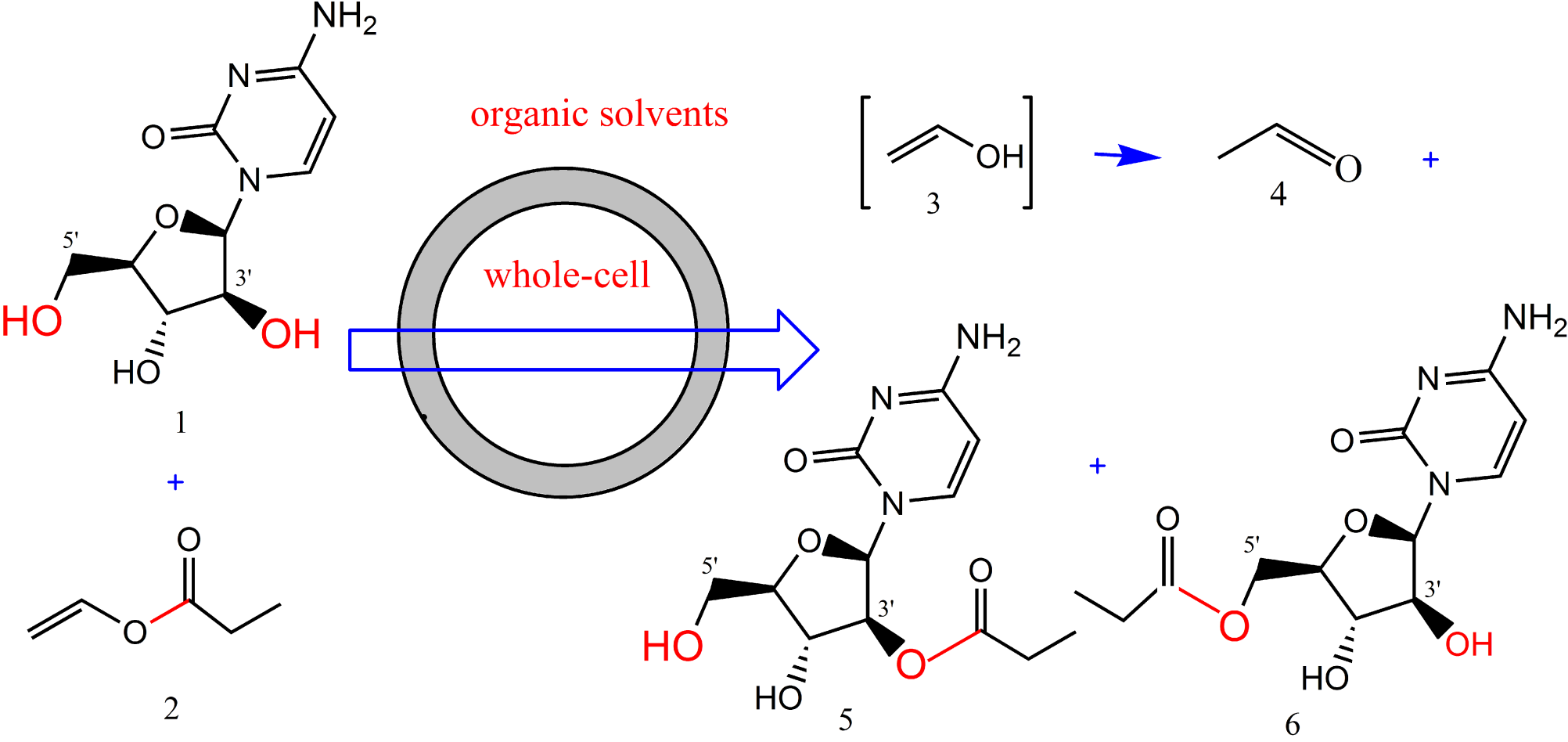


Fig.2


organic solvents
whole-cell
NH2
N
O
N
O
5'
3'
HO
OH
HO
1
+
O
O
2
[
OH
]
3
4
+
NH2
N
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N
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5'
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NH2
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O
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OH
HO
6