RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

RSC Advances

RSCPublishing

ARTICLE

Performance in Synthetic Applications of a Yeast Surface Display-Based Biocatalyst

Cite this: DOI: 10.1039/x0xx00000x

J. M. Eby,[a] and S. W. Peretti[a]

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

This work demonstrates the effectiveness of yeast surface display (YSD) as a scaffold for biocatalysts in hydrophobic, non-aqueous environments. Two lipases, *Candida antarctica* lipase B (CalB) and *Photobacterium lipolyticum* sp. M37 lipase (M37L), were immobilized independently by surface display on *Saccharomyces cerevisiae.* The two YSD biocatalysts were employed to synthesize esters of butanol and saturated fatty acids of varying length (8 to 16 carbons) in heptane. Effects of fatty acid chain length and temperature on the esterification reaction were examined. The YSD catalysts synthesized butyl decanoate in 10 repeated batches with little loss in activity. Compared to a commercial immobilized lipase (Novozym 435), the activity of both YSD lipases was lower on a mass loading basis, but higher when normalized on estimates of protein loading. Initial-rate kinetics of the butyl decanoate reaction were measured for the CalB-displaying yeast. Kinetics and apparent activity of M37L in the multi-batch experiments depend heavily on water concentration; kinetics for M37L could not be elucidated with initial-rate methods. The difference between CalB and M37L in water requirements illustrates a critical parameter for optimization of lipase activity in non-aqueous environments. The activity of both lipases in a completely hydrophobic environment is a step towards more economical biocatalysis of industrial esterification.

Introduction

Esters are valuable commodities with applications in the fragrance, personal care, fine chemical, and energy industries. They are most often produced from crude alcohols and organic acids or esters using an acidic or basic inorganic catalyst. Production of vinyl acetate, for example, involves reaction temperatures in excess of 100 °C and corrosive materials[1]. Downstream cleanup is required to remove the acid/base and their byproducts, making purification a major cost driver[2,3]. Aggressive removal of water and excess reagents is required to achieve commercially relevant conversions, further increasing process complexity and cost. Methods utilizing supercritical solvents and ionic liquids[4,5], though promising, are still many years away from being commercially viable.

Enzymatic esterification is an alternative to traditional catalysis that has lower energy requirements (lower temperatures and pressures), generates less waste (no excess reagents or byproducts), and represents "greener" (biodegradable, non-toxic) catalysis. Lipases catalyze a wide range of hydrolytic and synthetic reactions, and are widely used in industry[6]. They evolved as esterases[2], and readily cleave the ester bonds in triglycerides, often the first step in biodiesel production. Lipases are chiral catalysts and can catalyze the hydrolytic resolution of racemic mixtures[7,8]. In addition, absent water, they can catalyze the formation of ester bonds through esterification of a carboxylic acid or trans-esterification of a donor ester[9,10].

Lipase activity requires some structural flexibility. In nature, lipases are activated by interfacial contact[11], which causes a small conformational change in the enzyme's tertiary structure[12,13]. Many enzymes are functional in organic solvents if they are in an active configuration when water is removed[14]; lipases are similar[6,15]. A small amount of water may be required to hydrate the enzyme[16], but the amount is small enough to have little or no effect on the reaction equilibrium[17].

Lipases have demonstrated activity in organic solvents such as hexane and heptane and aqueous solutions as free (soluble) and immobilized enzymes[15,18,19]. Catalyst recycling is facilitated by immobilization, and immobilization methods often have positive impacts on thermal and physical stability[20]. Moreover, purified enzyme cost is a barrier to industrial implementation of biocatalysis. Purification and immobilization account for as much as 80% of the cost of the enzyme[21], so reduction of these costs significantly lowers the barrier for wider industrial application. Yeast surface display (YSD) represents a high

degree of process integration, combining production, purification, and immobilization of recombinant protein into a single operation[22,23].

Surface display (SD) has been applied to several chemistries, including chiral resolution by hydrolysis[24], cellulose degradation[25], and synthesis of esters[23,26,27]. However, reports of SD-catalyzed syntheses are few, and many require reaction times on the order of days[27] or relatively polar reactants[28-31].

Here, we demonstrate the ability of two lipases—lipase B from *Candida antarctica* (CalB) and lipase from *Photobacterium lipolyticum* M37 (optimized for yeast expression, ycM37L)—in surface display systems to synthesize fatty acid esters from fatty acids and alcohols. A systematic characterization of YSD esterification dependence on water content, fatty acid chain length, and temperature was performed using Novozym 435 (N435) as a reference standard, with an additional focus on the stability of YSD activity to repeated usage. Despite surface protein loading levels that are approximately three orders of magnitude below that of Novozym 435, these YSD-catalyzed reactions reach significant conversion levels in a matter of hours.

Results

Temperature dependence of the synthesis reaction

The temperature optima for YSD biocatalyzed synthesis of butyl dodecanoate (laurate; Figure 1), butyl decanoate (caprate, Figure 2), and butyl octanoate (caprylate; Figure 3) were determined. Equimolar solutions of butanol and either octanoic or dodecanoic acid were stirred at 30, 40, 60, or 80 °C for six hours. Conversion of the fatty acid to its butyl ester was determined at the end point.

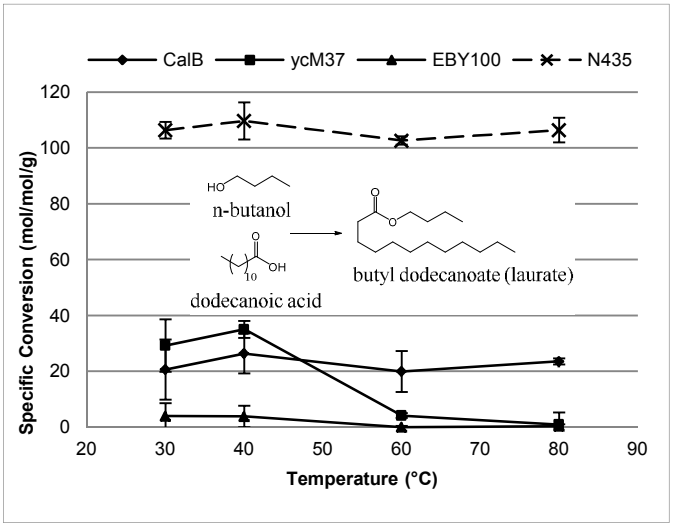

Figure 1: Synthesis of butyl dodecanoate with the YSD biocatalyst. Solutions of butanol and dodecanoic acid (both 100 mM in heptane) were combined with dry yeast biocatalyst (0.4% w/v). The reactions were stirred in a temperature-controlled water bath for six hours at 200 rpm. The values represent the average of three experiments, with standard deviations (vertical bars).

The optimum temperature for the CalB YSD biocatalyst was near 60 °C for octanoic and decanoic acid, but no clear optimum appeared for dodecanoic acid. The conversion at 60 °C was more than twice that at 30 °C for octanoic acid, but constant for dodecanoic acid. A slight decrease in conversion at 80 °C suggests potential thermal deactivation.

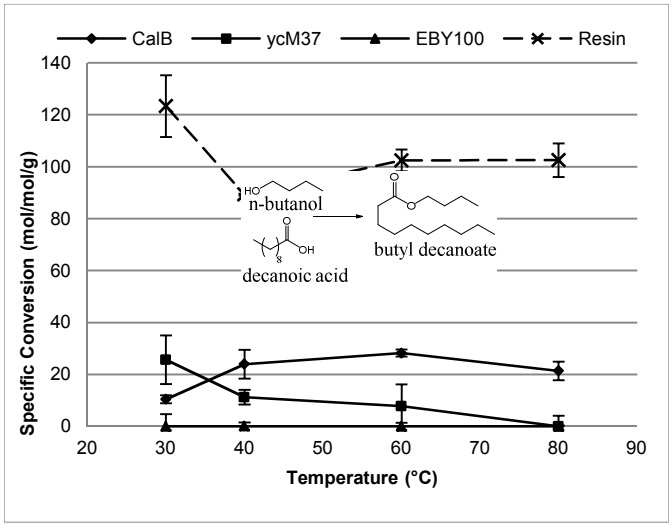

Figure 2: Synthesis of butyl decanoate with the YSD biocatalyst. Solutions of butanol and decanoic acid (both 100 mM in heptane) were combined with dry yeast biocatalyst (0.4% w/v). The reactions were stirred in a temperature-controlled water bath for six hours at 200 rpm. The values represent the average of three experiments, with standard deviations (vertical bars).

The optimum temperature for the ycM37L YSD biocatalyst was near 40 °C, above which temperature a steady decrease in activity was observed. At 80 °C, essentially no product was formed, which suggests lower thermal stability than that of CalB. This result is unsurprising given the psychrophilic nature

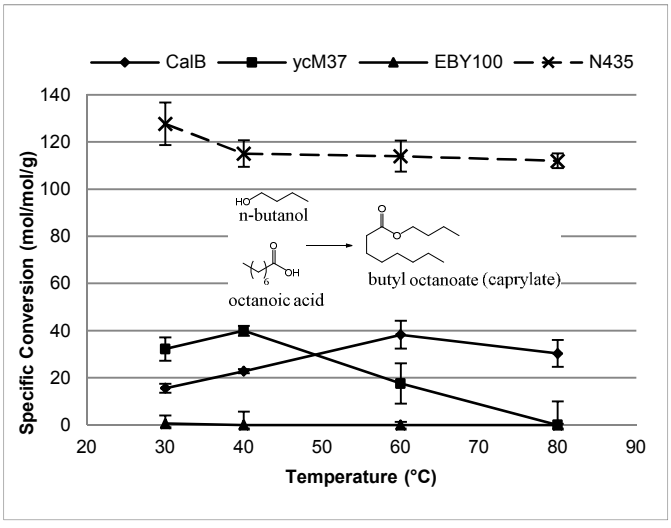

Figure 3: Synthesis of butyl octanoate with the YSD biocatalyst. Solutions of butanol and octanoic acid (both 100 mM in heptane) were combined with dry yeast biocatalyst (0.4% w/v). The reactions were stirred in a temperature-controlled water bath for six hours at 200 rpm. The values represent the average of three experiments, with standard deviations (vertical bars).

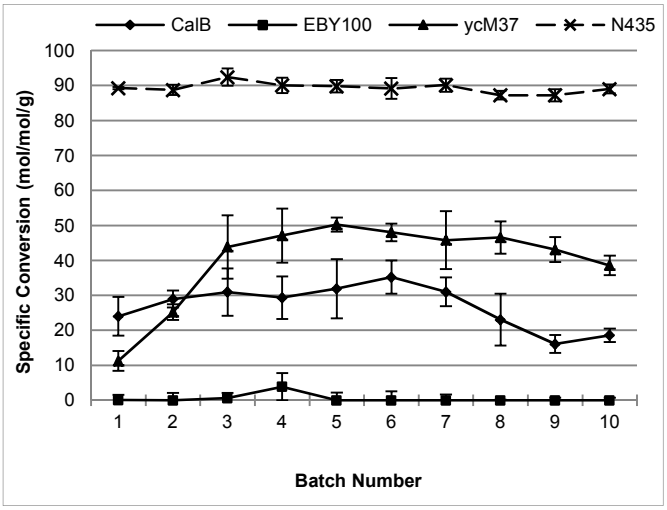

Figure 4: Synthesis of butyl decanoate with the YSD biocatalyst in multiple batch reactions at 40 °C. Solutions of butanol and decanoic acid were combined (to 100 mM in heptane) with dry catalyst at 0.5% w/v. After stirring at 200 rpm for six hours, the catalyst was removed from the reaction by centrifugation and the liquid fraction was sampled. The catalyst was used in the subsequent batch without further treatment.

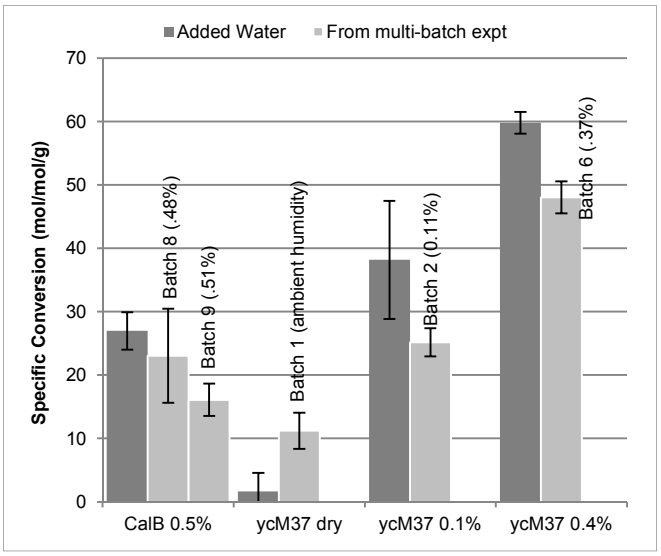

Figure 5: Changes in apparent activity of the YSD biocatalyst at different water concentrations. The activity of the biocatalyst in the synthesis of butyl decanoate was measured with water added in concentrations that matched the theoretical water production of selected batches from the multi-batch synthesis experiments

of the source organism.

The N435 resin reached equilibrium within six hours at every temperature tested, and no thermal deactivation was observed in the single batches used here. While changes in the fatty acid concentration were observed in some of the untransformed EBY100 samples, no product was detected in any of the samples.

Re-use of the YSD Biocatalyst

A significant aspect of the commercial application of enzyme catalysts is robustness. To demonstrate this, the YSD biocatalysts were subjected to ten butyl decanoate synthesis batch reactions at 40 °C. The temperature was chosen to prevent thermal deactivation of ycM37L. The catalysts were separated from the reactor medium by centrifugation and used in subsequent batches without further processing. Both YSD catalysts produced the ester product in all batches (Figure 4). The ycM37L biocatalyst went through an initial 2-batch activation period, after which it maintained fairly steady activity through the end of the trial. The CalB biocatalyst held at or above the activity of its first batch through 8 batches, after which a gradual decrease to 60% of the peak activity was observed. A similar gradual decline was observed in the final two batches for ycM37L (to 80% of maximum). N435 retained its activity with little variation through ten batches. Some small decrease in the fatty acid concentration in EBY100 trials was observed, but no product ester was formed.

Dispersion of the CalB and ycM37L yeast catalysts in the reaction began changing visibly at the fifth batch. In the initial batches, the cells had been well-suspended, resulting in a turbid reaction medium with few visible particles. After five batches, the cells began to aggregate and fall out of suspension without vigorous mixing. This phenomenon is examined in more detail in the Discussion. Most of the aggregates could be re-dispersed in fresh reagent solution, but this became more difficult as the number of batches increased. A similar effect was observed in experiments with water added. The changes in dispersion were hypothesized to be the result of water produced by the reaction. Incomplete mixing, as indicated by poor YSD dispersion, is proposed to be the cause of the changes in activity observed in ycM37L and CalB. No changes in dispersion or activity were observed in yeast without displayed lipase.

A small amount of water is often necessary for enzymatic activation in organic solvents[32], but the trials were initiated with dry catalyst (at equilibrium with the ambient humidity). The water content and its impact on the esterification activity needed to be titrated, so the butyl decanoate reaction was run again with dry catalyst (freshly prepared, not equilibrated with ambient humidity) and water was added to match the theoretical production at batches 2, 6, and 8 (Figure 5). The specific conversion does not match the theoretical water content exactly, but a clear trend is visible. Therefore, the lag in activity for ycM37L is due to a lack of water necessary for enzyme flexibility. The decrease in activity for ycM37L and CalB is the result of accumulated water—produced by the reaction—partitioning to the hydrophilic cell surface and dramatically reducing the quality of mixing. In other experiments with 0.5% water added directly to the YSD catalyst, little (if any) dispersion was observed, and the activity was uniformly low (data not shown).

Kinetics of butyl decanoate synthesis

Initial-rate kinetics of YSD-biocatalyzed butyl decanoate syntheses in heptane were measured at 40 °C. Butanol and decanoic acid were combined in equimolar solutions at concentrations between 25 and 250 mM. The concentration of decanoic acid was monitored by GC for 30-60 minutes. Initial rate data (Figure 6) were used to generate a Lineweaver-Burke plot (Figure 7) for synthesis catalyzed by YSD CalB. The v_{max} for the reaction is 1.9 mM/min, with a K_m near 70 mM.

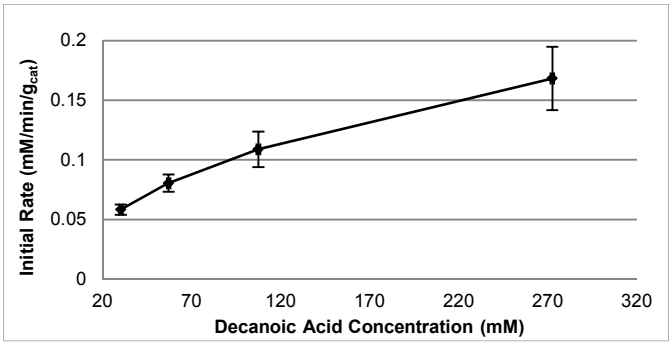

Figure 6: Plot of initial rate (V_0) vs. decanoic acid concentration for YSD CalB-catalyzed synthesis of butyl decanoate. Substrates (equimolar butanol and decanoic acid) and biocatalyst (0.5% w/v) were equilibrated at 40 °C, combined, and stirred at 200 rpm. Values and error bars are the averages and standard deviations, respectively, of three samples.

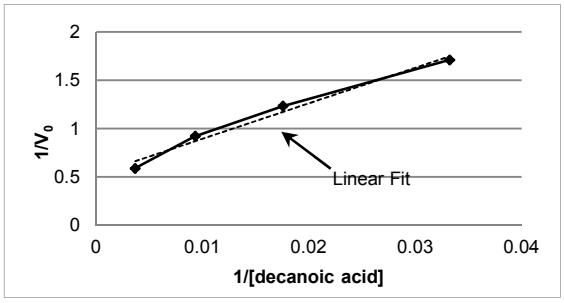

Figure 7: Lineweaver-Burke plot of inverse initial rates vs. inverse substrate concentrations from (Figure 5). The linear least-squares regression has an R^2 of 0.98. The values for V_{max} and K_m were derived from the equation of the trend line.

The rate of synthesis catalyzed by YSD ycM37L was effectively zero without water. That is, no decanoic acid was consumed (Figure 8A). When water was added, to 0.4% v/v as in Figure 5, non-zeroth-order behavior was observed (Figure 8B). At all substrate concentrations tested, a lag time of approximately 15 minutes was observed where the rate was near zero. This addition of water gives rise to behavior that is not consistent with standard enzyme kinetic mechanisms.

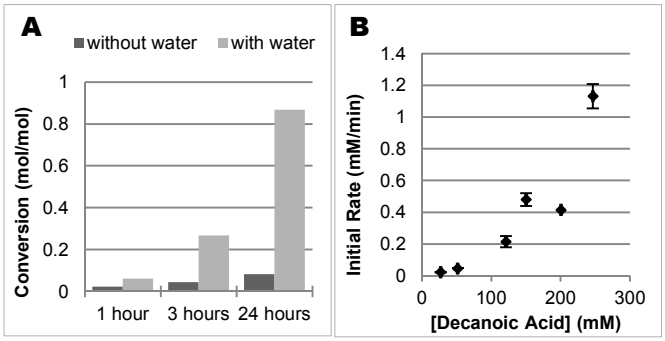

Figure 8: Butyl decanoate synthesis with YSD ycM37L. A. Without water, the reaction was too slow to measure initial rates. Water was added to 0.4% v/v, and equilibrium was reached within 24 hours. B. Plot of initial rate (V_0) vs. decanoic acid concentration for YSD ycM37L-catalyzed synthesis of butyl decanoate. For all trials, substrates (equimolar butanol and decanoic acid) and biocatalyst (0.5% w/v) were equilibrated at 40 °C, combined, and stirred at 200 rpm. Where indicated, water was slowly added to 0.4% v/v to initiate the reaction.

Effects of Alcohol Hydrophobicity and Catalyst Loading

To examine the effects of alcohol hydrophobicity on the esterification reaction, decanoic acid was esterified with 1-hexanol and 1-octanol (Figure 9). While a general trend is observed—lower conversion with longer alcohol—the similarity between the results with hexanol and octanol suggest that a threshold of solubility or diffusion has been reached. The solubility of each component in water is presented in Table 1.

Table 1: Solubility of decanoate ester reactants in water

Reactant	Solubility in water[53] (g/kg at 25 °C)
1-Butanol	79
1-Hexanol	5.9
1-Octanol	0.46
Decanoic acid	0.15[a]

[a] Solubility at 20 °C

The effects of catalyst loading on butyl decanoate synthesis were determined. The conditions from batch 6 of the re-use experiment (Figure 4) were approximated and run in parallel

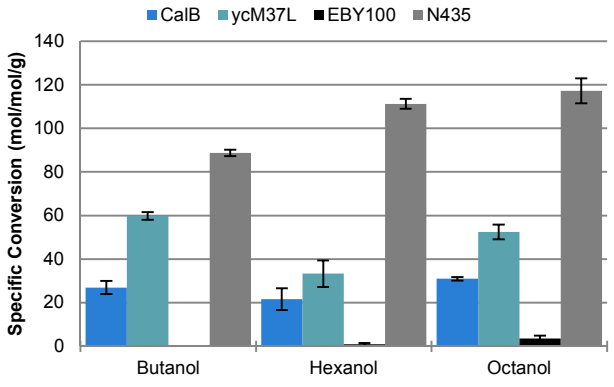

Figure 9: Synthesis of decanoate esters with various alcohols. Solutions of alcohol and decanoic acid (both 100 mM in heptane) were combined with dry catalyst, 0.4 % w/v. Water was slowly added to 0.4% v/v, unless otherwise indicated. *From Figure 5, 0.5% w/v.

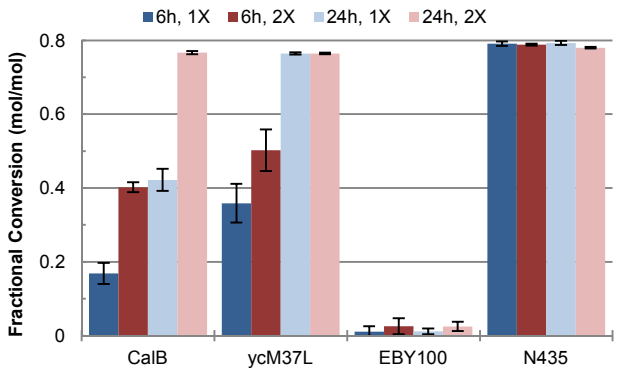

Figure 10: Butyl decanoate synthesis with doubled catalyst loading at 40 °C. Solutions of butanol and decanoic acid were combined (to 100 mM in heptane) with dry catalyst at 0.4% w/v (1X) or 0.8% w/v (2X). Water was slowly added (to 0.5% v/v) and the reaction was stirred at 200 rpm. Samples were taken after 6 and 24 hours.

with identical conditions but with doubled catalyst loading (Figure 10). Doubling the amount of catalyst increases the speed with which the YSD biocatalyst converts the fatty acid by a factor of at least 1.4 (ycM37L), up to a factor of 2 (CalB). The final equilibrium conversion appears unaffected, and no changes were observed in the control or the N435 standard.

Discussion

Comparison of catalyst protein loading

The YSD catalysts and Novozym 435 control were used in the esterification reactions with equal mass loadings. However, apparent differences in activity, stability, and specificity may be due, to a degree, to disparities in enzyme loading on the respective supports. Protein loading for Novozym 435 is not available from the manufacturer or vendors, but a published estimate[33] of 30 mg enzyme per gram of catalyst will be considered for scale. Previous estimates for the enzyme copy number in the Aga2 system were on the order of 50,000[34], which supports our measurements (lit.,[35] 2-5 x 10^5). Given the molecular weights of CalB and ycM37L (33 and 38 kDa, respectively), and assuming 20 billion cells per gram of dry weight[36], 70% of which are expressing the lipases[35], the protein loading for the YSD system was measured. The protein loading in the YSD system (37 µg/gdw (g dry weight) for CalB and 19 µg/gdw for ycM37L) is three orders of magnitude lower than in Novozym 435. If the YSD biocatalyst activity is only an order of magnitude lower (i.e., equilibrium in ~24 hours vs. 1-2 hours for N435 [data not shown]), this would suggest that the lipase in the YSD system is more efficiently accessed. The YSD system has a higher surface-to-volume ratio, and most of the protein is exposed to the solvent, rather than absorbed in a pore in the commercial resin. Increasing the catalyst loading increases the rate of conversion, as expected (Figure 10). Expressing more protein on the surface of the cell via YSD should, therefore, increase the specific activity of the biocatalyst. The current system—a single-copy episomal plasmid engineered for α-agglutinin fusion—is convenient for the facility of its characterization, but other display systems or hosts (e.g., *Pichia pastoris*) might yield higher activity. Novozym 435's higher loading might be a buffer against thermal degradation and protein desorption, which would be a benefit, but excess, inaccessible protein also adds expense.

Specificity for the fatty acid and alcohol

Both ycM37L and CalB appear to convert fatty acids with chain lengths of C12 and shorter to their respective butyl esters with equal ease[35]. The preference (for CalB) persists at higher temperatures (see Figure 1 vs. Figure 2 and Figure 3). The water requirement for ycM37L makes a similar analysis impossible (no water was added), although some water was clearly present, likely absorbed from the atmosphere. With acyl donor chains longer than dodecanoic acid, the activity drops significantly[35]. Analysis of the binding pocket of CalB reveals a channel that is the length of a 12-carbon chain[37]. A similar analysis has not been performed on the structure of M37L, and no study of its acyl donor size specificity for esterification could be found in the literature. However, the profile for hydrolysis of *p*-nitrophenyl esters by M37L in an aqueous environment has been reported[38] and follows a similar trend, though the drop-off is more pronounced between C8 and C12. Studies of the specificity of Novozym 435 suggest weak or no preference for fatty acids shorter than C12[39-41], although there are some reports of preference for shorter acyl donors in more polar solvents[42]. While it is often referred to as *C. antarctica* lipase, its behavior appears to differ from that of the wild-type CalB used in this study. The difference may be attributable to protein loading or surface charge of the support. The weak correlation between longer aliphatic alcohols and lower esterification activity suggests that turnover of the fatty acid is the rate-limiting step in the esterification reaction. The relative hydrophobicity of the reactants (Table 1) compared to the yeast surface implies a diffusion limitation, the mechanism for which would take significant effort to clarify. An upper limit on the accessibility of longer fatty acids to the active site might explain the plateau in CalB activity for dodecanoic acid in Figure 1.

Temperature optima for ester synthesis

The lipase-catalyzed esterification of octanoic (Figure 3) and dodecanoic acid (Figure 1) to their respective butyl esters was carried out at temperatures from 30 to 80 °C. For either fatty acid, the optimum temperature for synthesis (i.e., that at which the highest conversion was reached in six hours) was 60 °C for the CalB biocatalyst and 40 °C for ycM37L, except in the butyl decanoate reaction without water, where the apparent optimum temperature was 30 °C. The difference could be due to batch-to-batch variation in the amount of ambient moisture absorbed, further demonstrating the sensitivity of the system to water content. A relatively low T_{opt} is expected for ycM37L because it comes from a psychrophile; however, the esterification optimum is 15 degrees higher than that previously reported for hydrolysis[38,43]. The ycM37L biocatalyst also experiences a

sharp decline in activity at higher temperatures, losing 50% of its activity at 60° and producing no product at 80°. The support (yeast cell) is expected to confer some thermal stability, likely resulting in the observed $T_{opt,}$ but the enzyme is still sensitive to thermal denaturation.

The YSD CalB biocatalyst also sees some loss of activity at the highest temperature, but half as much as ycM37L (25% vs. 50%). This agrees with previous reports of the thermal stability of CalB in non-aqueous environments[10,19]. The ability of the enzyme (CalB) to operate at higher temperatures makes it advantageous in systems where the reactants are solid or are insoluble at lower temperatures (for example, hexadecanoic acid in heptane). However, in the three systems studied here, ycM37L reaches an equal conversion at a lower temperature.

Intriguingly, N435 did not exhibit the same temperature dependence. At temperatures that do not deactivate the enzyme, the rate is such that the reaction reaches equilibrium within six hours. At higher temperatures, though, a dip in activity is expected. However, if N435 (purportedly CalB) only loses 25% of its activity at 80 ˚C, it could conceivably reach equilibrium before the chosen end point.

Re-use of the YSD biocatalyst

The lipase catalysts were applied to ten batches of the butyl decanoate synthesis reaction (Figure 4). The reaction temperature was chosen to maximize conversion without denaturing ycM37L. Both catalysts maintained activity through all batches, but the ycM37L biocatalyst took two batches to reach a stable activity level. This effect can be ascribed to an initial lack of water necessary for enzyme flexibility, water that was produced by the reaction in sufficient quantities to fully activate the enzyme in two batches. The effect could be reproduced by adding water to identical reactions starting with dry catalyst (Figure 5).

While a small amount of water is necessary for peak activity, repeated use of the catalysts without drying resulted in accumulation of water in and around the hydrophilic yeast cells. Wetted cells do not disperse evenly in the solvent (heptane); aggregation and precipitation of the cells was observed to gradually worsen, starting around batch 5. Eventually, the catalyst clumps to a degree that it cannot be dispersed by mechanical stirring, and the cells cling to the walls of the reaction vessel (glass vials). A concomitant decrease in observed activity is the result. The negative control, which did not produce any product (or water), did not experience the same problems with dispersion (Figure 11). The acrylic resin-supported lipase did not vary in the course of the ten batches. Because the resin is hydrophobic, it would not accumulate water in the same way as the yeast, so none of the water-related problems with mixing and activity were expected. Mitigating the effects water in the reaction would be critical for industrial implementation of the YSD biocatalyst. At the experimental scale, equilibrating the catalyst to its peak water activity and adding molecular sieves to scavenge free water would be plausible. At a larger scale, catalyst drying between batches might be necessary.

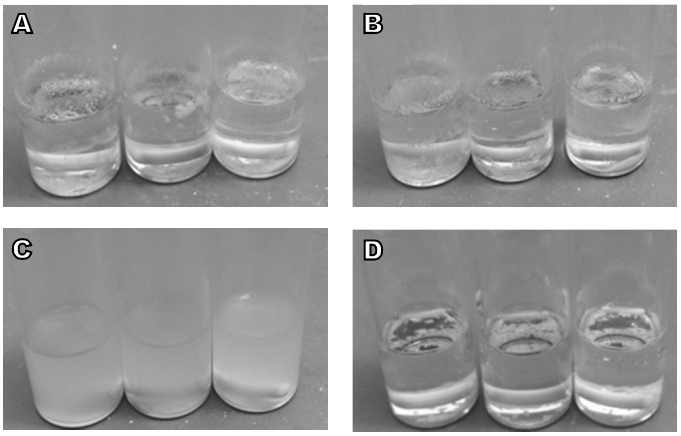

Figure 11: State of the catalyst after 10 batches of butyl decanoate synthesis. YSD biocatalysts displaying CalB (A) and ycM37L (B) aggregate and fall out of suspension after several batches. Control yeast cells (C) do not produce product or visibly change in their dispersion. At the beginning of the experiment, both YSD biocatalysts looked like the control. The Novozym 435 control (D) does not undergo any visible changes

The lyophilized yeast cells are hygroscopic, as evidenced by the difference in activity between freshly prepared catalyst and catalyst that has been repeatedly exposed to the ambient humidity in the lab ("dry" trial, Figure 5). The hydrolytic activity of the two batches of catalyst was the same within 5%, (data not shown). To achieve and maintain optimum activity, therefore, the water activity of the enzyme needs to be carefully controlled[17], and removal of some water from the reaction is recommended.

The stability of ycM37L in a nonaqueous environment has not been reported, but cross-linked enzyme aggregates steadily lost activity in hydrolytic assay containing *p*-nitrophenyl octanoate[50]. Applications of YSD to synthetic reactions have had mixed results. With CalB displayed on *Saccharomyces*, the catalyst lost activity within 5 cycles in toluene[27]. A different system—*Rhizomucor* lipase displayed on *Pichia*—had much better stability in biodiesel reactions, only losing 20% of its activity after ten batches[51]. Notably, filamentous systems employing *Aspergillus* (but no CalB) had excellent stability, producing methyl esters at more than 90% conversion after ten[52] to 15[26] batches. The YSD system described here compares favourably to other SD schemes in terms of activity retention, even robust filamentous systems.

Kinetics of butyl decanoate synthesis

YSD has been applied broadly, as described elsewhere[44], but characterization of YSD catalyst kinetics are absent in the literature. Given the strong dependence of kinetics on the immobilization support[45], comparisons to studies with free enzyme offer little insight. CalB immobilized on polypropylene had K_m values for octanoic acid and octanol (2.5 mM and 2.1

mM, respectively[54]) that were much lower than the 70 mM K_m reported here. It is unlikely that the kinetic parameters derived from these experiments reflect the intrinsic properties of the enzyme. Rather, this *apparent* K_m captures the effectiveness of a lipase that is limited by its environment (the cell surface). Based on the relative insensitivity to alcohol length, the inhibition is likely slow diffusion of the fatty acid. However, a recent kinetic study of N435 demonstrated a higher inhibition constant for the fatty acid in synthesis of hexyl octanoate—in addition to lower specificity for octanoic acid[55]—which might suggest that dead end decanoic acid-enzyme complexes play a part here.

CalB does not require additional water, so the kinetics observed in "dry" reactions are free of the influence of lipase "lid" opening or closing, provided the enzyme is hydrated, and that the amount of water produced by the reaction is negligible. Since ycM37L is apparently inactive without adding water, even a small addition (0.4% v/v is ~ 220 mM) dramatically changes the observed activity. While the yeast cells are hygroscopic and can be expected to absorb a significant portion of the water added, the effects of the additional water were too complex to represent by zeroth-order initial rate experiments. At low substrate concentrations, it is possible that hydrolysis is competing with the ester synthesis reaction, and the individual reactants' effects could not be deconvoluted from the cumulative rate.

Experimental

Strains and media

The host for the recombinant production and display of CalB is *S. cerevisiae* EBY100[46]. Yeast are propagated in 2X YAPD media (20 g/L yeast extract, 100 mg/L adenine hemisulfate, 40 g/L peptone, 40 g/L glucose[47]) at 30 °C. Yeasts carrying the surface display construct are propagated in synthetic complete (SC) media lacking tryptophan, containing glucose (20 g/L) for outgrowth and galactose (20 g/L) for induction. Plasmid DNA is screened and stored in a lab strain of *Escherichia coli* DH5α. Bacteria are grown in LB[48] containing ampicillin at 37 °C.

Cloning

Cloning materials were purchased from New England Biolabs. Construction of the yeast surface display vectors has been described elsewhere[35,46]. Briefly, the gene for wild-type CalB was amplified by PCR from a pET vector. The gene for ycM37L was optimized for yeast expression and synthesized by GENEWIZ, and amplified by PCR from their pUC vector. The PCR products were digested with BamHI and NheI and ligated into similarly digested vectors.

Production of the biocatalyst

Yeast are made electrocompetent in-house[49], transformed by electroporation, and recovered in YAPD containing 0.5M sorbitol. Putative transformants are screened and selected with fluorescently activated cell sorting by means of a C-terminal *c-myc* epitope in the fusion protein. Cultures of the sorted cells are grown in SC (glucose) to an optical density of ~ 5 in baffled flasks at 30 °C. The cells are harvested by centrifugation (4,500 *g,* 4°C, 30 minutes) and re-suspended in SC (galactose with 0.1% glucose) in a 2 L fermenter at 20 °C. The fermenter is stirred at 250 rpm with 4 L/min sparged air. After 18 hours, the cells are harvested by centrifugation (3,000 *g*, 4 °C, 60 minutes) and washed with cold, distilled water. Cells were suspended in distilled water, frozen, and dried. Lyophilization took place at ambient temperature in a SpeedVac (Thermo Fisher) for >8 hours.

Chemicals

Media components are biology grade and were purchased from Fisher (sugars and yeast nitrogen base) or Sigma-Aldrich (amino acids). Heptane, lauric acid, pentanol, and hexanol were purchased from VWR. The other fatty acids and butanol were purchased from Fisher. Butanol and octanol were purchased from Sigma-Aldrich. All organics were GC grade or better. Lipase from *C. antarctica* immobilized on acrylic resin was purchased from Sigma-Aldrich.

Esterification reactions

Dry catalyst was weighed into clean 1.5 dram glass vials. Solutions of one fatty acid and one alcohol (1 mL of each, both 200 mM in heptane) were added to the vials. To reduce pipetting errors and deviation due to evaporation, the reactant solutions were dispensed at room temperature. The vials were sealed and moved to a magnetically stirred heat block with digital temperature control. The reactions were stirred at 200 rpm for six hours, beginning when the reaction was first heated. Samples of 50 µL were added to 1 mL of heptane and analyzed by GC-FID. If the reaction took place at elevated temperature, the vials were briefly cooled in an aluminum bead bath before sampling. For repeated-use experiments, the liquid was aspirated with a pipette and any suspended catalyst was removed by centrifugation (17,000 *g* for 15-30s). The supernatant was decanted and fresh reactant solution was used to re-suspend the cells.

Substrates were prepared for the kinetics experiments by diluting equal volumes of 0.500 M solutions of butanol and decanoic acid in heptane. The substrates and biocatalyst were incubated separately at the reaction temperature (40 °C) for 15 minutes before combining. The reactions were stirred at 200 rpm. Water was added, if necessary, with an automatic pipette (Viaflo) dispensing at the slowest setting to reduce droplet size.

The samples were analyzed on a Shimadzu GC2010 equipped with an autosampler and a DB-5 capillary column (30m x 0.25 µm x 0.25 µm). One µL of each sample was injected at a 30:1 split ratio with helium as the carrier gas. The injector and detector were held at 300 °C. The oven temperature was held at 90 °C for 2 minutes, ramped to 160 °C at 20 °/min, to 250 °C at 5 °/min, and to 300 °C at 25 °/min. The concentration of the

fatty acid was determined by comparing the FID peak area to standard curves prepared in heptane.

Conclusions

Biocatalysts based on yeast surface display of ycM37L and CalB can effectively catalyze the synthesis of fatty acid esters of aliphatic alcohols. In six-hour batches at relatively mild temperatures (30-60 °C), the YSD catalyst's activity is 20-50% that of a commercial lipase (N435), despite three orders of magnitude differences in protein loading. The two lipases exhibit similar specificity for acyl donors of C12 and shorter. The optimum temperature for synthesis with ycM37L is 40 °C; with CalB, 60 °C. Both biocatalysts demonstrated long-term, multi-batch stability in the synthesis of butyl decanoate, and the need for control of water concentration in the reaction was evident. This system represents an inexpensive, green alternative to conventional synthesis methods.

Acknowledgements

The authors gratefully acknowledge Sonia Herrero from Professor Margo Daub's lab at NCSU for donating a lab strain of E. coli; Professor Balaji Rao's lab for donating the pCT-EGFP vector, the stocks for EBY100, and time on the cell sorter; and Kerri Cushing for donating the pET22-CalB vector. This work (JE) was supported in part by NSF award 0832498 through the Center for BioEnergy Research and Development, by the GAANN Interdisciplinary Doctoral Program in Molecular Biotechnology, award P200A090129-11, and by the Eastman Chemical Center of Excellence. The authors declare no conflicts of interest.

Notes and references

[a] North Carolina State University, Department of Chemical and Biomolecular Engineering, 911 Partners Way, Raleigh, NC 27695-7905.
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

1 G. Roscher, in *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA, 2000.
2 L. Fjerbaek, K. V. Christensen and B. Norddahl, *Biotechnol. Bioeng.,* 2009, **102**, 1298-1315.
3 C. Le Berre, P. Serp, P. Kalck and G. P. Torrence, in *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA, 2000.
4 J. Gao, Z. Luo and F. Luo, *Carbohydr. Polym.,* 2012, **89**, 1215-1221.
5 D. C. Steytler, P. S. Moulson and J. Reynolds, *Enzyme Microb. Technol.,* 1991, **13**, 221-226.
6 P. Villeneuve, J. M. Muderhwa, J. Graille and M. J. Haas, *J. Molec. Catal. B,* 2000, **9**, 113-148.
7 S. Chen and J. Fang, *J. Org. Chem.,* 1997, **62**, 4349-4357.
8 G. Kirchner, M. P. Scollar and A. M. Klibanov, *J. Am. Chem. Soc.,* 1985, **107**, 7072-7076.
9 O. Kirk, F. Björkling, S. E. Godtfredsen and T. O. Larsen, *Biocatal. Biotransfor.,* 1992, **6**, 127-134.
10 N. N. Gandhi, S. B. Sawant and J. B. Joshi, *Biotechnol. Bioeng.,* 1995, **46**, 1-12.
11 A. M. Brzozowski, U. Derewenda, Z. S. Derewenda, G. G. Dodson, D. M. Lawson, J. P. Turkenburg, F. Bjorkling, B. Huge-Jensen, S. A. Patkar and L. Thim, *Nature,* 1991, **351**, 491-494.
12 B. Withers-Martinez, F. Carrière, R. Verger, D. Bourgeois and C. Cambillau, *Structure,* 1996, **4**, 1363-1374.
13 U. Derewenda, A. M. Brzozowski, D. M. Lawson and Z. S. Derewenda, *Biochemistry,* 1992, **31**, 1532-1541.
14 A. M. Klibanov, *Trends Biochem. Sci.,* 1989, **14**, 141-144.
15 P. E. Sonnet and G. G. Moore, *Lipids,* 1988, **23**, 955-960.
16 P. J. Halling, *Enzyme Microb. Technol.,* 1994, **16**, 178-206.
17 A. Yoshida, S. Hama, K. Nakashima and A. Kondo, *Enzyme Microb. Technol.,* 2011, **48**, 334-338.
18 G. D. Yadav and A. H. Trivedi, *Enzyme Microb. Technol.,* 2003, **32**, 783-789.
19 S. Shankar, M. Agarwal and S. P. Chaurasia, *Indian J. Biochem. Biophys.,* 2013, **50**, 570-576.
20 M. Kato, J. Fuchimoto, T. Tanino, A. Kondo, H. Fukuda and M. Ueda, *Appl. Microbiol. Biotechnol.,* 2007, **75**, 549-555.
21 A. Illanes, *Enzyme biocatalysis: principles and applications*, Springer, New York, 2008.
22 T. Matsumoto, H. Fukuda, M. Ueda, A. Tanaka and A. Kondo, *Appl. Environ. Microbiol.,* 2002, **68**, 4517-4522.
23 A. Kondo and M. Ueda, *Appl. Microbiol. Biotechnol.,* 2004, **64**, 28-40.
24 S. H. Lee, J. Choi, M. Han, J. H. Choi and S. Y. Lee, *Biotechnol. Bioeng.,* 2005, **90**, 223-230.
25 Y. Fujita, S. Takahashi, M. Ueda, A. Tanaka, H. Okada, Y. Morikawa, T. Kawaguchi, M. Arai, H. Fukuda and A. Kondo, *Appl. Environ. Microbiol.,* 2002, **68**, 5136-5141.
26 S. Tamalampudi, S. Hama, T. Tanino, M. R. Talukder, A. Kondo and H. Fukuda, *J. Molec. Catal. B,* 2007, **48**, 33-37.
27 T. Tanino, T. Aoki, W. Chung, Y. Watanabe, C. Ogino, H. Fukuda and A. Kondo, *Appl. Microbiol. Biotechnol.,* 2009, **82**, 59-66.
28 S. Han, Z. Pan, D. Huang, M. Ueda, X. Wang and Y. Lin, *J. Molec. Catal. B,* 2009, **59**, 168-172.
29 Z. Pan, Z. Yang, L. Pan, S. Zheng, S. Han and Y. Lin, *J. Ind. Microbiol. Biotechnol.,* 2014, **41**, 711-720.
30 Z. Jin, J. Ntwali, S. Han, S. Zheng and Y. Lin, *J. Biotechnol.,* 2012, **159**, 108-114.
31 Z. Jin, S. Liang, X. Zhang, S. Han, C. Ren, Y. Lin and S. Zheng, *Biotechnol. Bioprocess. Eng.,* 2013, **18**, 365-374.
32 A. Zaks and A. M. Klibanov, *J. Biol. Chem.,* 1988, **263**, 8017-8021.
33 Z. Cabrera, G. Fernandez-Lorente, R. Fernandez-Lafuente, J. M. Palomo and J. M. Guisan, *J. Molec. Catal. B,* 2009, **57**, 171-176.
34 S. Shibasaki, M. Ueda, T. Iizuka, M. Hirayama, Y. Ikeda, N. Kamasawa, M. Osumi and A. Tanaka, *Appl. Environ. Microbiol.,* 2001, **55**, 471-475.
35 J. Eby and S. W. Peretti, *RSC Adv.,* 2015, **5**, 19166 - 19175.
36 B. Aguilar-Uscanga and J. M. François, *Lett. Appl. Microbiol.,* 2003, **37**, 268-274.

37 J. Pleiss, M. Fischer and R. D. Schmid, *Chem. Phys. Lipids,* 1998, **93**, 67-80.
38 H. S. Ryu, H. K. Kim, W. C. Choi, M. H. Kim, S. Y. Park, N. S. Han, T. K. Oh and J. K. Lee, *Appl. Microbiol. Biotechnol.,* 2005, **70**, 321-326.
39 M. Ardhaoui, A. Falcimaigne, S. Ognier, J. M. Engasser, P. Moussou, G. Pauly and M. Ghoul, *J. Biotechnol.,* 2004, **110**, 265-272.
40 P. Degn, L. H. Pedersen, J. ø Duus and W. Zimmermann, *Biotechnol. Lett.,* 1999, **21**, 275-280.
41 S. Patkar, J. Vind, E. Kelstrup, M. W. Christensen, A. Svendsen, K. Borch and O. Kirk, *Chem. Phys. Lipids,* 1998, **93**, 95-101.
42 N. R. Pedersen, R. Wimmer, J. Emmersen, P. Degn and L. H. Pedersen, *Carbohydr. Res.,* 2002, **337**, 1179-1184.
43 S. Jung, D. G. Jeong, M. S. Lee, J. Lee, H. Kim, S. E. Ryu, B. C. Park, J. H. Kim and S. J. Kim, *Proteins,* 2008, **71**, 476-484.
44 Y. Liu, R. Zhang, Z. Lian, S. Wang and A. T. Wright, *J. Molec. Catal. B,* 2014, **106**, 17-25.
45 C. Oliveira, M. F. Rosa, M. R. Aires-Barros and J. M. S. Cabral, *J Molec. Catal. B,* 2001, **11**, 999-1005.
46 E. T. Boder and K. D. Wittrup, *Nat. Biotechnol.,* 1997, **15**, 553-557.
47 D. R. Gietz and R. A. Woods, in *Methods in Enzymology*, ed. Christine Guthrie and Gerald R. Fink, Academic Press, 2002, p. 87-96.
48 J. Sambrook, *Molecular cloning: a laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor N.Y., 2001.
49 D. M. Becker and V. Lundblad, in *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., 2001.
50 J. Han and H. Kim, *J. Microbiol. Biotechnol.,* 2011, **21**, 1159-1165.
51 D. Huang, S. Han, Z. Han and Y. Lin, *Biochem. Eng. J.,* 2012, **63**, 10-14.
52 T. Adachi, J. Ito, K. Kawata, M. Kaya, H. Ishida, H. Sahara, Y. Hata, C. Ogino, H. Fukuda and A. Kondo, *Appl. Microbiol. Biotechnol.,* 2008, **81**, 711-719.
53 "Aqueous Solubility and Henry's Law Constants of Organic Compounds," in *CRC Handbook of Chemistry and Physics, 95th Edition* (Internet Version 2015), W. M. Haynes, ed., CRC Press/Taylor and Francis, Boca Raton, FL.
54 M. Martinelle and K. Hult, *Biochim. Biophys. Acta*, 1995, **1251**, 191-197.
55 C. Lopresto, V. Calabrò, J. Woodley and P. Tufvesson, *J. Molec. Catal. B*, 2014, **110**, 64-71.