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Effect of solvent systems on *Brevibacterium pusillum* for Didanosine synthesis

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ABSTRACT

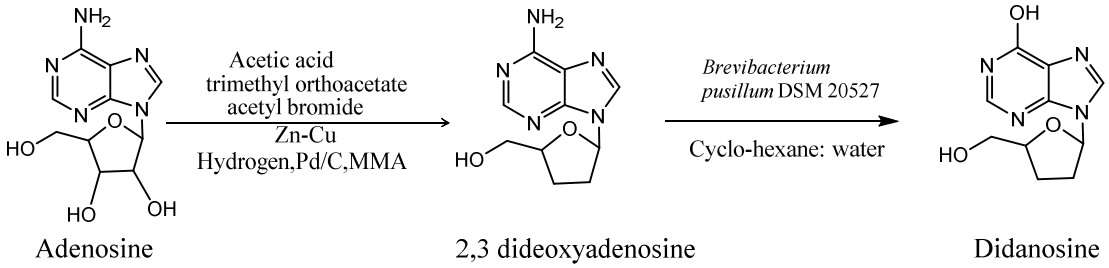
A whole cell mediated deamination of 2, 3-dideoxyadenosine to didanosine has been demonstrated using actinomycetes strain, Brevibacterium pusillum DSM 20527. The reaction was carried out in co-solvent system consisting of water (pH adjusted to 7.4) and cyclohexane. Adenosine is used as the starting material which is converted to 2, 3 dideoxyadenosine which in turn is converted to 2, 3-dideoxyinosine (Didanosine) using biotransformation reaction. Impurity generation during the process of synthesis was found to get reduced under co-solvent system. Also, Brevibacterium pusillum showed tolerance towards various solvents. Our work shows biocatalytic synthesis of Didanosine with less impurity generation.

Keywords: Biocatalysis, antiviral drug, 2, 3-dideoxyadenosine, 2, 3-dideoxyinosine.

INTRODUCTION

Biocatalysis for industrial chemistry and pharmaceuticals is experiencing significant growth over last couple of years. With recent advent of biotechnology, biocatalysis has become industrially an attractive and useful proposition. Biocatalysis has several advantages but it comes with its share of drawbacks also as exemplified by use of organic solvents for biocatalysis using live microbial cells, since organic solvents are toxic to the microbial cells as they cause damage to bacterial cell membrane [24, 25]. Organic solvent tolerant bacteria are very useful for several industrial applications [26, 32]. Biocatalysis in non-aqueous media is industrially very attractive [30]. The solvent engineering is a prerequisite for biocatalysis [29]. Enzymes from solvent tolerant bacteria are very useful for synthesis of industrially significant products [31].

In our work, we have tested Didanosine synthesis under various solvent systems as a model to test the activity and efficiency of *Brevibacterium pusillum.* Our objective was also to improve the existing synthesis method of Didanosine. Didanosine (2,3-dideoxyinosine) is a nucleoside reverse transcriptase inhibitor, effective against HIV and used in combination with other antiretroviral drug therapy as part of highly active antiretroviral therapy. Chemically, the synthesis of 2, 3-dideoxyinosine is carried out using inosine as starting material and by using protecting and deprotecting agents which resulted in low yield [1, 7]. Recently, development in whole cell biocatalysis has opened up the possibility towards synthesis of 2, 3-dideoxyinosine via transphosphorylation process [8 - 12], fermentation [13] as well as chemo-enzymatic process with adenosine deaminase [14 - 17]. Various methods have been reported for the synthesis of 2, 3 dideoxyadenosine in past [18-21]. Major drawbacks were the expensive raw materials, undesirable by product and less yield [22, 23]. An attempt has been made towards synthesis of 2, 3 dideoxyadenosine followed by didanosine synthesis using *Brevibacterium pusillum* DSM 20527 (Scheme 1) under aqueous and organic co-solvent system.



Scheme 1: Chemo-fermentation synthesis of Didanosine

MATERIALS AND METHODS

Chemicals and Microorganism

Adenosine, trimethyl orthoacetate, acetyl bromide were procured from Sigma Aldrich, Bengaluru, India. 25% *w/w* monomethyl amines from Balaji Amines Limited, India, glacial acetic acid, sodium carbonate, ethylenediamine tetraacetic acid (EDTA), methylene from Avra Laboratories Pvt. Ltd., Hyderabad, India. Glycerol, Tris (hydroxymethyl) aminomethane (TRIS) and magnesium sulfate hepta hydrate were purchased from Merck. Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, ammonium acetate, methanol, toluene, methylene dichloride (MDC), , n-propanol, n-hexane, n-heptane, dimethyl formamide, methyl–iso-butyl ketone, isopropyl ether, isopropyl alcohol, 2-methoxy ethanol, ethyl acetate, cyclohexane, 1,4 dioxane, acetonitrile, 2-butanol, 1-butanol, tert-butanol sodium chloride, sodium hydroxide (NaOH) and dimethyl sulfoxide were procured from Spectrochem Pvt. Ltd., India. Ammonia solution 30%, conc. HCl and orthophosphoric acid were procured from SD Fine Chemicals Ltd., India. Meat extract powder, peptone (bacteriological grade) and yeast extract were procured from Himedia Laboratories Ltd., India, and Chaitanya Biologicals Pvt. Ltd., India respectively.

Brevibacterium pusillum DSM 20527 was obtained from Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH.

Analytical Methods

HPLC

High performance liquid chromatography analysis was performed on Waters alliance 2645 High performance liquid chromatography instrument connected with UV detector at 254 nm using Thermo Scientific, Hypersil BDS C-18 (5 µm particle size, 250 x 4.6 mm length) eluted with gradient mobile phase system containing mobile phase A (8 volumes of methanol and 92 volumes of 3.86 g/L ammonium acetate solution, adjusted to pH 8.0 with concentrated ammonia) and mobile phase B (30 volumes of methanol and 70 volumes of 3.86 g/L ammonium acetate solution, adjusted to pH 8.0 with concentrated ammonia) at a flow rate of 1 mL/min .The retention time were found to be 6.5 min, 13.5 min, 15 min and 26.0 min for adenine, adenosine, 2,3-dideoxyinosine and 2,3 dideoxyadenosine respectively.

NMR spectroscopy

The ^{1}H NMR spectra was recorded in DMSO-d_6 on a Bruker Avance 300 spectrometer. The chemical shifts are reported in δ ppm relative to TMS (δ 0.00) and DMSO-d_6 as internal standards respectively.

Mass spectrometry

Electron Spray Ionization-Mass spectra (ESI-MS) of isolated compounds were measured using Agilent 1100 LC/MSD Trap SL instrument.

Specific optical rotation

Specific optical rotation of isolated compounds were measured using Perkin-Elmer 243 polarimeter (Uberlingen, Germany).

Synthetic procedures

Synthesis of 2, 3 dideoxy adenosine

To a solution of 100 g adenosine (0.37 moles) in 150 ml of acetic acid and 250 ml of acetonitrile, 60 mL of trimethylorthoacetate was added and stirred for 3 h at 50 ^{o}C. Reaction mixture was cooled and 120 mL acetyl bromide was added. Reaction mixture was neutralized with 30% sodium carbonate solution and the layers were separated. Aqueous layer was further extracted with 200 mL of acetonitrile. Freshly prepared Zn-Cu couple was added to the acetonitrile layer and stirred. 0.1 M EDTA solution was added to the reaction mass which was extracted with MDC. Organic layer was distilled and acetonitrile was added to the concentrated reaction mass which was

hydrogenated with 5 % w/w palladium on carbon. Reaction mass was filtered off and the solvent was distilled under vacuum. Crude was dissolved in methanol and 25 % monomethyl amine was added and stirred till the completion of reaction. Methanol from the reaction mixture was distilled off and purified using 20 % w/v sodium hydroxide and isopropyl alcohol. The organic layer was distilled off and a white solid was obtained. A yield of 45 % with HPLC purity of 98.88% 2, 3 dideoxy adenosine was obtained. ESI-MS was found to be 236 (M + H), $[\alpha]^{25}$ was found to be - 25.6 (*c* = 1, H_2O). ^{1}H NMR(300MHz, DMSO-d_6, δ/ppm): 1.88-1.95(1H, *m*), 2.20 - 2.30(1H, *m*), 3.49-3.53(1H, *d*), 3.67-3.71(1H, *d*), 4.31-4.38 (1H, *m*), 4.57(1H, *s*), 5.18(1H, *s*), 5.69 (1H, *s*), 5.86-5.87(1H, *d*), 7.28(2H, *s*), 8.12-8.14 (1H, *d*), 8.33-8.36 (1H, *d*), ^{13}C NMR(75MHz, DMSO-d_6, δ/ppm): 25.93, 31.94, 63.20, 81.90, 84.60, 119.35, 139.25, 149.10, 152.65, 156.23

Preparation of Brevibacterium pusillum DSM 20527 whole cell biomass

Brevibacterium pusillum lyophilized cells were first cultivated in a 50 mL of sterile liquid medium (pH 7.0) containing meat extract (1%), peptone bacteriological grade (1%), yeast extract (0.5%) and NaCl (0.5%) and cultivated aerobically at 30 – 35 °C for 24 h at 250 rpm. A 1% of seed II of *Brevibacterium pusillum DSM 20527* was inoculated into a 20 L fermentor containing 15 L sterile liquid medium (pH 7.0 – 7.5) cultivated aerobically at 30 – 35 °C for 24 h at 250 RPM. The growth medium was harvested at the time of early stationary phase by centrifugation at 9600 RPM at 10 - 15 ^{o}C for 20 min.

Synthesis of 2, 3-dideoxyinosine

The thawed whole cells of *Brevibacterium pusillum* DSM 20527 (2%) was added to a 5L fermenter containing 100 g of 2, 3 dideoxyadenosine in 2000 ml of demineralized water (pH of the mixture was adjusted to 7.0 using dil. HCl slowly under cold condition) and cyclohexane in the ratio 9: 1 v/v. The reaction mixture was then incubated at 30-35 °C for 16 h. The progress of reaction was monitored by HPLC. After the completion of reaction, the reaction mass was filtered off to remove cell debris and the filtrate was distilled off to obtain crude didanosine. The crude didanosine was stirred in 3 volumes of water for 6 h, filtered off and dried under vacuum to obtain pure 2, 3-dideoxyinosine. The product was analyzed by HPLC, MS, SOR and ^{1}H NMR (85 g, 85% isolated yield, purity > 99%). ESI-MS was found to be 237 (M + H) and 259 (M + Na). The $[\alpha]^{D}_{25}$ was found to be 27.2 (*c* = 1, H_2O), ^{1}H NMR(300MHz, DMSO-d_6, δ/ppm): 1.99 -2.07 (2H, *m*), 2.48 - 2.13 (2H, *m*), 3.53-3.49 (1H, *m)*, 3.66-3.61 (1H, *m*), 4.15-4.07 (1H, *m*), 4.97 (1H, *br*), 6.22 -6.19 (*dd*, *J* = 6.9 and 3.3 Hz), 8.05 (1H, *s*), 8.34 (1H, *s*), 12.34 (1H, br, *s*), ^{13}C NMR(75MHz, DMSO-d_6, δ/ppm): 26.93, 32.14, 63.50, 81.80, 83.60, 121.87, 137.25, 145.60, 148.70, 155.53.

RESULTS AND DISCUSSION

2, 3 dideoxy adenosine was synthesized using adenosine as a starting material, trimethyl orthoacetate, glacial acetic acid followed by elimination using zinc-copper couple and ene-reduction using 5% palladium on carbon which was further converted to 2, 3-dideoxyinosine using whole cells of *Brevibacterium pusillum* DSM 20527. The role of various solvents were studied.

Solvent study

Various solvents like methanol, toluene, n-propanol, n-hexane, n-heptane, dimethyl formamide, methyl–iso-butyl ketone, isopropyl ether, isopropyl alcohol, 2-methoxy ethanol, ethyl acetate, cyclohexane, 1, 4 dioxane, dimethyl sulfoxide, acetonitrile, 2-butanol, 1-butanol and tert-butanol were tested towards the synthesis of 2, 3-dideoxyinosine. A 10% of solvent with 90% of buffer with 10% of whole cell biomass of *Brevibacterium pusillum* DSM 20527 were used in solvent screening studies. Cyclohexane was best among the various solvents screened and purity obtained was more than 99%. Process related known impurities were detected by HPLC analysis in both the solvent systems cyclohexane-buffer & buffer. These impurities might be from starting materials, by-products, synthetic intermediates, degradation products etc. The profile of impurities in solvent system of cyclohexane and buffer obtained from HPLC analysis have been shown in table 1, the details of the impurities can be found in the supplementary table 1. It can be seen from the table that the purity of didanosine obtained under solvent system cyclohexane and buffer is more than 99% as compared to purity of 97.99% recovered using only buffer solvent system. Our observations indicate that there is a decrease in impurities of didanosine, depending on the polarity of organic solvents used in the process. Non-polar solvents were found to cause more reduction of impurities as compared to polar solvents.

Our work not only shows the solvent system giving the highest yield of didanosine bio-catalytically, but also sheds light on *Brevibacterium pusillum* DSM 20527's solvent tolerance.

The conversion of 2, 3-dideoxyadenosine under various solvent systems were monitored by TLC. It was observed that 10% cyclohexane and 90% buffer solvent system gives the maximum conversion of 2, 3- dideoxyadenosine to 2, 3-dideoxyinosine by *Brevibacterium pusillum* DSM 20527 within 24 hours (data not shown).

Table 1: Comparison of purity obtained using Cyclohexane & Buffer solvent system (Refer Supplementary table for impurities' details)

HPLC Peaks	%Area (Cyclohexane & buffer)	%Area (buffer)
Imp-A	0.06	0.54
Imp-B	0.05	0.32
Imp-C	-	-
Imp-D	-	-
Imp-E	0.09	0.72
Imp-F	0.05	-
Didanosine	99.42	97.99
Imp-G	-	0.17
Imp-H	-	-

Brevibacterium pusillum has industrial applications for example, in zylitol production [28]. Genus Brevibacterium as a whole, has ample industrial utilizations [27]. We have shown that *Brevibacterium pusillum* DSM 20527 is tolerant towards a range of solvents, however its activity varies with each solvent, as observed by the rate of conversion of 2,3- dideoxyadenosine. The whole cell biomass of *Brevibacterium pusillum* DSM 20527 produced by fermentation showed good conversion rate when reaction was performed in cyclohexane among the solvents tested.

CONCLUSION

Brevibacterium pusillum is an industrially useful microbe and our research shows that it can work in the presence of organic solvents as is evidenced by better and quicker biocatalytic synthesis of didanosine in the presence of cyclohexane. Aqueous and organic solvent system also showed reduction in impurity generation. Our research findings are a contribution towards the understanding of organic chemicals in biocatalysis.

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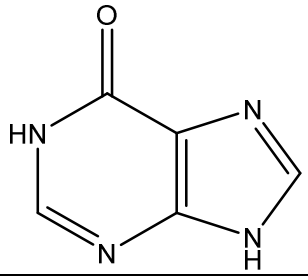
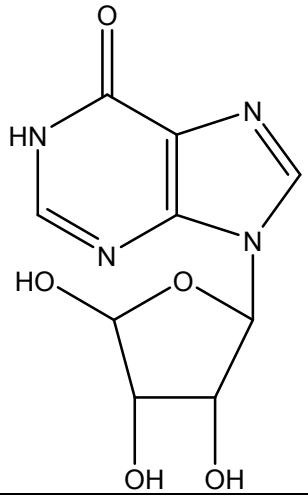
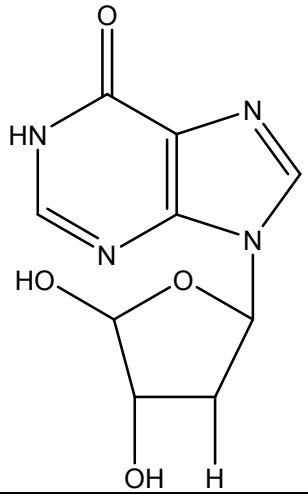
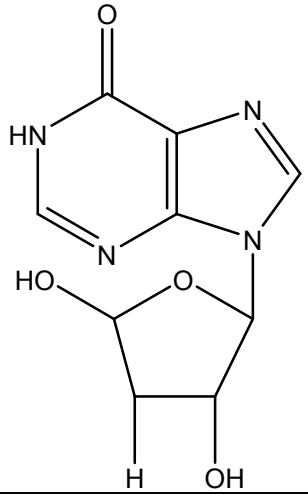
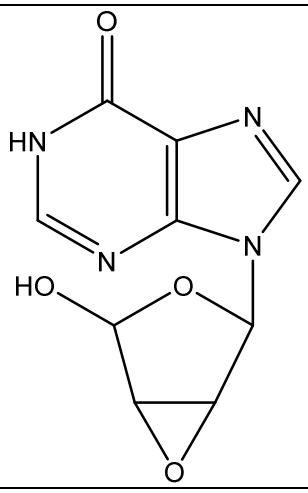
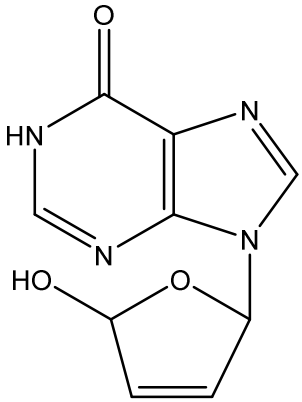
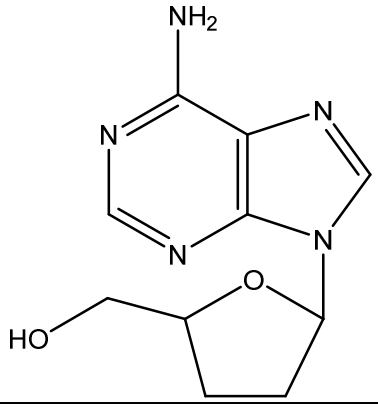
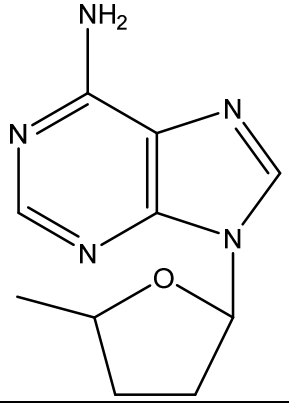
Supplementary table 1: Details of impurities in Didanosine

S.N	IMPURITIES	NAME	STRUCTURE	REFERENCE
1	IMP-A	1, 7-dihydro-6*H*-purin-6-one (hypoxanthine)		International Pharmacopeia 4th edition, Vol. 1
2	IMP-B	9-β-D-ribofuranosyl-1, 9-dihydro-6*H*-purin-6-one (inosine)		International Pharmacopeia 4th edition, Vol. 1
3	IMP-C	9-(2-deoxy-β-D-*erythro*-pentofuranosyl)-1, 9-dihydro-6*H*-purin-6-one (2'-deoxyinosine)		International Pharmacopeia 4th edition, Vol. 1
4	IMP-D	9-(3-deoxy-β-D-*erythro*-pentofuranosyl)-1, 9-dihydro-6*H*-purin-6-one (3'-deoxyinosine)		International Pharmacopeia 4th edition, Vol. 1

5	IMP-E	9-(2, 3-anhydro-β-D-ribofuranosyl)-1, 9-dihydro-6*H*-purin-6-one (2', 3'-anhydroinosine)		International Pharmacopeia 4th edition, Vol. 1
6	IMP-F	9-(2, 3-dideoxy-β-D-*glycero*-pent-2-enofuranosyl]-1, 9-dihydro-6*H*-purin-6-one; (2', 3'-didehydro-2', 3'-dideoxyinosine)		International Pharmacopeia 4th edition, Vol. 1
7	IMP-G	9-(2, 3-dideoxy-β-D-*glycero*-pentofuranosyl)-9*H*-purin-6-amine (2', 3'-dideoxyadenosine)		International Pharmacopeia 4th edition, Vol. 1
8	IMP-H	9-(2, 3, 5-trideoxy-β-D-*glycero*-pentofuranosyl)-9*H*-purin-6-amine (2', 3', 5'-trideoxyadenosine)		International Pharmacopeia 4th edition, Vol. 1