Solvent Tolerant Enzyme and Microbial Systems for Biotransformation Processes

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Introduction

The use of enzymes and genetically engineered biocatalysts for the synthesis of chiral synthons or intermediates as precursors and/or starting materials in asymmetric syntheses is receiving increased attention in the chemical and pharmaceutical industries and academic research laboratories [1]. Biocatalysis offers an attractive green chemistry route to producing chemical intermediates. This has led to the scale-up of an increasing number of biotransformations [2, 3]. An important development in the pharmaceutical industries is the increased demand for chiral drugs in enantiomerically pure form, which has in turn resulted in an increase in the application of biocatalysis in synthetic organic chemistry research over the past decade [4].

The synthetically useful Baeyer-Villiger (BV) oxidation reaction has been carried out using Novozyme-435 from *Candida antartica* lipase B [5] and recombinant whole cells from *Acinetobacter* and *Pseudomonas* species expressing monooxygenases [6]. The BV oxidation reactions involve the insertion of an oxygen atom into the carbon chain of an organic compound adjacent to a carbonyl group effecting a transformation of ketone to an ester in aliphatic substrates or lactones in alicyclic substrates [7]. Monooxygenase-mediated BV oxidations have been reviewed in the literature [2, 8], while more recently the scope and limitations of both biocatalytic methods and organometallic approaches to enantioselective Baeyer-Villiger oxidation have been reported [9].

Water-soluble compounds for biotransformations are easy to handle, while hydrophobic compounds pose a major problem due to their inaccessibility to the biocatalyst [7]. Organic solvents in which the hydrophobic compounds are soluble provide a means of overcoming this problem, with the added advantage of portioning out both substrates and products that can inhibit the biocatalysts in the aqueous phase [7]. However, proteins (enzymes) and microbes are unstable when used for biotransformation processes in organic solvents with low logarithm of the partition coefficient in a standard octanol-water two-phase system [7, 10]

Here, we present the initial results on screening and identification of potential biocompatible organic solvents using a recombinant *Escherichia coli* BL21 (DE3)(pMM4) strain over-expressing cyclohexanone monooxygenase as a model organism for the BV oxidation.

Material and Methods

Micro-organism

A recombinant *Escherichia coli* BL21 (DE3)(pMM4) overexpressing cyclohexanone monooxygenase (CHMO) from *Acinetobacter sp*. NCIB 9871 was used organism. The vector pMM4 with the gene of interest consists of the expression plasmid pET 22b(+) and the 1.6 kb CHMO gene which encodes cyclohexanone monooxygenase (CHMO). The plasmid is 7.02 Kb and has a gene encoding β-lactamase (*bla*) for ampicillin resistance. The CHMO gene follows a T-7 promoter. The expression vector also contains a *lacI* gene, which enables control of gene expression and induction with isopropylthio-β-D-galactoside (IPTG). The *E. coli* construct was obtained from the Chemical and Biochemical Engineering Department at the University of Iowa, and is available from New England Biolabs

It was maintained on LB agar plates (1% bacto-peptone, 0.5% bacto-yeast extract, 1% sodium chloride, 1.5% bacto-agar) containing 200 µg/ml ampicillin. To start a new culture freshly prepared LB agar plates were streaked with single colonies from the stock culture and incubated at 30^{o}C for 24 hours.

Media and growth conditions

A colony from an LB agar plate was used to inoculate 20-mL of liquid LB medium and incubated on a shaker overnight at 37^{o}C and 250 rpm. 1.5-mL of the overnight grown culture was used to inoculate 50-mL of sterilized liquid LB medium containing 200µg/ml of ampicillin in a 250-mL Erlenmeyer flask. The ampicillin stock solution was filter-sterilized using 0.2-µm membrane filters prior to addition to the medium. The culture was shaken at 250 rpm and 37 °C for 3 hours, and 30 µL of 84 mM of isopropyl-thio- ß -D-galactoside (IPTG) stock solution added to give a final concentration of 0.025 mM in the medium. The incubation was continued for three additional hours and was followed by addition of equimolar β-cyclodextrin (142 µL of 17.6mM stock solution) to each of the shake-flasks. Ten appropriately labeled shake-flasks were used.

Three of the shake-flasks after addition of β-cyclodextrin served as controls. Neat methylcyclohexanone (122 µL) was added to three of the shake-flasks immediately following addition of the β-cyclodextrin to give a final substrate concentration of 20 mM. The remaining four shake-flasks had methylcyclohexanone and either cyclohexane or methyl-cyclohexane added sequentially after the addition of the β-cyclodextrin to give 20 mM and 5% v/v final concentrations of substrate and solvent respectively in the medium. The shake-flasks were then shaken at 150 rpm and room temperature for 48 hours. Samples were withdrawn from the shake-flasks for analyses at 6hours intervals.

Chemicals

The substrate, methyl methylcyclohexanone, and the organic solvents (cyclohexane and methyl-cyclohexane) were from Sigma-Aldrich (U.S.A). The bacto-peptone, bacto-yeast extract, sodium chloride, bacto-agar, ampicillin, isopropyl-thio-ß-D-galactoside (IPTG) and β-cyclodextrin were from Fisher Scientific (U.S.A). All chemicals and organic solvents were used as received without purification.

Analytical Methods

Cell growth was monitored by measuring the optical density at 600nm. Biomass was determined by centrifuging 1-ml samples in eppendorf tubes at 10,000g and 25^{o}C for 10minutes using centrifuge, the cell pellet washed thrice with de-ionized distilled water and dried to constant weight in an oven at 105^{o}C. The supernatant was removed and stored at –18^{o}C.

Results and Discussion

Effect of organic solvents

The time course of absorbance and cell dry weight for the controls, shake-flasks with added methylcyclohexanone substrate and shake-flasks with substrate and 5%v/v of cyclohexane and methyl cyclohexane as solvents in the biotransformation are presented in Figures1- 4.

Effect of Cyclohexane

Figures 1 and 2 show the effect of cyclohexane on the absorbance and cell dry weight.

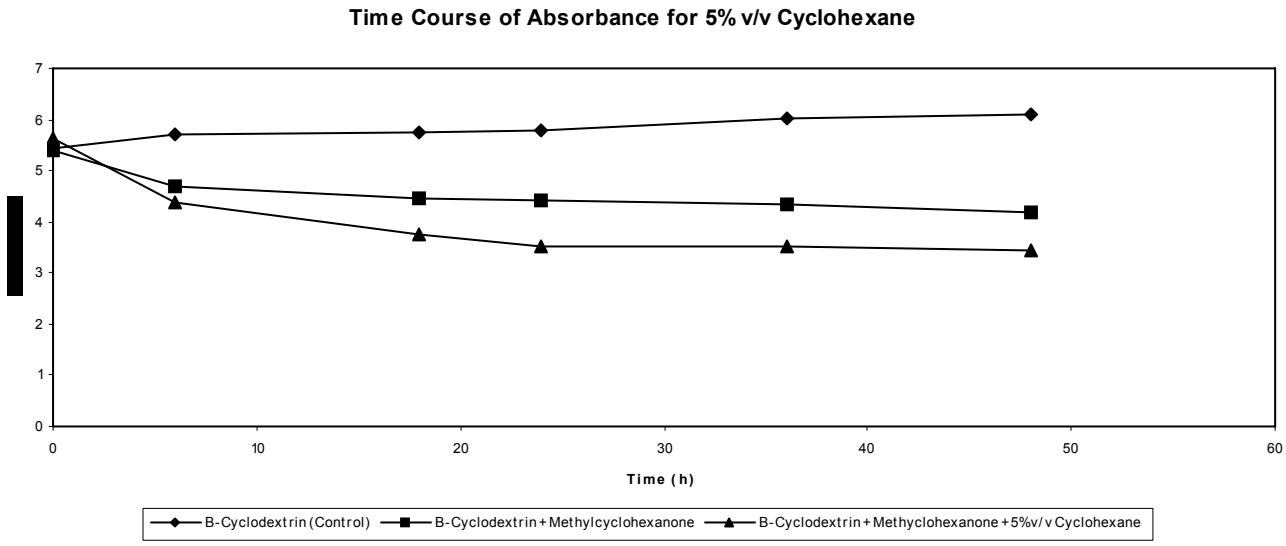

Figure 1: Time course of cell absorbance for 5% cyclohexane

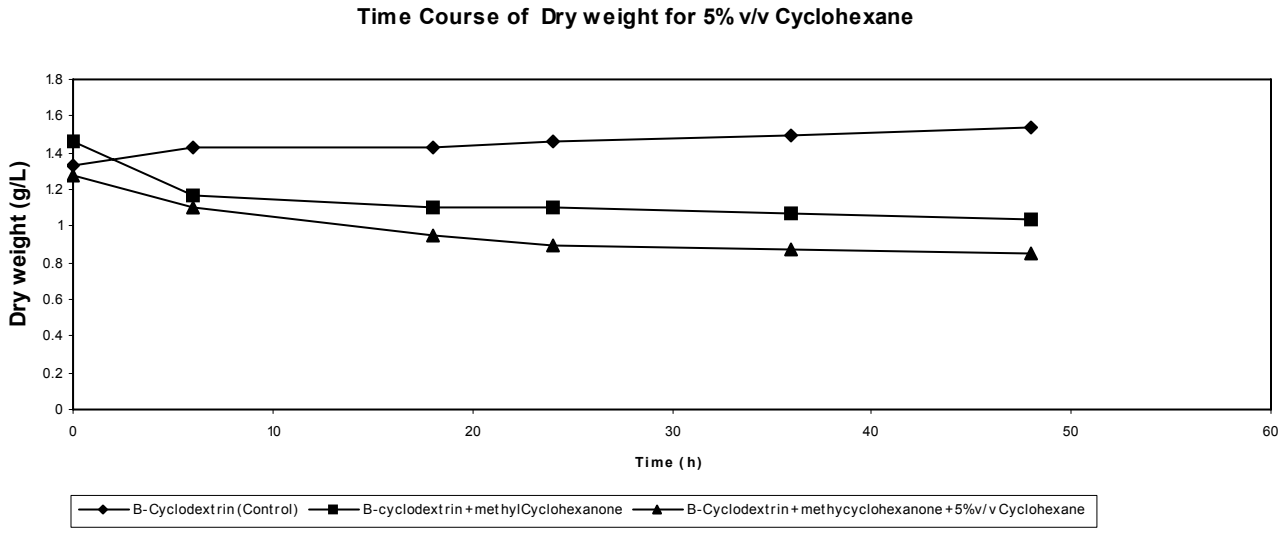

Figure 2: Time course of cell dry weight for 5% cyclohexane.

The data show that absorbance and cell dry weight of the control culture increased by approximately 15% over the 48-hour period. The data also show a general decrease of absorbance and cell dry weight with time for the shake-flasks with methyl cyclohexanone substrate and those with both substrate and 5%v/v of cyclohexane. The decrease in both the

absorbance and cell dry weight over the 48-hour period were 30% and 38% for the cultures containing the substrate and those with both substrate and solvent respectively.

The data in Figures 1 and 2 showing 45% higher decrease in the absorbance and cell dry weight of cultures with 5%v/v cyclohexane compared to the control may be attributed to the toxicity of the substrate. The addition of β-cyclodextrin therefore did not eliminate the substrate toxicity

The results also show that addition of both substrate and 5%v/v cyclohexane led to 53% decrease in both absorbance and cell dry weight compared to the control. The contribution to the decrease of absorbance and cell dry weight by the solvent addition was therefore only 8%. The substrate was thus far more toxic than the solvent which may be considered as fairly biocompatible.

Effect of methyl-cyclohexane

Figures 3 and 4 show the effect of methyl cyclohexane on the absorbance and cell dry weight

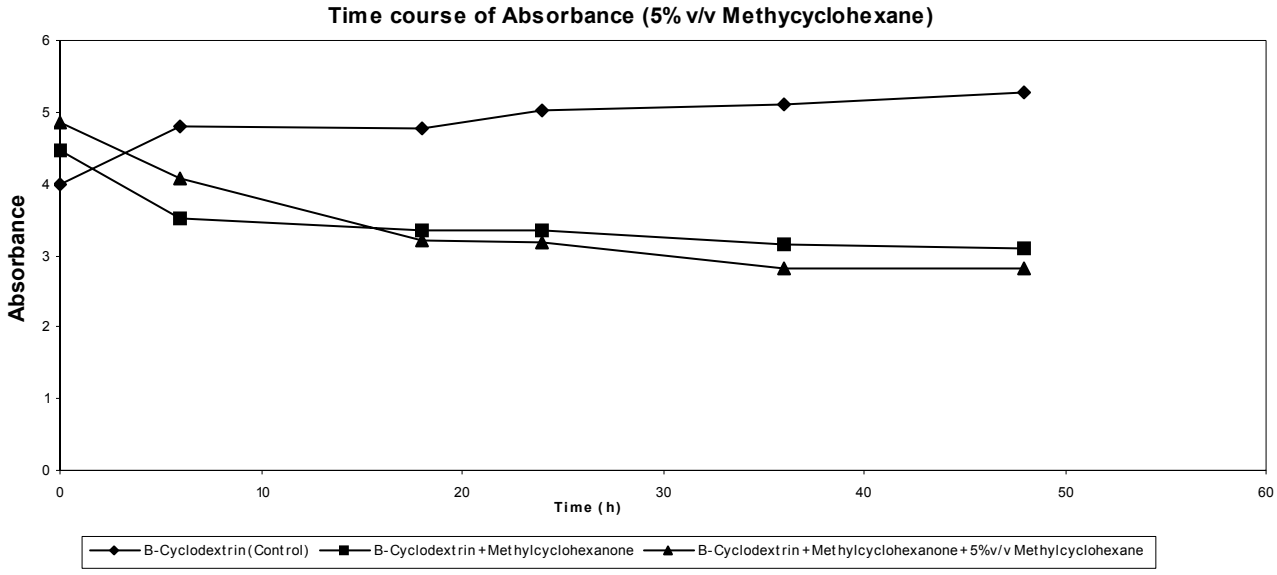

Figure 3: Time course of cell absorbance for 5% methyl cyclohexane

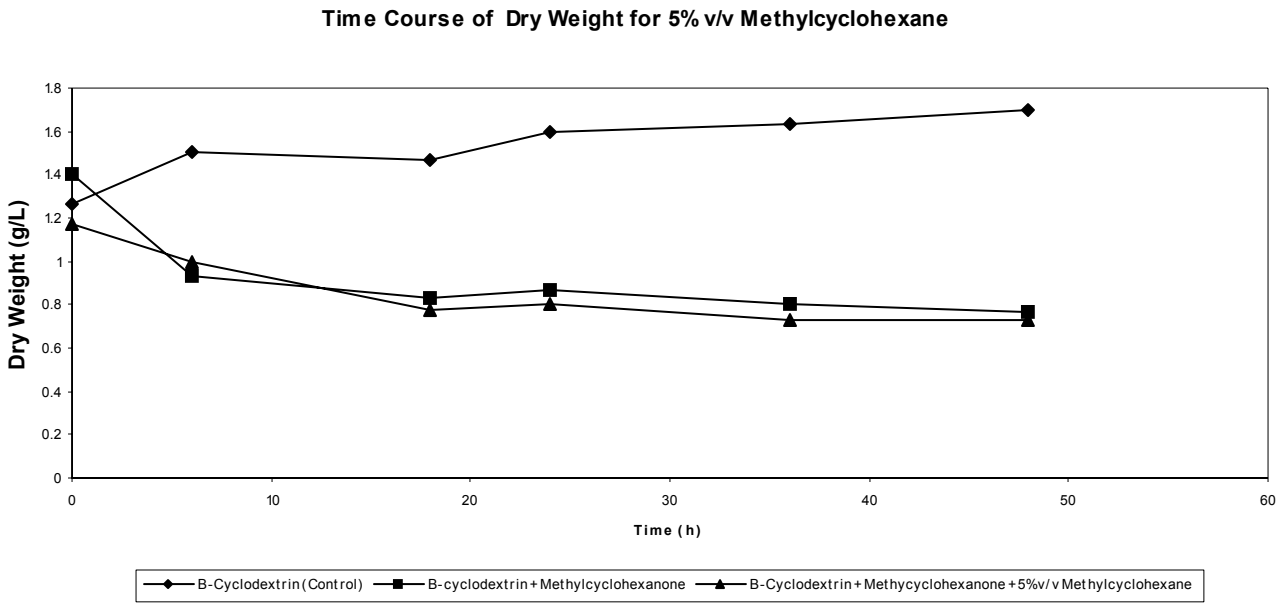

Figure 4: Time course of cell dry weight for 5% methyl cyclohexane

. The control was the same as that used for studying the effect of cyclohexane addition. The data also show a general decrease of absorbance and cell dry weight with time for the shake-flasks with methylcyclohexanone substrate and those with both substrate and 5%v/v of methyl-cyclohexane. The decrease in both the absorbance and cell dry weight over the 48-hour period were 31% and 42% for the cultures containing the substrate and those with both substrate and solvent respectively,

The data in Figure 3 and 4 showing 46% higher decrease in the absorbance and cell dry weight of cultures with 5%v/v methyl-cyclohexane compared to the control may also be attributed to the toxicity of the substrate as was the case when 5%v/v cyclohexane was used as solvent.

The results also show that addition of both substrate and 5%v/v cyclohexane led to 57% decrease in both absorbance and cell dry weight compared to the control. The contribution to the decrease of absorbance and cell dry weight due to solvent addition was therefore, only 11%. Again the substrate was far more toxic than the solvent which may be considered as fairly biocompatible.

Comparison of solvents

Figures 5 and 6 compare the effect on the biotransformation by addition of methyl cyclohexane and cyclohexane at 5% v/v concentration.

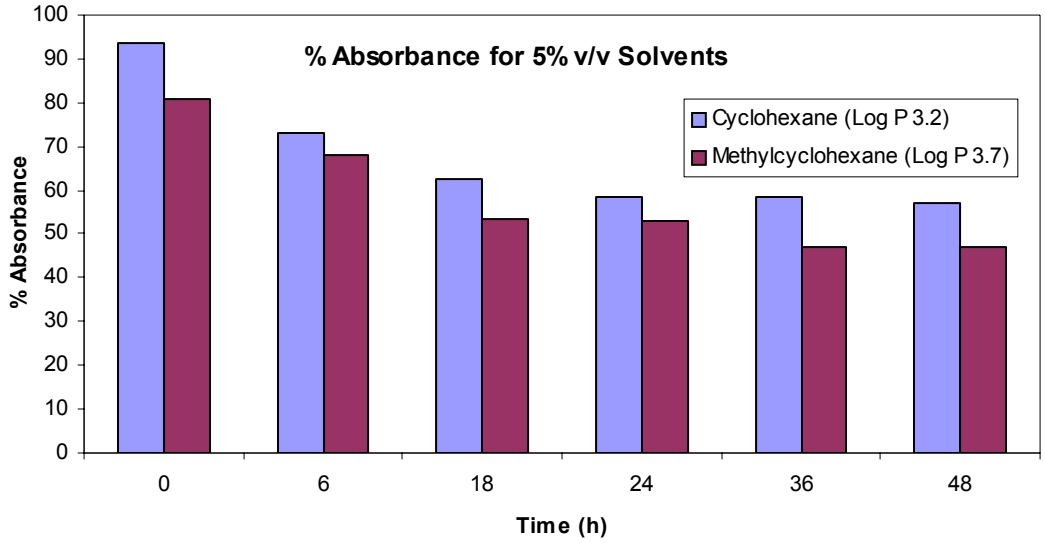

Figure 5: Time course of cell absorbance using organic solvents

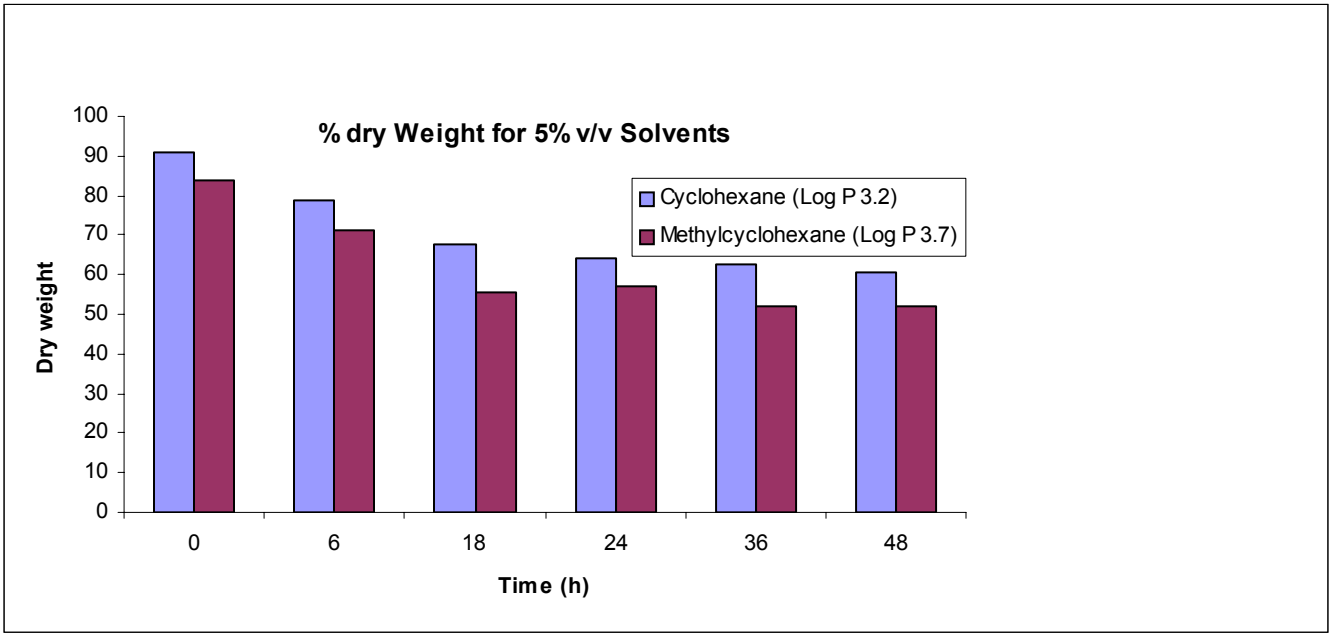

Figure 6: Time course of cell dry weight using organic solvents

The absorbance and cell dry weight of, the cultures containing cyclohexane were approximately 16% greater than those with methyl cyclohexane over the 48-hour period.

Since methyl cyclohexane with a log P value of 3.7 was expected to be more biocompatible than cyclohexane with a log P value of 3.2 [10], therefore, the absorbance and cell dry weight obtained were the opposite of what was expected.

The unexpected higher values of absorbance and cell dry weight using the cyclohexane may be due to the presence of inhibitory/toxic impurities in the methyl cyclohexane since it was used as received without purification.

Planned research now underway includes:(1) Determination of the cell viability with time by plating appropriate dilution of biotransformation samples on the agar plate and counting the colony forming units with incubation at 37^{o}C and (2) monitoring of substrate disappearance and product formation with time using different solvents with log P value between 3.2 and 6.6.

Acknowledgments

We thank our research partner at University of Iowa, Department of Chemical and Biochemical Engineering for providing *Escherichial coli* BL21 (DE3)(pMM4). This work was supported by a grant from the Center for Environmentally Beneficial Catalysis (CEBC), a National Science Foundation-Engineering Research Center (NSF-ERC)

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