REPORT DOCUMENTATION PAGE

1. AGENCY USE ONLY (Leave blank)  2. REPORT DATE  3. REPORT TYPE AND DATES COVERED
   October 30, 1997  Final Progress 6/20/94 – 6/19/97

4. TITLE AND SUBTITLE
   Enzyme Design for Nonaqueous Solvents

6. AUTHOR(S)
   Jonathan S. Dordick¹
   Douglas S. Clark²

7. PERFORMING ORGANIZATION NAMES(S) AND ADDRESS(ES)
   ¹Department of Chemical & Biochemical Engineering,
   125 Chemistry Bldg, The University of Iowa, Iowa City, IA
   52242.  ²Department of Chemical Engineering, University
   of California at Berkeley, Berkeley, CA 94720–9989.

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
   U.S. Army Research Office
   P.O. Box 12211
   Research Triangle Park, NC 27709-2211

11. SUPPLEMENTARY NOTES
   The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as
   an official Department of the Army position, policy or decision, unless so designated by other documentation.

12. DISTRIBUTION / AVAILABILITY STATEMENT
   Approved for public release; distribution unlimited.

ABSTRACT (Maximum 200 words)

   From a biotechnological perspective there are many advantages of employing enzymes in organic as opposed to aqueous media. While there is now little question that enzymes can function in organic media, reaction rates are typically quite low. Relatively few kinetic studies have been carried out, and detailed investigations of enzyme structure and dynamics are fewer still. The overall goal of this ARO-sponsored research has been to identify the factors that govern enzyme activity and specificity in organic solvents, and apply this knowledge in the design of enzyme catalysts with optimal (i.e., aqueous-like) activity in nonaqueous media. In addition, this research set out to expand on our recent success in activating enzymes for use in organic solvents - in some cases activations of greater than 1000-fold have been achieved - and to understand fundamentally the factors that govern enzyme activity and enzyme activation in organic solvents. The knowledge gained in this investigation can be applied to a more general design of enzyme catalysts with optimal (i.e., aqueous-like) activity and tailored selectivity in nonaqueous media.

14. SUBJECT TERMS
   enzymes in organic solvents, enzyme activation in dehydrated media, solvent effects on enzyme mechanisms, subtilisin catalysts

15. NUMBER OF PAGES
   9

16. PRICE CODE
   U

17. SECURITY CLASSIFICATION OR REPORT
   UNCLASSIFIED

18. SECURITY CLASSIFICATION OF THIS PAGE
   UNCLASSIFIED

19. SECURITY CLASSIFICATION OF ABSTRACT
   UNCLASSIFIED

20. LIMITATION OF ABSTRACT
   UL

NSN 7540-01-280-5500

Enclosure 1

Standard Form 298 (Rev. 2-88)  Prescribed by ANSI Std. 298-18
298-102
<table>
<thead>
<tr>
<th>Block 1</th>
<th>Agency Use Only (Leave blank)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block 2</td>
<td>Report Date. Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least year.</td>
</tr>
<tr>
<td>Block 3</td>
<td>Type of Report and Dates Covered. State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).</td>
</tr>
<tr>
<td>Block 4</td>
<td>Title and Subtitle. A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.</td>
</tr>
<tr>
<td>Block 5</td>
<td>Funding Numbers. To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:</td>
</tr>
<tr>
<td></td>
<td>C - Contract  PR - Project</td>
</tr>
<tr>
<td></td>
<td>G - Grant  TA - Task</td>
</tr>
<tr>
<td></td>
<td>PE - Program  WU - Work Unit</td>
</tr>
<tr>
<td></td>
<td>Element  Accession No.</td>
</tr>
<tr>
<td>Block 6</td>
<td>Author(s). Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).</td>
</tr>
<tr>
<td>Block 7</td>
<td>Performing Organization Name(s) and Address(es). Self-explanatory.</td>
</tr>
<tr>
<td>Block 8</td>
<td>Performing Organization Report Number. Enter the unique alphanumeric report number(s) assigned by the organization performing the report.</td>
</tr>
<tr>
<td>Block 9</td>
<td>Sponsoring/Monitoring Agency Name(s) and Address(es). Self-explanatory.</td>
</tr>
<tr>
<td>Block 10</td>
<td>Sponsoring/Monitoring Agency Report Number. (If known)</td>
</tr>
<tr>
<td>Block 11</td>
<td>Supplementary Notes. Enter information not included elsewhere such as; prepared in cooperation with...; Trans. of...; To be published in... When a report is revised, include a statement whether the new report supersedes or supplements the older report.</td>
</tr>
<tr>
<td>Block 12a</td>
<td>Distribution/Availability Statement. Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NORFORD, REL, ITAR).</td>
</tr>
<tr>
<td></td>
<td>DOD - See DoD 4230.25, “Distribution Statements on Technical Documents.”</td>
</tr>
<tr>
<td></td>
<td>DOE - See authorities.</td>
</tr>
<tr>
<td></td>
<td>NTIS - Leave blank.</td>
</tr>
<tr>
<td>Block 12b</td>
<td>Distribution Code.</td>
</tr>
<tr>
<td></td>
<td>DOD - Leave blank</td>
</tr>
<tr>
<td></td>
<td>DOE - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports</td>
</tr>
<tr>
<td></td>
<td>NASA - Leave blank.</td>
</tr>
<tr>
<td></td>
<td>NTIS - Leave blank.</td>
</tr>
<tr>
<td>Block 13</td>
<td>Abstract. Include a brief (Maximum 200 words) factual summary of the most significant information contained in the report.</td>
</tr>
<tr>
<td>Block 14</td>
<td>Subject Terms. Keywords or phrases identifying major subjects in the report.</td>
</tr>
<tr>
<td>Block 15</td>
<td>Number of Pages. Enter the total number of pages.</td>
</tr>
<tr>
<td>Block 16</td>
<td>Price Code. Enter appropriate price code (NTIS only).</td>
</tr>
<tr>
<td>Block 17 - 19</td>
<td>Security Classifications. Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.</td>
</tr>
<tr>
<td>Block 20</td>
<td>Limitation of Abstract. This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.</td>
</tr>
</tbody>
</table>
ENZYME DESIGN FOR NONAQUEOUS SOLVENTS

JONATHAN S. DORDICK, PH.D. AND DOUGLAS S. CLARK, PH.D.

OCTOBER 30, 1997

U.S. ARMY RESEARCH OFFICE

CONTRACT/GRANT NUMBER: DAAH04-94-G-0307

UNIVERSITY OF IOWA, DEPARTMENT OF CHEMICAL AND BIOCHEMICAL ENGINEERING
AND

UNIVERSITY OF CALIFORNIA, BERKELEY, DEPARTMENT OF CHEMICAL ENGINEERING

APPROVED FOR PUBLIC RELEASE;
DISTRIBUTION UNLIMITED

THE VIEWS, OPINIONS, AND/OR FINDINGS CONTAINED IN THIS REPORT ARE THOSE OF THE AUTHORS AND SHOULD NOT BE CONSTRUED AS AN OFFICIAL DEPARTMENT OF THE ARMY POSITION, POLICY, OR DECISION, UNLESS SO DESIGNATED BY OTHER DOCUMENTATION.
1. FORWARD

From a biotechnological perspective there are many advantages of employing enzymes in organic as opposed to aqueous media. While there is now little question that enzymes can function in organic media, reaction rates are typically quite low. Relatively few kinetic studies have been carried out, and detailed investigations of enzyme structure and dynamics are fewer still. The overall goal of this ARO-sponsored research has been to identify the factors that govern enzyme activity and specificity in organic solvents, and apply this knowledge in the design of enzyme catalysts with optimal (i.e., aqueous-like) activity in nonaqueous media. In addition, this research set out to expand on our recent success in activating enzymes for use in organic solvents - in some cases activations of greater than 1000-fold have been achieved - and to understand fundamentally the factors that govern enzyme activity and enzyme activation in organic solvents. The knowledge gained in this investigation can be applied to a more general design of enzyme catalysts with optimal (i.e., aqueous-like) activity and tailored selectivity in nonaqueous media.

2. TABLE OF CONTENTS (IF MORE THAN 10 PAGES)

   Not Applicable

3. LIST OF APPENDIXES, ILLUSTRATIONS AND TABLES

   Not Applicable

4. BODY OF REPORT

   A. STATEMENT OF THE PROBLEM STUDIED

   The goals of this ARO-sponsored research were as follows:

   1. **To investigate the effect of solvent properties (e.g., hydrophobicity, polarity, hydration) on enzyme activity and specificity.**

   2. **To tailor and improve enzyme activity in organic solvents by a) protein engineering, b) solvent engineering, and c) catalyst engineering (e.g., use of supports, additives, and natural stabilizers).**

   3. **To control enzyme specificity by protein and solvent engineering.**

   4. **To develop rational and predictive guidelines for biocatalyst optimization in organic solvents.**

   Much of this work was performed using the serine proteases subtilisin (Carlsberg and BPN') and α-chymotrypsin, as well as the non-serine protease, thermolysin. Instead of a broad summary of the results and impact of this work, we provide a complete compilation of the abstracts of publications that have resulted from this research. A detailed bibliography of these publications is given in Section C below.
B. SUMMARY OF PUBLISHED RESULTS


The catalytic efficiencies ($k_{cat}/K_m$) of subtilisin Carlsberg and $\alpha$-chymotrypsin in anhydrous organic solvents are dramatically increased when each enzyme is lyophilized in the presence of excess salts. For example, a biocatalyst powder containing 98% (w/w) KCl, and 1% (w/w) each of phosphate buffer and subtilisin was 3,750-times more active in n-hexane for the transesterification reaction between N-Ac-L-Phe-OEt and 1-propanol than the enzyme prepared without KCl. This activation was primarily due to a large increase in the catalytic turnover ($k_{cat}$) and provides for catalytic efficiencies in n-hexane to within an order of magnitude of that obtained for hydrolytic reactions in aqueous solutions. The activation was also observed in other organic solvents as well as with other salts indicating that the effect may be general. Enzymatic catalysis in the gas phase was not dramatically affected by the presence of salt in the lyophilized mixture suggesting that salt does not act as a lyoprotectant, but rather protects the enzyme from direct interaction (and inactivation) by the organic solvent. We hypothesize that a highly polar salt matrix is formed that excludes direct solvent contact with the enzyme and helps to maintain the native structure of the enzyme.


$\alpha$-Chymotrypsin (CT) is easily extracted from an aqueous phase into isoctane containing a low concentration of surfactant (≤ 2 mM AOT) via the formation of ion pairs between the protein and the surfactant. The low surfactant concentration eliminates the formation of reversed micelles. Subsequent drying of the enzyme-containing isoctane phase results in an extensively dehydrated soluble enzyme in isoctane. This soluble enzyme preparation can then be dissolved in a wide variety of organic solvents. Soluble CT is highly active in isoctane, with a $k_{cat}/K_m$ for the transesterification of Ac-L-Phe-OEt with n-propanol of 3,020 M$^{-1}$s$^{-1}$ which is ca. 15% of that in aqueous buffer for Ac-L-Phe-OEt hydrolysis. The soluble enzyme is ca. 2,500-fold more active than CT suspended in isoctane. Spectral analysis of soluble CT in organic solvents do not reveal any significant changes as compared to soluble CT in water, hence the soluble enzyme retains its native secondary and tertiary structure. The highly active soluble CT was used for thermodynamically-controlled peptide synthesis resulting in efficient synthesis of dipeptides in yields approaching 100%. In addition to CT, subtilisin Carlsberg was dissolved in isoctane in a similar manner and shows a value of $k_{cat}/K_m$ for the transesterification of Ac-L-Phe-OEt and n-propanol of 68% of that for hydrolysis in aqueous buffer.


A novel type of organosoluble biocatalyst, representing a non-covalent complex of enzyme with a sugar-based amphiphilic polymer is described. The subtilisin Carlsberg-palmitoyl poly(sucrose acrylate) complex was found to be soluble and catalytically active in a number of organic solvents of different nature.

Hydrophobic interactions are important in numerous biological processes; however, the nature and extent of hydrophobic interactions in nonaqueous enzymology remain poorly defined. We have estimated the free energies of enzyme-substrate hydrophobic interactions for a model reaction catalyzed by subtilisin BPN' (from Bacillus amyloliquefaciens). Transition state stabilization of subtilisin in water has contributions from both ground state destabilization of hydrophobic substrates in water and intrinsic enzyme-substrate hydrophobic interactions. Both contributions are evident even in hydrophobic organic solvents, and can be modified by protein engineering of the enzyme's binding site as well as by changing the hydrophobicity of the reaction medium. We have also developed a method to estimate the hydrophobicity of the enzymic transition state involving systematic variation of the substrate and solvent hydrophobicities. The observed binding pocket hydrophobicities were directly affected by replacing the Gly166 residue, located at the back of the hydrophobic S1 binding pocket of subtilisin BPN', with more hydrophobic amino acids such as alanine and valine. Thus, the observed S1 binding pocket hydrophobicities of the wild-type, G166A, and G166V mutants were measured to be 1.2, 1.8, and 2.6 log P units, respectively. Our method of calculating effective binding pocket hydrophobicity was found to be applicable to other enzymes including horseradish peroxidase and α-chymotrypsin. The measurements of the binding pocket hydrophobicities have significant implications toward tailoring enzyme function in aqueous as well as nonaqueous media.


Comparing the behavior of freely suspended and immobilized enzyme in organic media with low water contents can yield insights into interactions among the biocatalyst, solvent, and support that influence protein structure and function. Immobilized chymotrypsin has higher activity than the suspended enzyme (by one to two orders of magnitude) in anhydrous organic solvents ranging from nonpolar n-octane to polar acetonitrile. In anhydrous tetrahydrofuran (THF), glass-adsorbed chymotrypsin is ca. ten-times more active than the suspended enzyme, and electron spin resonance (ESR) spectra of an active-site spin label reveal greater local flexibility. Upon adding up to 0.5% v/v water, increased catalytic efficiency of the immobilized enzyme is accompanied by a sharp rise in active-site polarity but no apparent change in active-site conformation or dynamics. Under the same conditions the activity of the suspended enzyme also increases; however, the active-site polarity remains nearly constant while the spin label reflects increasing molecular flexibility. For both preparations, further changes in protein structure occur as enzyme activity increases with 0.5 to 7% v/v added water. Computer simulations of the room-temperature ESR spectra suggest that different initial conformational states contribute to the different behavior of the two enzyme systems over the entire range of added water. These findings show that the structural properties of suspended and immobilized enzyme can differ markedly and that these differences are important to enzyme activity in organic media.

Calculation of kinetic constants of an enzymatic reaction in organic solvents requires knowledge of the functional active-site concentration in organic solvents, and this can be significantly different than that in water. An experimental method for active-site titration of serine proteases in organic media has been developed based on the kinetics of inhibition by phenylmethanesulfonyl fluoride (PMSF), a serine-specific inhibitor (or suicide substrate). This kinetic approach is fundamentally different from other techniques that require complete titration of all accessible enzyme active sites. This active site titration method was applied to subtilisins BPN' and Carlsberg, and α-chymotrypsin, and resulted in fractions of active sites that ranged from 8 to 62% (of the fraction active in water) depending on the enzyme, the method of enzyme preparation, and the organic solvent used. The active site concentration of subtilisin BPN' and Carlsberg increased with increasing hydrophobicity of the solvent, and with increasing solvent hydration in tetrahydrofuran. The dependence of the fraction of active sites on the nature of the organic solvent appears to be governed largely by solvent-induced inactivation caused by direct interaction of a hydrophilic solvent with the enzyme.


The rational control over enzyme-catalyzed regioselectivity has been studied using sucrose acylation by vinyl esters in organic media as a model. Subtilisins BPN' and Carlsberg preferentially acylate at the 1'-hydroxyl of sucrose with some acylation observed at the 6-hydroxyl. The preference for the 1'-hydroxyl is strongly affected by the hydrophobicity of the organic solvent and the chain length of the vinyl ester. Increasingly hydrophobic solvents and longer chain lengths lower the favorable formation of the 1'-acylation and improve 6-acylation. Molecular modeling of sucrose in the binding pocket of subtilisin BPN' shows that the 1'-acylation is favored in solvents that can solvate sugars (such as pyridine) as the glucose moiety is exposed to the medium, whereas 6-acylation leaves the entire sucrose molecule buried within the enzyme's binding pocket. Thus, 1'-acylation is stericly more favorable than 6-acylation. Increasingly hydrophobic solvents affect regioselectivity by changing the degree of solvation of the glucose moiety in the medium and forcing the sucrose 1'-ester completely into the binding pocket. In a related modeling, the vinyl ester chain length was shown to modulate regioselectivity by controlling the bond angles between the resulting acyl- enzymes and the sucrose thereby affecting the positioning of the sucrose in the binding pocket of subtilisin BPN'. This study shows that control over enzymic regioselectivity can be achieved by rational choices of substrate and solvent.


Enzyme structure and function have been studied for subtilisin BPN' solubilized in organic solvents by ion pairing with low concentrations of an anionic surfactant (Aerosol OT) in the absence of reversed micelles. Soluble subtilisin shows strikingly different behavior in octane and tetrahydrofuran (THF). In octane, the $k_{cat}/K_{m}$ for the transesterification of N-Acetyl L-Phenylalanine Ethyl Ester (APEE) is 370 M$^{-1}$s$^{-1}$, within one order of magnitude of the enzyme's hydrolytic activity in water. Moreover, the
observed half-life of the soluble enzyme in octane is nearly three orders of magnitude greater than in water, presumably due to the absence of autolysis in the organic solvent. In contrast, the catalytic efficiency of the enzyme dissolved in the polar solvent THF is 0.04 M⁻¹s⁻¹, and the enzyme loses 99% of its activity within 10 min. Comparable enzyme inactivation could also be observed in octane, but only at elevated temperatures such as 70°C. Therefore, the mechanisms of deactivation of the soluble enzyme were investigated in both octane and THF. Kinetic and spectroscopic (CD and EPR) studies support the existence of multiple inactive forms of the soluble enzyme in THF at 25°C and in octane at 70°C. Notably, in both cases a denatured form can be renatured in anhydrous octane at 25°C, the first demonstration of enzyme renaturation in a bulk organic solvent. A model explaining the THF- and thermally-induced inactivation processes of soluble subtilisin BPN' is proposed, and the apparent reasons for the exceptionally high activity and stability of the soluble enzyme in octane are discussed.


The activity and substrate specificity of subtilisin-catalyzed acylation of nucleosides in organic solvents can be controlled by lyophilizing the enzyme from an aqueous solution containing the substrate. This “molecular imprinting” technique was examined using thymidine as a model nucleoside, and the resulting subtilisin preparation was up to 50-fold more reactive toward thymidine acylation in nearly anhydrous tetrahydrofuran than subtilisin lyophilized from aqueous buffer in the absence of the nucleoside. Although several compounds, including thymine and ribose, improved the rate of thymidine acylation, the thymidine-imprinted enzyme was the most efficient catalyst for this reaction. Furthermore, it was possible to alter the substrate selectivity of subtilisin by lyophilizing the enzyme in the presence of a different nucleophilic substrate. For example, imprinting made possible the discrimination between structurally different (i.e., sucrose versus thymidine) as well as structurally similar (i.e., thymidine versus deoxyadenosine) nucleophiles. Molecular modeling studies of the interaction of thymidine or the unrelated sucrose with subtilisin revealed that structural changes upon imprinting in the serine protease’s catalytic triad may be responsible for the observed activation and selectivity changes. Further use of molecular dynamics indicated that structural changes in the catalytic triad occur during imprinting, and that these changes may be the major factor that contributes to imprinting-induced substrate selectivity. This contrasts with the previously held notion that imprinting influences mainly substrate binding.


The transition state for subtilisin-catalyzed transesterification was probed by high-pressure kinetic studies in solvents spanning a wide range of dielectric constants. The electrostatic model of Kirkwood described the solvent effects and gave a lower limit of 31±1.5 Debye for the dipole moment of the transition state. This value remained constant in a wide range of polar and apolar solvents, indicating that the catalytic triad of subtilisin is remarkably robust. Despite the highly polar transition state, substantial rate enhancements relative to the uncatalyzed reaction were measured in highly apolar solvents such as hexane; this is the first report of such an extreme disparity between transition-state and solvent polarities. Moreover, the solvent dependence of the activation volume implies a low effective dielectric of the polypeptide chain in the active site, and substantial penetration of the active site by solvent. Kirkwood’s model
was also used to quantify the effect of an active-site mutation on the transition-state dipole moment. These results illustrate that the electrostatic model combined with high-pressure kinetics can provide unique information on the basic properties of enzyme reaction processes, and can be useful in predicting solvent effects on enzyme reaction rates.


Multinuclear NMR spectroscopy has been used to study water bound to subtilisin Carlsberg suspended in tetrahydrofuran (THF), with the water itself employed as a probe of the hydration layer's physicochemical and dynamic characteristics. The presence of the enzyme did not affect the intensity, chemical shift or linewidth of water (up to 8% v/v) added to THF, as measured by $^{19}$O- and $^2$H-NMR. This finding suggests that hydration of subtilisin can be described by a three-state model that includes tightly bound, loosely bound, and free water. Solid-state $^2$H-NMR spectra of enzyme-bound D$_2$O support the existence of a non-exchanging population of tightly bound water. An important implication is that the loosely-bound water is the same as free water from an NMR viewpoint. This loosely-bound water must also be the water responsible for the large increase in catalytic activity observed in previous hydration studies.


The dramatic activation of serine proteases in nonaqueous media resulting from lyophilizing in the presence of KCl, first reported by Khmelnitsky et al. (1994), is shown to be unrelated to relaxation of potential substrate diffusional limitations. Specifically, lyophilizing subtilisin Carlsberg in the presence of KCl and phosphate buffer in different proportions, ranging from 99% (w/w) enzyme to 1% (w/w) enzyme in the final lyophilized solids, resulted in biocatalyst preparations that were not influenced by substrate diffusion, and thereby retained their kinetically-controlled properties. This result was evident through use of a classical analysis whereby the initial rates of catalysis as a function of active enzyme in the biocatalyst preparation, normalized per constant volume of catalyst material, were measured for all biocatalyst preparations studied. Plots of initial reaction rates as a function of the percent of active subtilisin in the biocatalyst preparations were linear for all biocatalyst preparations. Thus, enzyme activation (reported by Khmelnitsky et al. (1994) to be as high as 3750-fold in hexane for the transesterification of N-Ac-L-Phe-OEt with n-PrOH) is a manifestation of intrinsic enzyme activation and not relaxation of diffusional limitations resulting from diluted enzyme preparations. Similar activation is reported for thermolysin, a non-serine protease, thereby demonstrating that enzyme activation due to lyophilizing in the presence of KCl may be a general phenomenon.

C. LIST OF PUBLICATIONS


D. LIST OF PARTICIPATING SCIENTIFIC PERSONNEL AND ADVANCED DEGREE EARNED

Yuri Khmelnitsky - Postdoctoral scientist (1992-1994), U. Iowa
Jungbae Kim - Ph.D. awarded, 5/95, U. Iowa
Pramod Wangikar - Ph.D. awarded, 5/95, U. Iowa
Valerie Suzawa - Ph.D. awarded 8/95, U. C. Berkeley
Joseph Rich - Ph.D. awarded 8/96, U. Iowa
Bruce Bedell - M.S. Candidate (1995 - present), U. Iowa
Peter Michels - Ph.D. awarded 10/96, U. C. Berkeley
Christopher Lee - M.S. awarded 12/96, U. C. Berkeley
Vadim Mozhaev - Postdoctoral scientist (1996 - present), U. Iowa
Michael Ru - Ph.D. Candidate (1996 - present), U. C. Berkeley
5. REPORT OF INVENTIONS ONLY


6. BIBLIOGRAPHY

Not Applicable

7. APPENDICES

Not Applicable