27 January 2015

Final Report

Optimizing Immobilized Enzyme Performance in Cell-Free Environments to Produce Liquid Fuels

Grant No. DE-SC0006520

Funding Period Request: February 1, 2011 - August 31, 2014

by

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Abstract

Limitations on biofuel production using cell culture (*Escherichia coli*, *Clostridium*, *Saccharomyces cerevisiae*, brown microalgae, blue-green algae and others) include low product (alcohol) concentrations (≤0.2 vol%) due to feedback inhibition, instability of cells, and lack of economical product recovery processes. To overcome these challenges, an alternate simplified biofuel production scheme was tested based on a cell-free immobilized enzyme system. Using this cell free system, we were able to obtain about 2.6 times higher concentrations of iso-butanol using our non-optimized system as compared with live cell systems. This process involved two steps: (i) converts acid to aldehyde using keto-acid decarboxylase (KdcA), and (ii) produces alcohol from aldehyde using alcohol dehydrogenase (ADH) with a cofactor (NADH) conversion from inexpensive formate using a third enzyme, formate dehydrogenase (FDH). To increase stability and conversion efficiency with easy separations, the first two enzymes were immobilized onto methacrylate resin. Fusion proteins of labile KdcA (fKdcA) were expressed to stabilize the covalently immobilized KdcA. Covalently immobilized ADH exhibited long-term stability and efficient conversion of aldehyde to alcohol over multiple batch cycles without fusions. High conversion rates and low protein leaching were achieved by covalent immobilization of enzymes on methacrylate resin. The complete reaction scheme was demonstrated by immobilizing both ADH and fKdcA and using FDH free in solution. The new system without *in situ* removal of isobutanol achieved a 55% conversion of ketoisovaleric acid to isobutanol at a concentration of 0.5 % (v/v). Further increases in titer will require continuous removal of the isobutanol using our novel brush membrane system that exhibits a 1.5 fold increase in the separation factor of isobutanol from water versus that obtained for commercial silicone rubber membranes. These bio-inspired brush membranes are based on the presence of glycocalyx filaments coating the luminal surface of our vasculature and represent a new class of synthetic membranes. They, thus, meet the requirements/scope of the Bimolecular Materials program, Materials Science and Engineering Div., Office of Science, US DOE.

Goals and Plans:

The increasing demand to find more carbon neutral energy sources has motivated the search for biologically-derived fuel products. The largest concern facing alcohol-based biofuels is the ability to develop an efficient high-yield commercially available production process[1]. Exciting new biologically based methods for the production of high value liquid fuels have recently been reported[2].

The research funded by this grant for the past year focused on the bioconversion of acids to aldehydes to alcohol (butanol) using a two-enzyme system. While this enzymatic route offers great promise and excellent selectivity for the production of biofuels, enzymes exhibit slow kinetics, low volume capacity in solution and product feedback inhibition. These limitations have to be overcome so that biofuels can be produced economically. A novel approach is used here to address these limitations. Enzymes synthesized via recombinant DNA technology are immobilized on a solid substrate in order to stabilize them and allow the product to continuously be removed while retaining catalyst. This cell-free enzyme system will be coupled with a separation technique, possibly pervaporation, to constantly remove the desired butanol. Thus we address slow kinetics (genetic mutation, enzyme coupling and removal of inhibitory product), low volume capacity (immobilization and stabilization of enzymes) and product feedback inhibition (product removal) with our approach.

We offer an alternate simplified biofuel production approach to cell culture with the hope of overcoming all three limitations listed above, while speeding up the process considerably and possibly reducing the cost of fuel production. Our semi-*in vitro* partial cell-free scheme requires the following steps (**Fig. 1**):

(i) *E. Coli cells* Production/isolation of two critical enzymes (ketoacid decarboxilase, KdcA, and alcohol dehydrogenase, ADH) using standard fermentation.

(ii) *Starting Substrate: Streptomyces cinnamonensis* mutants overproduce 2-ketoisovaleric acid to titers of 2.4 g/L[3].

(iii) *Cell-free.* Simultaneous *in vitro* application of the two enzymes (KdcA and ADH) attached to solid substrates to convert acid (ketoisovaleric acid) to aldehyde (isobutyraldehyde) and then to alcohol (isobutanol, the fuel)

(iv) *Recovery:* Continuous removal and recovery of isobutanol in order to drive the reactions toward isobutanol[4].

This process is attractive because there are no cells that undergo product feed-back inhibition, enzymes are produced at high titer using standard fermentation *sans* feed-back limitations (step (i) above), protein engineering is used to stabilize the two enzymes to high product titer and high temperature (step (iii)), enzymes are stabilized through immobilization (step (iii)), and the reaction is driven toward product through its continual removal of alcohol (recovery) (step (iv)). We published three manuscripts and plan to publish two more. We first optimize the immobilization of two tetramer enzymes, β- galactosidase (control)

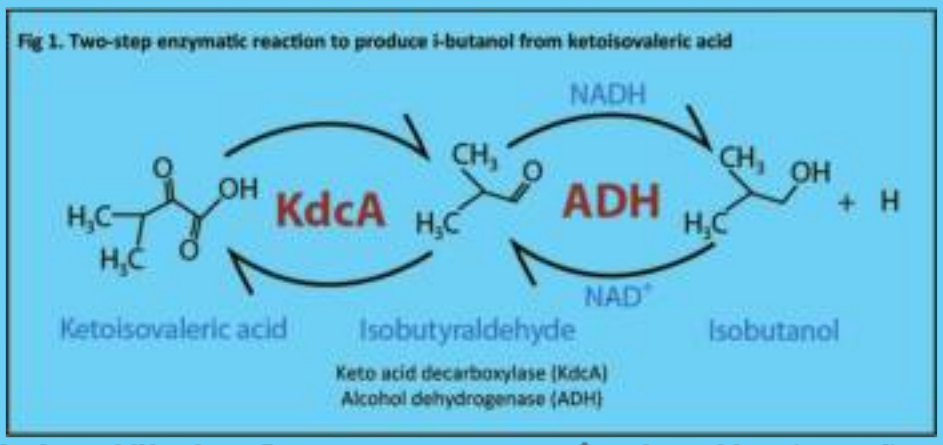


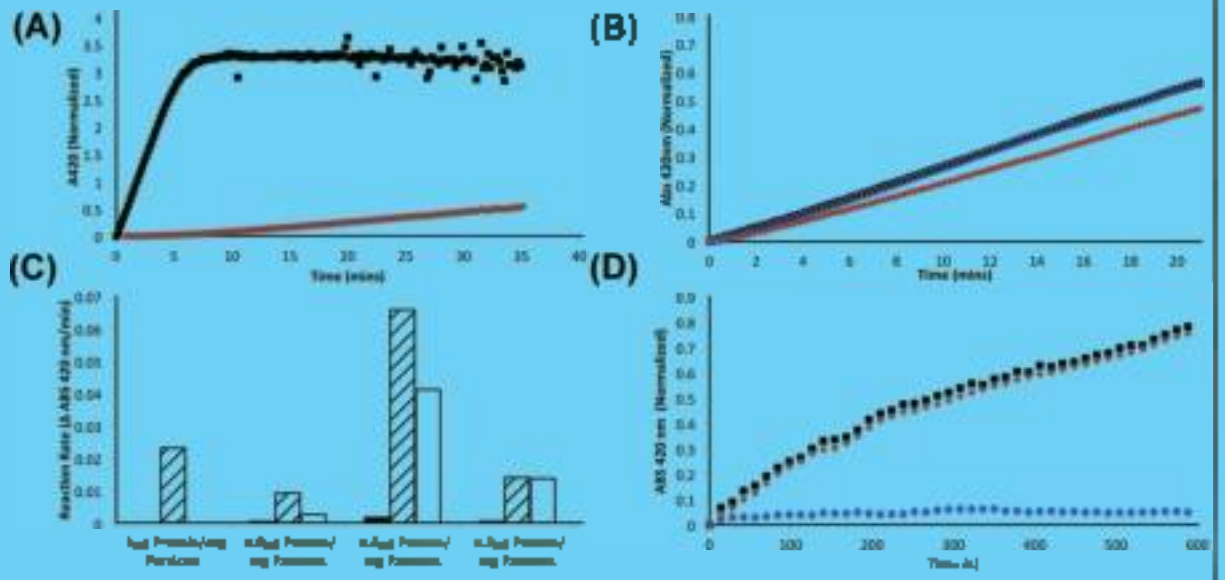
Fig 1. Two-step enzymatic reaction to produce i-butanol from ketoisovaleric acid

and ADH, in the first manuscript[5]. Two manuscripts, exploring the effects of hydrophobic[6] and hydrophilic[7] surfaces both using experiments and simulations. The fourth manuscripts will report on KdcA stabilization using protein engineering and a combination of the two enzymes (KdcA and ADH) into the complete scheme[8]. Finally, a new class of polymer brush membranes will be reported in a manuscript focused on product (isobutanol) removal using pervaporation[9,10].

Research Results:

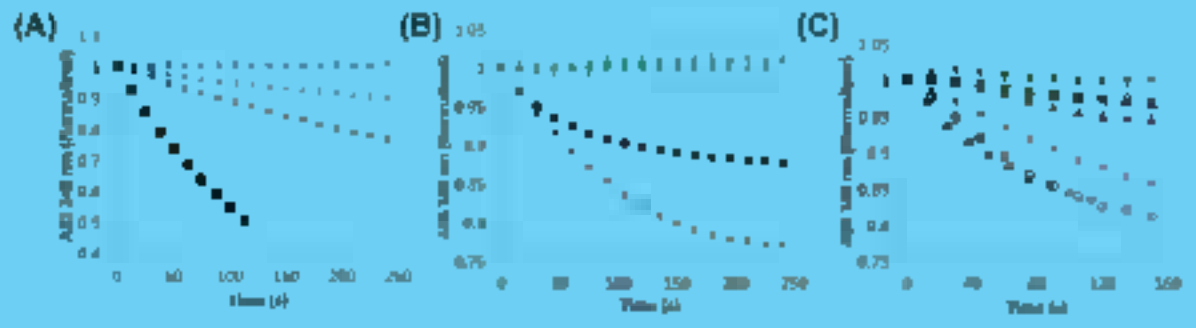
We demonstrated that the enzyme activity differs when immobilized on either 200 nm and 100 nm mean diameter silica particles. In addition, SBA-15 a mesoporous silica, provided a negative curvature surface in which confinement stabilized the protein and retained its activity. Further studies were performed to test the effect of surface coverage and particle loading. The particle system was tested with a model enzyme, β-galactosidase, and our two critical enzymes

Fig. 2. Normalized ß-gal kinetic activity in solution and immobilized passively (just adsorbed) or covalently on non-porous silica, and porous methacrylate (resin) particles. (A) ß-gal in solution (■), ß-gal passive immobilization on 200 nm nanoparticles (◆) (B) ß gal in solution (■), Free ß-gal in a physical mixture with particles (○), covalently immobilized ß-gal (◆). (C) Reaction rate of ß-gal when immobilized, pH 5 (solid), 6 (hatched), or 7 (empty) and varied surface concentration. (D) ß-gal in solution (■), ß-gal covalently attached to amino functionalized methacrylate resin (◆), and amino functionalized methacrylate resin with no ß-gal (●). All absorbance readings were normalized by their initial absorbance.



for alcohol production, alcohol dehydrogenase (ADH) and keto-acid decarboxylase (KdcA). This past year, we developed a viable method for immobilizing enzymes with multiple domains on commercial methacrylate resin. When immobilized, the enzyme must exhibit high activity or retain a large fraction of its solution activity and be able to remain immobilized and active through multiple reactions without leaching from the support. Of the four steps listed above on page 2, this past year we have:

Fig. 3 Normalized ADH kinetic activity in solution and immobilized passively (just adsorbed) or covalently on non-porous silica, porous SBA-15 and porous methacrylate (resin) particles. (A) ADH covalently attached on 200 (●) and 100 (▲) nm acid functionalized silica nanoparticles, in solution (■), and nanoparticles with no immobilized ADH (●). (B) ADH passively immobilized on SBA-15 (◆), ADH passively immobilized on SBA-15 after multiple washes (▲), SBA-15 particles with no immobilized ADH (●), ADH free in solution (■). (C) ADH covalently attached to amino functionalized methacrylate resin as a function of bound ADH concentration: methacrylate resin no ADH (●), 3 μg ADH/mg resin (■), 7 μg ADH/mg resin (▲), 15 μg ADH/mg resin (◆), 17 μg ADH/mg resin (○), equivalent ADH free in solution (■). All absorbance readings were normalized by their initial absorbance



1. Tested the reaction of a model multi-domain enzyme, β -galactosidase (β -gal), with a simple colorimetric assay –immobilized on various surfaces to identify the best surface and curvature (concave or convex) (**Fig. 2**).
2. Immobilize ADH onto the optimal surface obtained from (1) and compared its conversion efficiency with that in free solution for aldehyde to alcohol (**Figs. 3 & 4**).

Production and optimization of keto-acid decarboxylase (KdcA) One key component of the two-stage enzymatic reaction is the first enzyme of the reaction, KdcA, which is not commercially available. We have earlier cloned, overproduced and purified KdcA and successfully tested its activity alone and in combination with immobilized ADH and KdcA in free solution (**Fig. 5**). KdcA is unstable when immobilized and looses its activity.

Fig. 4. Long-term stability of immobilized ADH on methacrylate resin. (A) Percent conversion of isobutaldehyde to isobutanol over 19 days. (B) Gas chromatographs of reaction mixture (top), reaction mixture after incubation with methacrylate resin without immobilized ADH (middle) and reaction mixture after incubation with ADH immobilized on methacrylate resin (bottom). N=NADH; N+=NAD+; A=isobutaldehyde; B=isobutanol

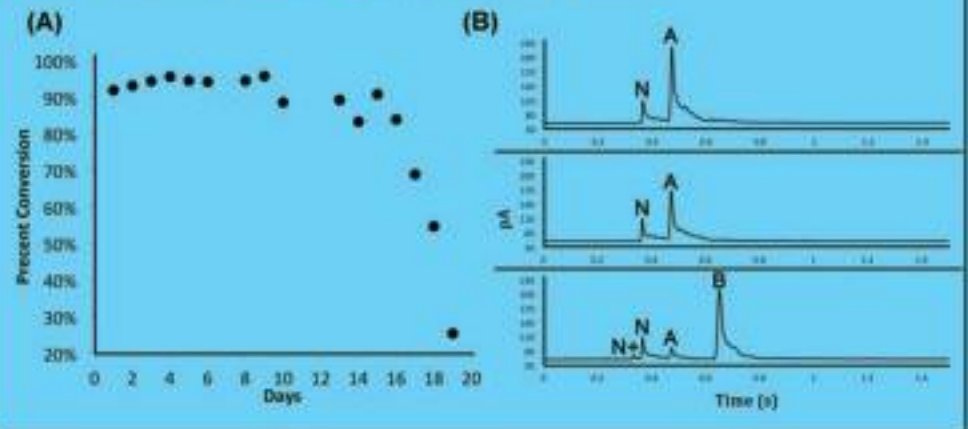
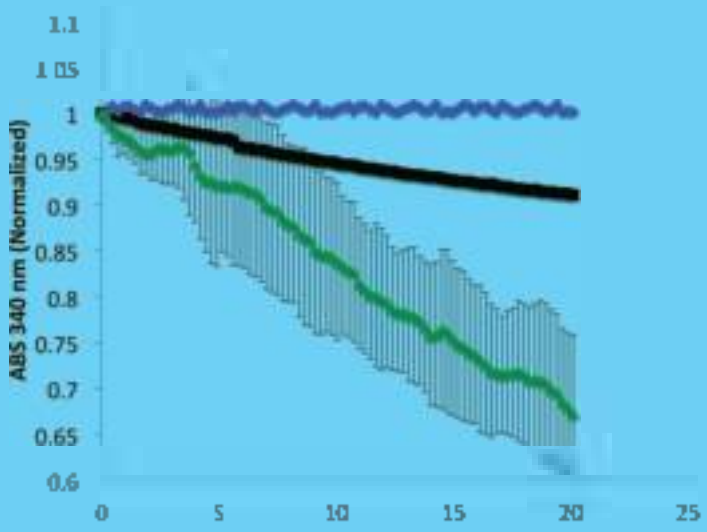


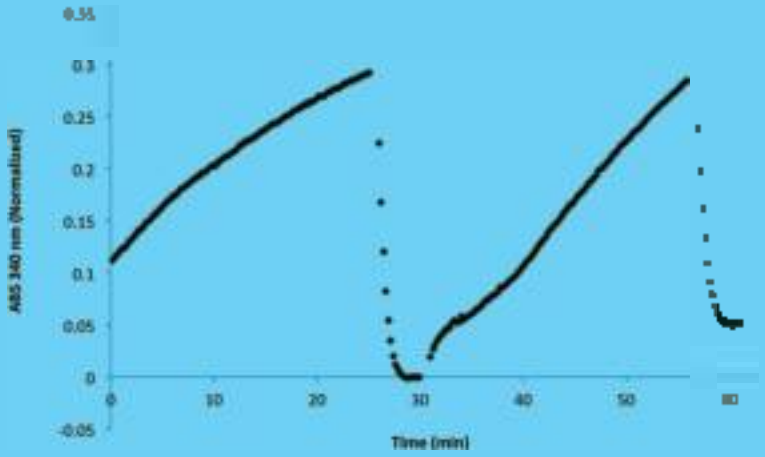
Fig. 5 Normalized KdcA in solution &ADH immobilized covalently on amino-functionalized methacrylate resin. KdcA enzymatic activity free in solution with methacrylate resin without immobilized ADH(■), immobilized ADH enzymatic activity without free KdcA after ketoisovaleric acid is added (◆), and immobilized ADH enzymatic activity with free KdcA after ketoisovaleric acid is added (▲)



Cofactor Recycling – Formate Dehydrogenase (FDH): In order for the *in vitro* immobilized enzyme system to be viable, a strategy for recycling the valuable and expensive co-factor needs to be developed. One suitable mechanism employs the enzymatic conversion of formate to CO_2 by formate dehydrogenase[11]. We will use this biologically inspired mechanism to regenerate the NADH co-factor

FDH was used to demonstrate cofactor recycling in solution. To start the reaction we added formate to the mixture of NAD+, ADH, and buffer. An increase in absorbance at 340 nm indicating that the NAD+ undergoes a reduction reaction with the aid of FDH to produce NADH is observed (**Fig. 6**). This reaction by adding isobutyraldehyde (25 min & 55 min) and additional formate (30 min) could be recycled.

Fig. 6 Normalized FDH and ADH Kinetic Activity in Solution. Sodium formate added at t = 0 & 30 min Isobutyraldehyde added at t = 25 & 55 min ABS 340 nm measures the concentration of NADH present in the system



KdcA Fusion Protein Development Super-folded GFP or previously stabilized MBD was genetically fused to wild-type KdcA, according to the procedure outlined in Section 3.2.7. GFP and MBD were selected because they have previously been shown to increase the solubility of proteins[12,17]. In order to understand the impact of the fused proteins on enzyme kinetics and determine which fusion protein exhibited the greatest improvement over wild-type, enzymatic activity was tracked by observing the consumption of ketoisovaleric acid over time. From these measurements, we were able to obtain the total consumption per time and enzyme concentration (**Fig. 7**). The maltose binding protein keto-acid decarboxylase (MK) fusion protein showed the best substrate consumption over time: this enzyme construct had the highest enzymatic activity (k_{cat} = $24 \pm 1.3 \times 10^4\ s^{-1}$) (**Table 1**). The his-tag green florescent protein keto-acid decarboxylase (HGK) and maltose binding protein keto-acid decarboxylase his-tag (MKH) fusion proteins had similar enzymatic activity (k_{cat} = $16 \pm 2.5 \times 10^4\ s^{-1}$ and $20 \pm 0.3 \times 10^4\ s^{-1}$ respectively). Wild-type KdcA had the worst activity ($k_{cat} = 5.8 \pm 0.02 \times 10^4\ s^{-1}$) and also exhibited substrate inhibition (**Fig. 7**).

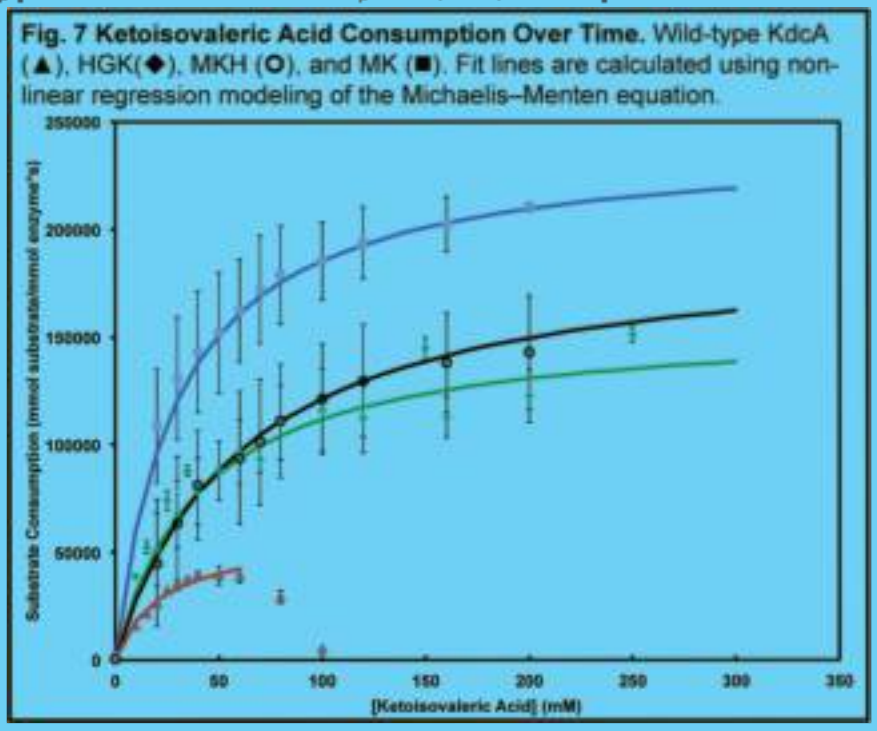


Fig. 7 Ketoisovaleric Acid Consumption Over Time. Wild-type KdcA (▲), HGK(◆), MKH (○), and MK (■). Fit lines are calculated using non-linear regression modeling of the Michaelis–Menten equation.

Table 1: Michaelis-Menten Kinetic Constants

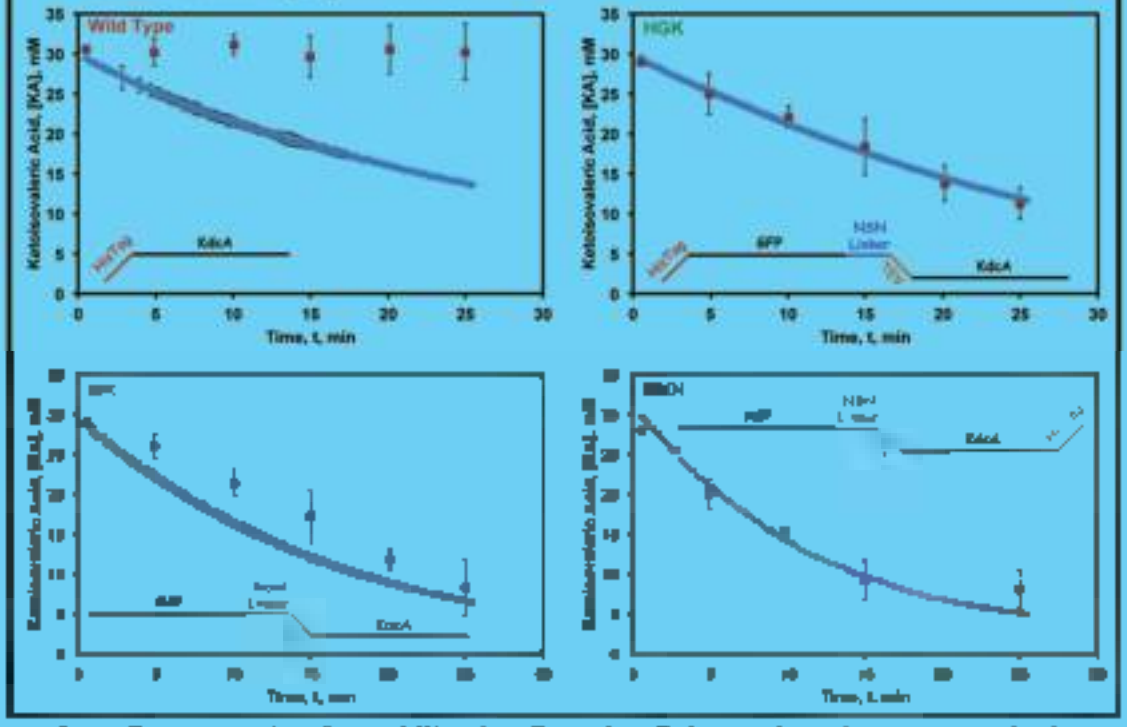
	Wild-type	HGK	MK	MKH
K_m (mM)	22 ± 8	41 ± 10	30 ± 11	60 ± 30
k_{cat} (1/s) ($\times 10^4$)	5.8 ± 0.02	16 ± 2.5	24 ± 1.3	20 ± 0.3

$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad k_{cat} = \frac{V_{max}}{[E]}$$

[E], enzyme concentration
[S], substrate concentration

KdcA Fusion Protein Immobilization Stability: The addition of GFP or MBD was enough to stabilize the KdcA for immobilization (**Fig.8**). We found that the MK11 fusion immobilized better and had higher activity than the other two constructs. This is likely due to the fact that there is a portion of protein on both sides of KdcA: MBD on one end, and the his-tag on the other. The immobilization reaction is between the epoxy and a free NH_2 group. By creating more solvent exposed sections on the two sides of the enzyme, we can bias the immobilization to those sites rather than to the KdcA structure, where it could disrupt the stability of the enzyme.

Fig. 8 KdcA Activity Immobilized on Amino-Epoxy Methacrylate (■) vs. Free in Solution (◆).



Low Concentration Immobilization Reaction Scheme: In order to assess the impact of cofactor recycling on the two-step immobilized enzyme system, we compared the ability of the reaction to go to completion in the absence and presence of NADH recycling (without and with addition of formate, respectively). When limited cofactor NADH (1.2 µmol) and 64 µmol of ketoisovaleric acid were reacted with immobilized MKH, immobilized ADH, and FDH in solution, without formate, only the intermediate (isobutyraldehyde) was produced (**Table 2**). The same system reacted with 1500 µmol formate isobutanol was produced. There was a 55 ± 3.5% conversion of acid to alcohol after 24 hours. From a mass balance, the cofactor was recycled about 30 times before the reaction reached steady state.

Table 2: Small-Scale Reaction with Immobilized ADH, Immobilized KdcA and FDH in Solution

	Keto-isovaleric Acid	NADH	Isobutyraldehyde	Isobutanol
Starting Reaction mixture	64 µmol	1.2 µmol	-------	-------
Reaction w/o Formate	Negligible	Negligible	62 ± 1.2 µmol	Negligible
Reaction w/ Formate	Negligible	0.73 ± 0.16 µmol	29 ± 2.2 µmol	35 ± 2.2 µmol

High Concentration Immobilization Reaction Scheme: To drive the reaction to completion, formate was added in greater excess and the reaction as a whole was scaled up to produce a larger theoretical percent of isobutanol. Ketoisovaleric acid (320 µmol) was reacted with 6 µmol of NADH and 1500 µmol of formate. If the reaction were to go to completion, a total of 2%

(v/v) of isobutanol would be produced. At 24 hours the reaction reached a steady state with 57 µmol of starting material, 162 µmol of intermediate, and 101 µmol of alcohol (**Table 3**). These concentrations were determined via absorbance at 340 nm for the starting material, and from gas chromatography peaks for the intermediate and product. The resulting concentration of isobutanol was 0.5% (v/v); this result demonstrates that the second reaction is reversible and the presence of a significant concentration of isobutanol and isobutyraldehyde inhibits the first reaction. By using an *in situ* removal technique, such as pervaporation, the reaction could be driven to completion.

Table 3: High Concentration Reaction Scheme with Immobilized ADH, Immobilized KdcA, and FDH in Solution

	Keto-isovaleric Acid	NADH	Isobutyraldehyde	Isobutanol
Starting Reaction mixture	320 µmol	4 µmol	-------	-------
Reaction Cofactor Recycling	57 ± 2.1 µmol	2 ± 1.1 µmol	162 ± 1.8 µmol	101± 1.8 µmol

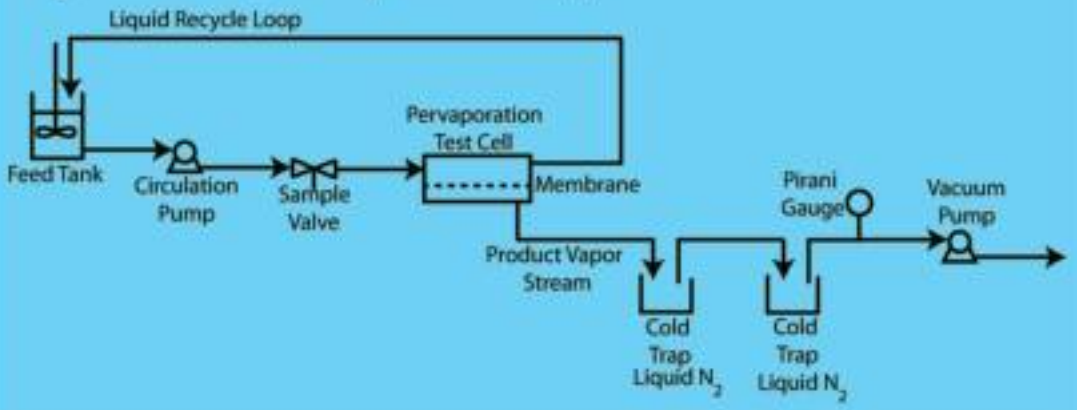
The reaction system produced a final concentration of 0.5% (v/v) isobutanol. Unreacted starting material (acid) and intermediate (aldehyde) were still present in the solution. The first reaction was the conversion of the acid to aldehyde (**Fig. 1**). This reaction produced CO_2, and is nearly irreversible because of the creation of a dissolved gas product. The second reaction in the system involves the conversion of the aldehyde to the alcohol. This reaction is a reversible reaction; in natural systems ADH is used as a dehydrogenase to remove the hydrogen and produce an aldehyde. Even with the driving force imposed by FDH, the reaction still will reach equilibrium. The reason the initial substrate was still present was likely due to the build-up of the aldehyde and alcohol, which are solvents that can harm the stability of the KdcA fusion protein.

***In Situ Removal of Isobutanol (Pervaporation)*:** The term "pervaporation" is derived from a combination of "permeation" and "vaporization". Pervaporation (PV) is a membrane separation technique that utilizes a non-porous, selectively permeable membrane to separate the components of a complex liquid feed mixture[14]. PV relies on the differences in membrane permeability and the thermodynamic activity of the components[15]. The process exploits the unique chemical potentials and partial pressures of each component of the mixture[16]. The mechanism by which a component is transported in PV is described by three consecutive steps:[16,17]

1. Sorption of a component on the upstream-feed side of the membrane.
2. Diffusion of the adsorbed species through the polymer matrix down a chemical potential gradient.
3. Desorption and evaporation of the component on the downstream side of the membrane.

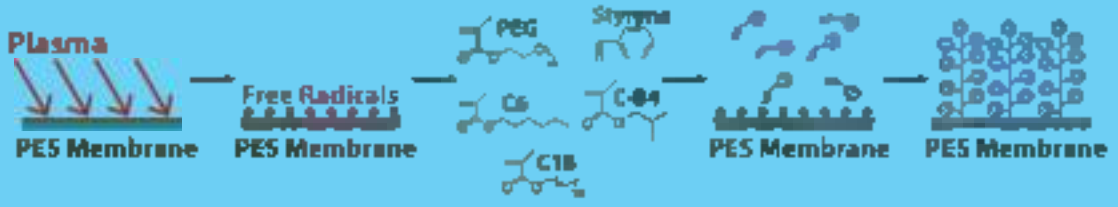
A custom PV system (**Fig. 9**) was used to quantify the flux of material that passed through each membrane. Gas chromatography (GC) analyses of the retentate and permeate were used to determine molar separation factor ($\alpha = [(X_{iso}/X_w)_{permeate}/(X_{iso}/X_w)_{retentate}]$ where X_{iso} and X_w are mole fractions for isobutanol and water, respectively). Commercial PDMS membranes, Sil5 and Sil20, have an active layer thickness of 5 and 20 microns, respectively, and serve as the industry gold standard for hydrophobic PV membranes.

Fig. 9 Laboratory Pervaporation Set-Up.



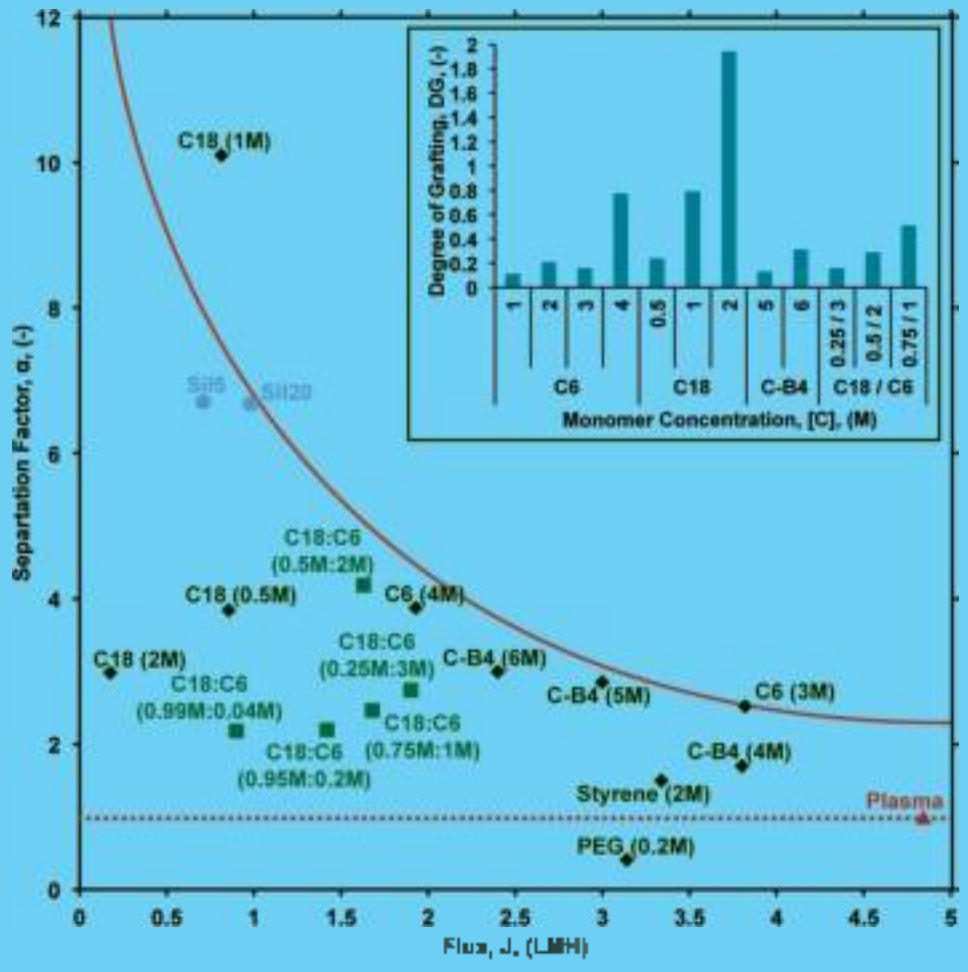
APP-induced graft polymerization was used together with our high throughput platform (**Fig. 10**)[18]. This approach enabled the creation of a library of PES nanofiltration membranes with different chemical and morphological properties and the comparison of their PV performance with commercial silicone rubber (PDMS) membranes. The following monomers were grafted: Isobutyl Methacrylate (C-B4), Hexyl Methacrylate (C6), Stearyl Methacrylate (C18), and Poly(ethylene glycol) methyl ether methacrylate (PEG) (n=45). The graft density of the polymerized surface layer was measured and was determinant for the separation of isobutanol from an aqueous solution

Fig. 10 Schematics and Process Diagram for Synthesizing and Testing Brush Membranes. Atmospheric pressure plasma-induced graft polymerization (APP) of vinyl monomer on poly(ether sulfone) (PES) membranes. Monomers polyethylene glycol (PEG), styrene, hexyl methacrylate (C6), isobutyl methacrylate (C-B4), and stearyl methacrylate (C18). Vinyl functional groups are labeled "R".



In **Fig 11**, the isobutanol selectivity over water is plotted against permeation flux of isobutanol for 10 single grafted monomers, 5 different grafted mixtures of C18 with C6 monomers, and 2 commercial membranes (Sil5 and Sil20). The C18 brush membrane had a flux, J, of 0.8 ± 0.15 LMH (L/m^2-h); this is comparable to the fluxes for Sil5 and Sil20 of J = 0.7 ± 0.06 LMH and 1 ± 0.11 LMH, respectively. However, the selectivity for the C18 brush membrane was α = 10.1 ± 0.86 compared with α = 6.7 ± 0.11 and 6.7 ± 0.05 for Sil5 and Sil20 respectively. All the other single-grafted brush membranes had much higher fluxes (J > 1.5 LMH), but with significantly lower selectivities (α < 4). Addition of C6 to C18 in any amount reduced α significantly. C6 (at 3 M monomer concentration) as a single graft exhibited the highest J (3.8 LMH) at α = 2.5. Further work is underway to determine if one can shift the C18 α-values to the right to achieve higher fluxes.

Fig. 11 Separation Factor (α) Versus Permeation Flux (J) for a Range of Different Brush Membranes. Commercial PDMS membranes (•) (Sil5 and 20), pure monomers (◆), C18 C6 monomer mixtures (■), and plasma only (▲); the red dotted line indicates no separation (α=1), the red solid line shows the lower limit of J versus α for performance of new membranes over existing membranes. Monomers: polyethylene glycol (PEG), styrene, hexyl methacrylate (C6), isobutyl methacrylate (C-B4), and stearyl methacrylate (C18). Insert: Degree of Grafting (DG) for vinyl monomers: Hexyl methacrylate (C6), stearyl methacrylate (C18), and isobutyl methacrylate (C-B4), and mixtures of C18/C6



Summary.

Our model enzyme system provided us with an increased understanding of what factors affect kinetic activity, especially during enzyme immobilization. With respect to immobilized enzyme systems, our experimental findings confirmed previous observations and expanded the knowledge and understanding of these systems. Thus, hydrophobic surfaces like carbon causes proteins to be more susceptible to denaturing because of surface-protein and protein-protein interactions. Concave geometries offer higher enzymatic conversion kinetics than free equivalent protein in solution due to their stabilization in confinement. Desorption and protein leaching can be avoided by immobilization through covalent bonds. Changing the reaction time for immobilization, pH of the solution, and amount of enzyme on the surface allowed us to obtain a wide range of enzyme orientations, degrees of surface coverage, and the ability to probe

both protein-protein and protein-surface interactions. Methacrylate surfaces with an extended tether provided the best support for β – galactosidase (β-gal), alcohol dehydrogenase (ADH), and keto-acid decarboxylase (KdcA) fusion proteins. The beads are easily removed from the reaction solution for replenishing the enzyme and offer a stable, reusable, and robust system.

Characterization of ADH and KdcA in solution provides us with a better understanding of the stability of the enzymes. Alcohol toxicity/inhibition can be detected not only at a cellular level (>2% (v/v)), but also at an enzyme level (>10% (v/v)) [Data not shown]. Formate dehydrogenase (FDH) can be used as a viable cofactor regeneration technique. The enzyme uses an inexpensive sacrificial substrate and creates CO_2 as a byproduct. FDH does not retain its activity when immobilized. As was the case with KdcA, FDH could benefit from being genetically fused to a stable protein or domain, like maltose binding domain.

Fusion proteins are a direct way of increasing a desired property of a protein. In our system, GFP and MBD were used to increase solubility. These highly stable proteins can be attached to an unstable protein, such as KdcA, to allow for better expression, increased solubility, and ultimately improved stability. The additional structure that MBD and GFP provide to the fusion protein helped to screen protein-protein interactions. The propensity of KdcA to self-aggregate diminished and the enzyme was able to perform better in solution. The enzyme fusion protein no longer exhibits substrate inhibition and is able to be easily immobilized without losing its activity.

The immobilized ADH and immobilized KdcA fusion reaction system with cofactor recycling could be used to produce isobutanol from ketoisovaleric acid. The reaction; however, did not go to completion. The aldehyde to alcohol reaction is reversible under limiting cofactor conditions. Additionally, the build-up of the intermediate (isobutyraldehyde) and product (isobutanol) decreased the activity of the immobilized KdcA fusion protein to the point that it could not convert the remaining substrate (ketoisovaleric acid). The immobilized enzyme reaction system could be pushed towards completion if we employ an *in situ* system to remove isobutanol.

Pervaporation is a solution-diffusion governed separation process that can be used to continuously remove isobutanol from the reaction system and also can be used to dehydrate isobutanol. We developed a new brush-like membrane that is capable of removing isobutanol (minor component) from water (major component). Using atmospheric plasma graft polymerization (APP), we grafted a variety of hydrophobic monomers to the surface of the poly(ether sulfone) (PES) membrane. The best monomer was an 18 repeat carbon chain. The flux we obtained was similar to the commercial gold standard membrane (PDMS); however, the separation factor was 1.5 times higher when using our new brush-like membrane.

List of people working on the project:

- Dr. Georges Belfort (PI), Professor, 5%
- Joseph Grimaldi, Graduate Student, 100%
- Dr. Cynthia Collins, Assistant Professor (Free to the project – assisting with the protein engineering of KdcA), 5%
- Dr. Sanat Kumar and Mithun Radhakrishna, Collaborators from Columbia University.

Collaboration

This project involves collaboration between our group at RPI (Dr. Georges Belfort, PI, and Joseph J. Grimaldi, PhD Student) and a molecular modeling group at Columbia University in NY City (Dr. Sanat Kumar PI, and Mithun Radhakrishna PhD Student). Both groups reinforce each other's research. We, at RPI, conduct laboratory experiments optimizing enzyme immobilization

and performance, while the Columbia University group conduct molecular simulations on proteins at interfaces in order to determine their structural and thermal stability. We have submitted one manuscript to an ACS peer-reviewed journal "Langmuir" and have met three times in NY City (Feb 23 2012), Troy (September, 2012) and Pittsburgh, PA in November, 2012 (at the AIChE Annual Meeting) to discuss our collaboration.

Unexpended Funds: 0%

Conference Presentations

Grimaldi J, Collins CH, Belfort G; "Optimizing immobilized enzyme performance in cell-free environments to produce liquid fuels"; 244th ACS National Meeting, Philadelphia, PA, August 19-23, 2012 Poster Presentation
"Acknowledgements: Funding and support from the U.S. Dept. of Energy DOE (DE-SC0006520)"

Grimaldi J, Collins CH, Belfort G; "Optimizing immobilized enzyme performance in cell-free environments to produce liquid fuels"; 2012 AIChE Annual Meeting, Pittsburg, PA, Oct. 28- Nov. 2, 2012 Oral Presentation
"Acknowledgements: Funding and support from the U.S. Dept. of Energy DOE (DE-SC0006520)"

Grimaldi J, Collins CH, Belfort G; "Optimizing immobilized enzyme performance: *in vitro* production of isobutanol"; 27th symposium of the Protein Society, Boston, MA July 20-24 2013
"Acknowledgements: Funding and support from the U.S. Dept. of Energy DOE (DE-SC0006520)"

Grimaldi J, Collins CH, Belfort G; "Optimizing immobilized enzyme performance: *in vitro* production of isobutanol"; ACS National Meeting, Dallas, TX March 16-20 2014
"Acknowledgements: Funding and support from the U.S. Dept. of Energy DOE (DE-SC0006520)"

Publications

Grimaldi, J., Imbrogno, J., Kilduff, J., Belfort, G. (2014) *Hydrophobic Brush Membranes for Filtration Based on Solution-Diffusion Mechanism with Aplications to Pervaporation (PV) & Reverse Osmosis (RO).* US Provisional Patent: 62/079,605.

Radhakrishna, M., Grimaldi, J., Belfort, G., Kumar, S.K. (2013) *Stability of proteins inside a hydrophobic cavity,* Langmuir, 29 (28), 8922–8.

Grimaldi, J., Collins, C.H., Belfort, G. (2014) *Towards Cell-Free Biofuel Production: Stable Immobilization of Oligomeric Enzymes,* Biotechnology Progress, 30(2) 324-31.

Grimaldi, J., Radhakrishna, M., Belfort, G., Kumar, S.K. (2014) *Protein Stability on Hydrophilic Surfaces,* Biophysical Journal, In press.

Grimaldi, J., Collins, C.H., Belfort, G. (2014) *Towards Cell-Free Biofuel Production: Development of a Novel Immobilized Enzyme System,* (In Preparation)

Grimaldi, J., Imbrogno, J., Sorci, M., Belfort, G. (2014) *Specialized Membranes for Pervaporation,* (In Preparation)

References:

1 Fischer, C., Kleinmarcuschamer, D. & Stephanopoulos, G. Selection and Optimization of Microbial Hosts for Biofuels Production. *Metabolic Engineering* **10**, 295-304, doi:10.1016/j.ymben.2008.06.009 (2008).

2 Arnold, F. H. The Race for New Biofuels. *Engineering and Science* **71**, 12-19 (2008).

3 Pospíšil, S., Kopecký, J., Přikrylová, V. & Spížek, J. Overproduction of 2-ketoisovalerate and monensin production by regulatory mutants of Streptomyces cinnamonensis resistant to 2-ketobutyrate and amino acids. *FEMS microbiology letters* **172**, 197-204 (2006).
4 Strathmann, H. & Gudernatsch, W. Continuous Removal of Ethanol from Bioreactor by Pervaporation. *Extractive Bioconversions*, 67-89 (1991).
5 Grimaldi, J., Collins, C. & Belfort, G. Toward Cell□Free Biofuel Production: Stable Immobilization of Oligomeric Enzymes. *Biotechnol. Prog.* **30**, 324-331 (2014).
6 Radhakrishna, M., Grimaldi, J., Belfort, G. & Kumar, S. K. Stability of proteins inside a hydrophobic cavity. *Langmuir* **29**, 8922-8928 (2013).
7 Grimaldi, J., Radhakrishna, M., Belfort, G. & Kumar, S. K. Stability of Proteins on Hydrophilic Surfaces. *Langmuir* ((In Reveiw)).
8 Grimaldi, J., Collins, C. & Belfort, G. Towards Cell-Free Biofuel Production: Development of a Novel Immobilized Enzyme System. *In Preparation* (2014).
9 Grimaldi, J., Imbrogno, J., Kilduff, J. & Belfort, G. A New Class of Synthetic Membranes: Organophilic Pervaporation Brushes for Biofuel Recovery. *Nature Materials* (Submitted).
10 Grimaldi, J., Imbrogno, J., Kilduff, J. & G, B. Hydrophobic Brush Membranes for Filtration Based on Solution-Diffusion Mechanism with Aplications to Pervaporation (PV) & Reverse Osmosis (RO). US Provisional Patent (2014).
11 Shaked, Z. e. & Whitesides, G. M. Enzyme-Catalyzed Organic Synthesis: NADH Regeneration by using Formate Dehydrogenase. *J. Am. Chem. Soc.* **102**, 7104-7105 (1980).
12 Wood, D. W. *et al.* Optimized Single□Step Affinity Purification With a Self□Cleaving Intein Applied to Human Acidic Fibroblast Growth Factor. *Biotechnol. Prog.* **16**, 1055-1063 (2000).
13 Gromek, K. A. *et al.* Improved Expression and Purification of Sigma 1 Receptor Fused to Maltose Binding Protein by Alteration of Linker Sequence. *Protein Expr. Purif.* **89**, 203-209 (2013).
14 Lee, Y. M., Bourgeois, D. & Belfort, G. in *Proceedings of the Second International Conference on Pervaporation Processes in the Chemical Industry.* (ed R. Bakish) 249-265 (Bakish Materials Corp.).
15 Böddeker, K. W. Terminology in Pervaporation. *Journal of Membrane Science* **51**, 259-272 (1990).
16 Bell, C., Gerner, F. & Strathmann, H. Selection of Polymers for Pervaporation Membranes. *Journal of Membrane Science* **36**, 315-329 (1988).
17 Smitha, B., Suhanya, D., Sridhar, S. & Ramakrishna, M. Separation of Organic-Organic Mixtures by Pervaporation--A Review. *Journal of Membrane Science* **241**, 1-21 (2004).
18 Gu, M., Kilduff, J. E. & Belfort, G. High Throughput Atmospheric Pressure Plasma-Induced Graft Polymerization for Identifying Protein-Resistant Surfaces. *Biomaterials* **33**, 1261-1270 (2012).