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Synthetic resin-bound truncated *Candida antarctica* lipase B for production of fatty acid alkyl esters by transesterification of corn and soybean oils with ethanol or butanol

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ABSTRACT

A gene encoding a synthetic truncated *Candida antarctica* lipase B (CALB) was generated via automated PCR and expressed in *Saccharomyces cerevisiae*. Western blot analysis detected five truncated CALB variants, suggesting multiple translation starts from the six in-frame ATG codons. The longest open reading frame, which corresponds to amino acids 35–317 of the mature lipase, appeared to be expressed in the greatest amount. The truncated CALB was immobilized on Sepabeads® EC-EP resin and used to produce ethyl and butyl esters from crude corn oil and refined soybean oil. The yield of ethyl esters was 4-fold greater from corn oil than from soybean oil and was 36% and 50% higher, respectively, when compared to a commercially available lipase resin (Novozym 435) using the same substrates. A 5:1 (v/v) ratio of ethanol to corn oil produced 3.7-fold and 8.4-fold greater yields than ratios of 15:1 and 30:1, respectively. With corn oil, butyl ester production was 56% higher than ethyl ester production. Addition of an ionic catalytic resin step prior to the CALB resin increased yields of ethyl esters from corn oil by 53% compared to CALB resin followed by ionic resin. The results suggest resin-bound truncated CALB has potential application in biodiesel production using biocatalysts.

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1. Introduction

Biodiesel is a renewable, domestic, and clean-burning advanced biofuel that is an alternative to conventional petroleum diesel fuel (Federal Register, 2010). Biodiesel consists of fatty acid alkyl esters produced by catalytic transesterification of triglycerides extracted from lipid-bearing plants and animals. In transesterification, an alcohol, such as methanol, ethanol, or butanol, is reacted with the triglycerides to produce fatty acid methyl, ethyl, or butyl esters (FAME, FAEE, or FABE), respectively. Commercially, methanol is generally used because it is less expensive than other alcohols. Even under ideal conditions, about 10% of the substrate is released as glycerol byproduct, which is unsuitable for internal combustion engines. Most current industrial transesterification processes use homogeneous chemical catalysts that are inexpensive and give high levels of conversion in short reaction times. However, chemical transesterification has several drawbacks: free fatty acids and water in the oil interfere with the reaction, the process is energy intensive, purification of glycerol is difficult, the catalyst must be removed from the products, and alkaline wastewater requires treatment (Fukuda et al., 2001; Vasudevan and Briggs, 2008). Heterogeneous biocatalysts, such as lipases, eliminate several of

Abbreviations: CALB, *Candida antarctica* lipase B; ORF, open reading frame; SBO, soybean oil; CO, corn oil; EE, ethyl ester.

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these drawbacks (Akoh et al., 2007; Fjerbaek et al., 2009; Nielsen et al., 2008). Enzymatic transesterification yields high purity product without generating alkaline wastewater streams, enables easy recovery of higher quality glycerol, and in many cases the catalyst is readily recovered and subsequently re-used (Ranganathan et al., 2008; Salis et al., 2009). A major barrier to commercialization of the enzymatic process is the cost of the enzymes, which could be reduced by producing large quantities of stable, active lipases using recombinant DNA technology from ethanologenic yeast strains available on-site at ethanol production facilities (Blank et al., 2006; Kato et al., 2007; Lutz, 2004; Magnusson et al., 2005; Patkar et al., 1998; Qian et al., 2009; Villeneuve et al., 2000).

Recently, the biodiesel industry has been plagued by economic obstacles. Of particular concern is the high cost of commodity vegetable oils, such as soybean oil in the United States (Moser, 2009; Paulson and Ginder, 2007; Vasudevan and Briggs, 2008). Because current processes for fuel ethanol production from corn starch yield substantial amounts of low-quality corn oil as a byproduct, producing biodiesel from corn oil extracted at an ethanol production facility would be an ideal option to reduce feedstock costs. Corn oil triglycerides may be converted into fatty acid ethyl esters and glycerol by transesterification with ethanol already available on-site. In principle, an integrated biorefinery combining starch ethanol and cellulosic ethanol production in one location may become cost-effective if biodiesel is also produced as a co-product in a single-step column transesterification of ethanol and corn oil catalyzed by low-cost resin bound lipases expressed in a recombinant yeast strain capable of cellulosic ethanol production.

The immobilization of biocatalysts contributes to the cost-effectiveness of most enzymatic production processes (Akoh et al., 2007; Bernardes et al., 2007; Guisán, 2006; Park et al., 2011; Shimada et al., 2002; Svendsen, 2000; Tan et al., 2010; Watanabe et al., 2002). Lipases may be immobilized on specialty resins to establish a continuous single-step column process for biodiesel production, even with a high content of free fatty acids in the oil. Sepabeads® EC-EP are polymethacrylate beads containing epoxy functional groups on their surfaces. These beads anchor lipase catalysts to their surfaces through covalent linkages with the epoxy functional groups and increase the stability of the enzyme. A polyhistidine tag on the lipase facilitates a nucleophilic substitution reaction, which causes immobilization to occur (Mateo et al., 2002; Torres et al., 2003).

Candida antarctica lipase B (CALB) is a widely used industrial biocatalyst (Blank et al., 2006; Magnusson et al., 2005; Patkar et al., 1998; Qian et al., 2009; Sharma et al., 2001). It has the α/β hydrolase fold typical of most lipases. The active site contains the oxyanion hole (Gly39 and Thr40), which stabilizes the transition states, and a catalytic triad consisting of Ser105, His224, and Asp187. The active site pocket is composed of two channels, the acyl- and the alcohol-binding sites. The alcohol-binding part contains the stereospecificity pocket, defined by Thr42, Ser47, and Trp104. In contrast to other lipases CALB does not display interfacial activation. The lid region (helix 7–9; aa 135–155) and helix 16/17 in the C-terminal part of the protein play a role in controlling access to the active site (Blank et al., 2006; Lutz, 2004; Magnusson et al., 2005; Qian et al., 2009; Uppenburg et al., 1995). Optimization of lipases and development of strains to express large quantities of these biocatalysts are essential for inexpensive production of biodiesel from multiple alcohols. The objective is to prepare a highly active, stable, low-cost lipase expressed at high levels in an ethanologenic yeast strain.

This work describes the production of an ORF for a synthetic truncated *C. antarctica* lipase B (CALB) enzyme using automated PCR amplification from a full-length synthetic CALB ORF assembled previously (Hughes et al., 2011). The amplified CALB ORF is cloned into pYES2 DEST 52, expressed at a high level in *Saccharomyces cerevisiae*, immobilized on Sepabeads® resin, and assayed for production of fatty acid ethyl and butyl esters from corn oil or soybean oil triglycerides and cellulosic ethanol or n-butanol.

2. Materials and methods

2.1. PCR amplification of an ORF for a truncated CALB enzyme

A gene open reading frame (ORF) encoding a synthetic truncated CALB enzyme was produced by automated PCR amplification of a portion of the sequence of a synthetic full-length CALB ORF produced previously by a stepwise PCR assembly strategy (Hughes et al., 2011). The amplified sequence included nucleotides 178–1026 (numbering starts at beginning of prepro sequence) of the GenBank sequence (accession number Z30645) for *C. antarctica* lipase B except that the full-length synthetic CALB used as PCR template had mutations at position 245 (C to T) and position 986 (T to A). The mutation at nucleotide 245 produces a new in-frame start codon. Nucleotides 178–180 (CTT) code for amino acid 35 of the mature protein (not including the prepro sequence in the numbering). The amplified sequence (nucleotides in the forward and reverse primers are underlined) was:
CTTCTCGTCCCCGGAACCGGCACCACAGGTCCACAGTCGTTCGACTC-GAACTGGATCCCCCTCTCA**ATG**CAGTTGGGTTACACACCCTGCTGGA-TCTCACCCCCGCCGTTC**ATG**CTCAACGACACCCAGGTCAACACGGAG-TAC**ATG**GTCAACGCCATCACCGCGCTCTACGCTGGTTCGGGCAACAA-CAAGCTTCCCGTGCTTACCTGGTCCCAGGGTGGTCTGGTTGCACAGT-GGGGTCTGACCTTCTTCCCCAGTATCAGGTCCAAGGTCGATCGACTT-**ATG**GCCTTTGCGCCCGACTACAAGGGCACCGTCCTCGCCGGCCCTCT-CGATGCACTCGCGGTTAGTGCACCCTCCGTATGGCAGCAAACCACCG-GTTCGGCACTCACCACCGCACTCCGAAACGCAGGTGGTCTGACCCAG-ATCGTGCCCACCACCAACCTCTACTCGGCGACCGACGAGATCGTTCA-GCCTCAGGTGTCCAACTCGCCACTCGACTCATCCTACCTCTTCAACG-GAAAGAACGTCCAGGCACAGGCCGTGTGTGGGCCGCTGTTCGTCAT-CGACCATGCAGGCTCGCTCACCTCGCAGTTCTCCTACGTCGTCGGT-CGATCCGCCCTGCGCTCCACCACGGGCCAGGCTCGTAGTGCAGACT-ATGGCATTACGGACTGCAACCCTCTTCCCGCCAATGATCTGACTCCC-GAGCAAAAGGTCGCCGCGGCTGCGCTCCTGGCGCCGGCAGCTGCAG-CCATCGTGGCGGGTCCAAAGCAGAACTGCGAGCCCGACCTC**ATG**CCC-TACGCCCGCCCCTTTGCAGTAGGCAAAAGGACCTGCTCCGGCATCGT CACCCCC.

The forward primer was: CACC**ATG**CTTCTCGTCCCCGGAACCGG-CACCACAGGTCCACAGTCGTTCGACTC. The primer brings in the first (artificial) ATG start codon. Subsequent in-frame ATGs are nucleotides 244–246 (T is a mutation; codon was ACG in GenBank sequence), 289–291, 322–324, 460–462, and 967–969).

The reverse primer was: GGGGGTGACGATGCCGGAGCAGGTC-CTTTTGCCTAC. PCR thermal cycling and purification of the PCR amplicon were performed on a robotic workcell using the automated protocols described previously (Hughes et al., 2011). The PCR mixture contained 38 μL H_2O, 10 μL 5× Phusion HF Buffer, 1 μL 10 mM dNTPs, 0.5 μL oligonucleotide mixture, and 0.5 μL Phusion Enzyme (Finnzymes Phusion High-Fidelity PCR kit; New England Biolabs, Ipswich, MA). The oligonucleotide mixture consisted of 10 μL (1 μg/μL) each of the full-length CALB template (Hughes et al., 2011), forward primer, and reverse primer (Sigma–Aldrich, St. Louis, MO, USA). The reaction was prepared in a Bio-Rad Hard-Shell 96-well PCR plate (Bio-Rad Laboratories, Hercules, CA) on ice, placed into a PTC-225 Tetrad (Bio-Rad Laboratories, Hercules, CA) at 98 °C for 2 min, and run using the following cycle: 98 °C for 40 s, 50 °C for 30 s, 72 °C for 45 s, repeated for 30 times, followed by 72 °C for 7 min and 4 °C for 5 min. The amplified DNA was separated using agarose gel electrophoresis and observed with an AlphaImager™ 3400 (Alpha Innotech Corporation, San Leandro, CA) using a trans-UV light. The 964-base-pair amplicon was isolated and purified by the GENECLEAN® II method (GENECLEAN® II kit, MP Biomedical,

Irvine, CA) as described previously (Hughes et al., 2011). The purified DNA was used for directional cloning into pENTR D TOPO® vector.

2.2. Directional cloning into pENTR D TOPO and plasmid preparation

The purified ORF for the truncated CALB enzyme, with a CACC sequence at the 3′ end to facilitate cloning into pENTR, was TOPO ligated directionally into pENTR D TOPO vector (Life Technologies Corporation, formerly Invitrogen, Carlsbad, CA) and the resulting reaction was transformed into TOP10 chemically competent *Escherichia coli* cells (Life Technologies Corporation, Carlsbad, CA), as described previously (Hughes et al., 2006). Volumes of 20 μL and 100 μL of the bacterial culture were spread onto two lysogeny broth kanamycin (LB Kan; Teknova, Inc., Hollister, CA) plates and incubated overnight at 37 °C. Selected bacterial colonies from the plates were picked into each well of an ABgene-0932 96-well deepwell plate filled with 1600 μL LB Kan medium and incubated overnight at 37 °C with shaking. The resulting colonies were subjected to a plasmid preparation procedure using QIAprep 96 Turbo Kit protocol (Qiagen, Valencia, CA) as described previously (Hughes et al., 2007).

2.3. Restriction enzyme digestion and DNA sequencing

Forty-four microliters of pENTR D TOPO® containing the ORF for the truncated CALB enzyme and 6 μL of the restriction enzyme mixture containing 0.5 μL *Bsr*GI restriction endonuclease, 0.5 μL BSA, and 5.0 μL Buffer 2 (New England Biolabs, Ipswich, MA) were combined in a Bio-Rad 96-well Hard-Shell plate and placed in an incubator at 37 °C overnight. The digestion reaction mixture was subjected to agarose gel electrophoresis to verify the presence of the inserts, and the inserts were sequenced as described previously (Hughes et al., 2006).

2.4. Gateway® cloning into pYES2 DEST 52 expression vector

Four microliters of pENTR D TOPO® containing the ORF for a truncated CALB enzyme, 1 μL of the destination vector pYES2 DEST 52 (Invitrogen, Carlsbad, CA), and 3 μL 1x Tris–EDTA pH 8 buffer (Sigma–Aldrich, St. Louis, MO) were combined in a 1.5-mL microfuge tube at room temperature. After the LR Clonase™ II enzyme mixture (Invitrogen, Carlsbad, CA) was thawed on ice for 2 min and mixed, 2 μL were added to the reaction mixture. The reaction mixture was treated as described previously (Hughes et al., 2007). The plasmid (pYES2 DEST 52 containing the amplified CALB ORF) was extracted using the QIAprep 96 Turbo Kit protocol (Qiagen, Valencia, CA).

2.5. Yeast transformation

Four isolated yeast colonies of PJ69-4 (S. Fields, Washington University, Seattle, WA) from frozen glycerol stock were inoculated into two different 125-mL baffled shake flasks containing 25 mL of autoclaved yeast extract Bacto-Peptone and dextrose (YPD) rich medium [10 g/L yeast extract, 20 g/L bacto-peptone, and 20 g/L dextrose (Sigma–Aldrich, St. Louis, MO)] and placed in a New Brunswick Innova 4230 Incubator/Shaker at 30 °C for 2 days with shaking. A 1.5-mL aliquot was taken from each flask and placed into a 1.5 mL microfuge tube and centrifuged at 15,000 × *g* for 5 min. The supernatant was removed, and 125 μL of EZ-transformation solution, 4 μL of plasmid (pYES2 DEST 52 containing the ORF for the truncated CALB enzyme), and 5 μL of carrier DNA (MP Biomedical, Irvine, CA) were combined and added to the pellet in the tube. The cells were resuspended by mixing and incubated at 42 °C for 30 min. Volumes of 20 μL and 100 μL were then spread onto two separate complete minimal (CM) 2% (w/v) glucose minus-URA selective medium plates (Teknova, Inc., Hollister, CA) and incubated at 30 °C for 2 days. CM 2% (w/v) glucose minus-URA medium consisted of the following: 1.4 g/L yeast synthetic drop-out medium supplement minus histidine, leucine, tryptophan, and uracil; 0.06 g/L L-leucine; 0.04 g/L L-tryptophan; 0.02 g/L L-histidine; 20 g/L dextrose; and 5 g/L ammonium sulfate (Sigma–Aldrich, St. Louis, MO).

2.6. Expression of truncated CALB in PJ69-4 yeast and yeast cell lysis

Precultures were prepared by inoculating 25 mL of liquid glucose minus-URA medium in 150-mL Erlenmeyer flasks with *S. cerevisiae* diploid strain PJ69-4 containing pYES2 DEST 52. After incubation for 2 days at 30 °C with shaking to absorbance equivalent to 1.5–1.8 at 660 nm, 20 mL of the preculture was transferred into 1 L of CM glucose minus-URA liquid medium or YPD rich medium consisting of 10 g/L yeast extract, 20 g/L Bacto Peptone, and 20 g/L glucose (Sigma–Aldrich, St. Louis, MO) in a 4 L Erlenmeyer. After incubation for 2 days at 30 °C with shaking to absorbance equivalent to 1.5–1.8 at 660 nm, three 1-mL samples were collected for Western blot analysis. The remaining culture was centrifuged in a Beckman Avanti J20 (Beckman Coulter, Inc. (Fullerton, CA) for 20 min at 2340 × *g* in a JS4.3 swing bucket rotor using 500-mL spin bottles. The supernatant was decanted and the pellet was transferred into CM galactose minus-URA medium consisting of 1.4 g/L yeast synthetic drop-out medium supplement minus histidine, leucine, tryptophan, and uracil; 0.06 g/L L-leucine; 0.04 g/L L-tryptophan; 0.02 g/L L-histidine; 20 g/L galactose; and 5 g/L ammonium sulfate (Sigma–Aldrich, St. Louis, MO) or YPG rich medium consisting of 10 g/L yeast extract, 20 g/L Bacto Peptone, and 20 g/L galactose (Sigma–Aldrich, St. Louis, MO) to activate the *GAL*1 promoter driving expression of the truncated CALB. The culture was incubated for 2 days at 30 °C with shaking. After incubation, sampling for Western blot analysis, and centrifugation, the pellets were resuspended in an equal volume of Yeast-Protein Extraction Reagent (Y-PER Plus; Thermo Fisher Scientific, Inc., Waltham, MA) and placed in a 60-cm^3 syringe having an 18-gauge needle. The syringe was filled with about 3.0 mL of acid-washed 425–600 μm (US sieve size 30–40) glass beads too large to pass through the needle. The syringe plunger was pushed in to drive the pellet solution through the beads and lyse the yeast cells. The plunger was pulled out and the solution that was driven through was poured back into the syringe. This process was repeated five times. A light microscope was used to confirm that the yeast cells were ruptured. One milliliter Qiagen Superflow nickel beads (Qiagen, Valencia, CA), capable of binding 20 mg of His-tagged protein per mL slurry, was added to the lysed yeast cells in a 50-mL tube and the mixture was incubated overnight at 4 °C with low-speed tumbling.

The syringe plunger treatment would not be used in large-scale extraction of the enzyme. If the cell cannot be genetically engineered so that the desired product is excreted into the environment, it will be disrupted by one of the methods that has been proven successful on a large scale, such as a bead mill, which is regarded as one of the most efficient disruption techniques (Chisti and Moo-Young, 1986).

2.7. Binding of truncated CALB to resin

After incubation, 2.5 mL of the lysed cells was placed into each of four 50-mL tubes, and centrifuged at 2340 × *g* for 15 min. The supernatant in each tube was drawn off and divided into a second set of four 50-mL tubes. The pellet remaining in each of the first

four tubes was resuspended in 2 mL of 20 mM PBS pH 7 (Mediatech, Inc. Manassas, VA) plus 2 M $(NH_4)_2SO_4$ (Sigma–Aldrich, St. Louis, MO) equilibrium buffer (resin-charging buffer),and centrifuged. The supernatant was drawn off and was divided among the four tubes containing the previous supernatant, and the process was repeated once more. To each of the four tubes containing pooled supernatant were added 5.0 mL of buffer and 1.5 mL of EC-EP Sepabeads® resin (Resindion (Binasco, MI-Italy; Silvia et al., 2006). To each of the 4 tubes containing pelleted cells were added 2.0 mL buffer, 1.5 mL resin, and 0.5 mL 300 mM EDTA (Sigma–Aldrich, St. Louis, MO) to elute the lipase from the Ni beads. Four tubes containing 2.5 mL of commercial CALB resin (acrylic resin with Novozym 435; Sigma–Aldrich, St. Louis, MO) and 2.5 mL of buffer and a tube with 1.5 mL Sepabeads resin and 2.5 mL buffer were also prepared. The tubes were tumbled overnight (16–18 h) at 30 °C. After incubation, tubes were centrifuged for 15–20 min at 2340 × *g*, and the supernatant was removed. The resins with bound lipase were used in the production of fatty acid alkyl esters. Four 50-mL conical tubes containing 1.5 mL of DIAION® weakly acidic cation-exchange resin (Tai-Young Chemical Co., Ltd., Taiwan) were also prepared to be used in sequence with the CALB resin.

2.8. Fatty acid alkyl ester production and analysis by gas chromatography

A 30:1, 15:1, or 5:1 (v/v) mixture of absolute ethanol or n-butanol (Sigma–Aldrich, St. Louis, MO) with corn oil (Lincolnland Agri-Energy, LLC, Palestine, IL) or ethanol with soybean oil (KIC Chemicals, Inc., New Paltz, NY) was prepared. The alcohol/oil mixture to be used was added in a 2:1 (v/v) ratio of mixture to resin to the appropriate tubes containing resin-bound lipase and to the tubes containing 1.5 mL of DIAION® ionic resin. All tubes were tumbled and incubated overnight (16 h) at 37 °C. After incubation, a 1:1 (v/v) ratio of hexane was added to each tube. The samples were vigorously shaken and then centrifuged for 15–20 min at 2340 × *g*. From each sample, 100 μL was collected from the hexane layer at the top and placed into a vial containing 1 mL of hexane. The hexane samples were analyzed for fatty acid alkyl ester production using an Agilent model 6890 GC-FID equipped with a model 6890 series injector and an Agilent D8-5HT (15 m × 0.32 mm i.d., 0.10 μm film thickness) column (Agilent, Santa, Clara, CA). The injection volume was 1 μL. The carrier gas was helium at a flow rate of 3 mL/min. The oven temperature was initially held at 50 °C for 1 min, increased to 180 °C at 15 °C/min, to 230 °C at 7 °C/min, and to 380 °C at 30 °C/min, and held for 10 min at 380 °C. One microgram of triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), and ethyl ester (EE) were used as standards using the same injection volume and GC conditions as the samples.

2.9. Western blot analysis

One-mL samples of culture were taken after incubation in YPD medium and after incubation in YPG medium and placed in a 1.5-mL microfuge tube, centrifuged for 2 min at 15,000 × *g*, and the supernatant was transferred to a new tube. Twenty milliliters 2× Tris–tricine loading buffer (pH 8.3; Invitrogen, Carlsbad, CA) plus 2 mL β-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA) were prepared and 40 μL were added to the pellet and supernatant samples. The samples were mixed thoroughly and heated at 95 °C for 10 min, then 10 μL of each sample and 3 μL of SeeBlue® Plus2 Pre-stained Standard (Invitrogen, Carlsbad, CA) were loaded onto a 4–20% Tris–tricine SDS-PAGE gel (Invitrogen, Carlsbad, CA) in an X-Cell® Novex box and run in 1× Tris–tricine running buffer (Invitrogen, Carlsbad, CA) at 125 V for 100 min on the Bio-Rad Power Pac Basic™. The PVDF membrane was prepared and processed as described previously (Hughes et al., 2011) using 30 μL of a solution prepared by reconstituting a 1-μg vial of Qiagen Penta-His antibody (Qiagen, Valencia, CA) with 1 mL 1× PBS (Mediatech, Inc. Manassas, VA) as the primary antibody. The PVDF membrane was air-dried overnight and analyzed by the AlphaImager™ System 3400 (Alpha Innotech Corporation, San Leandro, CA) to determine band density. The Integrated Density Value (IDV) of the 38-kD band (concentration = 0.27 μg/3 μL) in the SeeBlue Plus2 Standard was used to estimate protein concentration.

3. Results

3.1. Structure and expression of truncated C. antarctica lipase B (CALB)

A robotic workcell (Hughes et al., 2005, 2006, 2007) was programmed to perform a series of automated PCR cycling and PCR amplicon purification and isolation steps (Fig. 1) that produced a gene open reading frame encoding a synthetic truncated CALB by PCR amplification of a portion of the sequence of a synthetic gene ORF encoding a full-length CALB generated previously by a stepwise PCR assembly strategy (Hughes et al., 2011). The amplified sequence included nucleotides 178–1026 (numbering starts at beginning of prepro sequence) in the GenBank sequence (accession number Z30645) of the gene for *C. antarctica* lipase B, except that the full-length synthetic gene ORF that was used as the PCR template had mutations at position 245 (C to T) and position 986 (T to A) (Fig. 2A). The mutation at nucleotide 245 produced a new in-frame start codon. Nucleotides 178–180 code for amino acid 35 of the mature protein (not including the prepro sequence in the numbering). Following nucleotide 1026, the ORF sequence for truncated CALB also contained the nucleotides coding for *att*B, six histidine residues, and a stop codon. The nucleotides coding for *att*B are underscored with a dotted line in Fig. 2A.

The ORF for truncated CALB contained six in-frame ATG start codons, one created in front of nucleotide 178 and the five that were present in the ORF for synthetic full-length CALB that was used as the PCR template. The five ATG codons from the full-length ORF occur at nucleotides 244–246, 289–291, 322–324, 460–462, and 967–969. The ATG codon at nucleotides 244–246 was created by the C to T mutation at position 245 that resulted during the assembly of the full-length ORF. Use of all six start codons would express six synthetic truncated CALB proteins containing amino acids 35–317, 57–317, 72–317, 83–317, 129–317, and 298–317, respectively, of the mature CALB enzyme (with T53M and F304Y). In addition to the amino acid residues from the CALB sequence, all the proteins contained 29 residues of the *att*B sequence and 6 histidine residues at the C-terminal (Fig. 2B).

Five of these proteins were detected by Western blot analysis of the pellets from the galactose culture (Fig. 3; righthand gel) at 33.8 kD (total 319 amino acids including 1 artificial Met and 283 CALB residues), 31.4 kD (296 total; 261 CALB), 29.7 kD (281 total; 246 CALB), 28.4 kD (270 total; 235 CALB), and 23.5 kD (224 total; 189 CALB). The shortest protein (55 total; 20 CALB) was not seen on the Western blot gel. The five truncated CALB proteins detected on the Western blot all contained the lid region (amino acids 135–155). Four (33.8 kD, 31.4 kD, 29.7 kD, and 28.4 kD) of these five CALB proteins contained the catalytic triad consisting of Ser105, His224, and Asp187 (Fig. 2B). Only the 33.8-kD protein contained the oxyanion hole (Gly39 and Thr40) and the complete alcohol-binding pocket defined by Thr42, Ser47, and Trp104. On the Western blot of the supernatant from the galactose culture only the 33.8-kD variant was detected.

Almost no expression of CALB proteins was detected by Western blot analysis using the cultures grown on glucose (Fig. 3; lefthand gel). A trace amount of the 28.4-kD CALB protein was

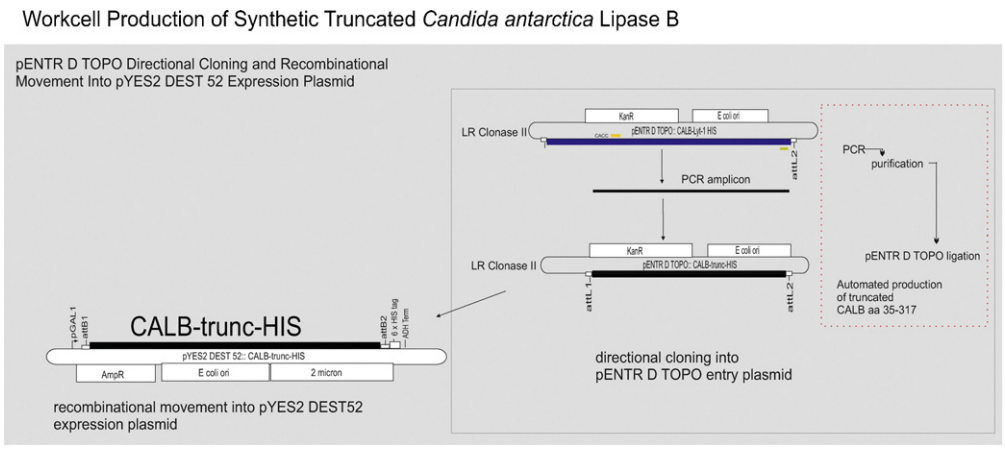

Fig. 1. A robotic workcell was programmed to perform the PCR amplification and amplicon purification and isolation steps depicted in the figure that produced a truncated synthetic version of *Candida antarctica* lipase B (CALB). The template was the full-length synthetic CALB-Lyt-1-HIS nucleotide sequence assembled previously (Hughes et al., 2011). The primers were selected to amplify a truncated CALB open reading frame that expressed amino acids 35–317 of the mature CALB enzyme and to add the CACC Gateway sequence and an ATG start codon with the forward primer. The purified amplicon was directionally cloned into pENTR D TOPO and recombinationally moved into pYES2 DEST 52 for expression in yeast.

seen only in the lanes with the pellet samples. When the cultures were switched from glucose to galactose, expression of the truncated CALB variants was driven by activation of the *GAL*1 promoter. The 33.8-kD CALB enzyme was expressed at a much higher level than any of the other four variants, with most of the enzyme localized to the cell pellet (4.85 mg/10 mL measured using the IDV compared to that of SeeBlue Plus2 Standard) and only a small amount present in the supernatant (1.5 mg/10 mL).

3.2. Fatty acid ethyl ester production from corn and soybean oils using resin-bound truncated CALB

The results for fatty acid ethyl ester production from corn oil and soybean oil (15:1 (v/v) ratio of ethanol to oil) using resin-bound truncated CALB from galactose rich medium (YPG) are presented in Table 1. A commercially available lipase resin (Novozym 435) was used for comparison. Although the specification for Novozym 435 resin gives the activity of the enzyme, in the present work all

A. **Truncated *Candida antarctica* Lipase B Open Reading Frame with Catalytic Triad Amino Acid Positions Ser105 Asp187 His224**

```
                                                                  cacc atg*ctt
ctc gtc ccc gga acc ggc acc aca ggt cca cag tcg ttc gac tcg aac tgg atc ccc ctc
tca aTg cag ttg ggt tac aca ccc tgc tgg atc tca ccc ccg ccg ttc atg ctc aac gac
acc cag gtc aac acg gag tac atg gtc aac gcc atc acc gcg ctc tac gct ggt tcg ggc
aac aac aag ctt ccc gtg ctt acc tgg tcc cag ggt ggt ctg gtt gca cag tgg ggt ctg
acc ttc ttc ccc agt atc agg tcc aag gtc gat cga ctt atg gcc ttt gcg ccc gac tac
aag ggc acc gtc ctc gcc ggc cct ctc gat gca ctc gcg gtt agt gca ccc tcc gta tgg
cag caa acc acc ggt tcg gca ctc acc acc gca ctc cga aac gca ggt ggt ctg acc cag
atc gtg ccc acc acc aac ctc tac tcg gcg acc gac gag atc gtt cag cct cag gtg tcc
aac tcg cca ctc gac tca tcc tac ctc ttc aac gga aag aac gtc cag gca cag gcc gtg
tgt ggg ccg ctg ttc gtc atc gac cat gca ggc tcg ctc acc tcg cag ttc tcc tac gtc
gtc ggt cga tcc gcc ctg cgc tcc acc acg ggc cag gct cgt agt gca gac tat ggc att
acg gac tgc aac cct ctt ccc gcc aat gat ctg act ccc gag caa aag gtc gcc gcg gct
gcg ctc ctg gcg ccg gca gct gca gcc atc gtg gcg ggt cca aag cag aac tgc gag ccc
gac ctc atg ccc tac gcc cgc ccc tAt gca gta ggc aaa agg acc tgc tcc ggc atc gtc
acc ccc ttg tac aaa gtg gtt cga tct aga ggg ccc ttc gaa ggt aag cct atc cct aac
cct ctc ctc ggt ctc gat tct acg cgt acc ggt cat cat cac cat cac cat taa
```

B. **All Possible Met Residues from Six In-Frame ATG Start Codons in CALB-trunc-6xHis**

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                Met*L L V P G T G T T G P Q S F D S N W I P L S Met Q L G Y T P C W I S P P P F Met L N
D T Q V N T E Y Met V N A I T A L Y A G S G N N K L P V L T W S Q G G L V A Q W G L T F F P S I R S K
V D R L Met A F A P D Y K G T V L A G P L D A L A V S A P S V W Q Q T T G S A L T T A L R N A G G L T
Q I V P T T N L Y S A T D E I V Q P Q V S N S P L D S S Y L F N G K N V Q A Q A V C G P L F V I D H A
G S L T S Q F S Y V V G R S A L R S T T G Q A R S A D Y G I T D C N P L P A N D L T P E Q K V A A A A
L L A P A A A A I V A G P K Q N C E P D L Met P Y A R P Y A V G K R T C S G I V T P L Y K V V R S R G
P F E G K P I P N P L L G L D S T R T G H H H H H H STOP
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Fig. 2. (A) The nucleotide sequence of the gene open reading frame (ORF) for the truncated synthetic CALB enzyme contained nucleotides 178–1026 of the GenBank sequence for this lipase (accession number Z30645). Nucleotides 178–180 (CTT) following the initial CACC ATG sequence code for amino acid 35 of the mature protein. The sequence included six in-frame ATG start codons (codon indicated 'aTg' is a mutation of nucleotide 245 to T from C in the GenBank sequence) potentially capable of expressing six truncated CALB proteins. The nucleotide sequence encoding the 29-residue *att*B sequence is underscored with a dashed line. (B) The amino acid sequence shows the six methionine residues corresponding to the six in-frame start codons indicated in the matching color matching the color. The positions of the amino acid residues for the catalytic triad are shown in bold type and underscored.

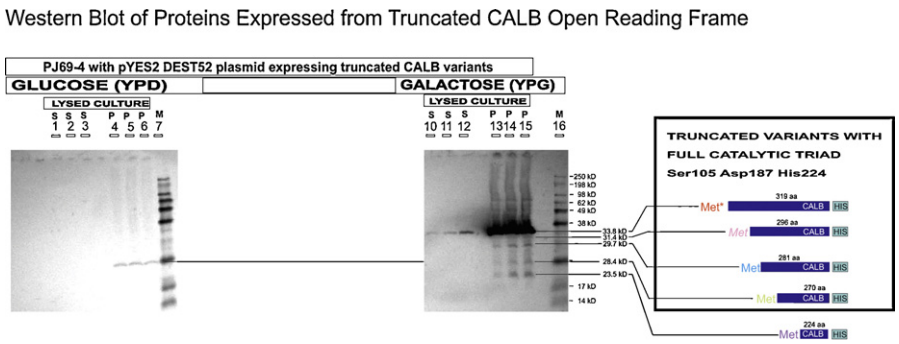

Fig. 3. Five of the 6 truncated CALB proteins that potentially could be expressed in PJ69-4 yeast after culture in galactose medium, using the in-frame start codons in the amplified gene open reading frame (ORF), were detected in galactose culture by Western blot analysis (righthand gel) at 33.8 kD (total length 319 amino acids = 1 artificial Met + 283 CALB + 29 *att*B + 6xHis residues), 31.4 kD (296 total; 261 CALB), 29.7 kD (281 total; 246 CALB), 28.4 kD (270 total; 235 CALB), and 23.5 kD (224 total; 189 CALB). The 33.8-kD recombinant protein contains amino acids 35–317 of the mature CALB enzyme. All of these CALB proteins except the 23.5-kD protein contain the complete catalytic triad consisting of Ser105, His224, and Asp187. Substantially more of the 33.8-kD protein is expressed than of the other 4 variants. Experimental conditions: 4–20% gradient Tris–tricine SDS-PAGE run at 110 V for 120 min; transferred 10 h 160 mA. M = SeeBlue® Plus2 Pre-stained Standard. S = supernatant; P = pellet.

comparisons were made based on concentrations in mg/10 mL of the enzymes determined from densitometry readings of the gels. If all of the protein were not active, the activity per milligram of protein as calculated in this work would appear lower than would be the case if all protein were contributing to activity. The yield of C16 and C18 ethyl esters from supernatant per mg of CALB enzyme ranged from 3.4- to 4.5-fold greater than the yield per mg of enzyme from the corresponding pelleted lysed cells with either corn oil or soybean oil. The results for total yield (supernatant plus pellet) of C16 plus C18 ethyl esters are shown in Fig. 4. The total yield of ethyl esters from corn oil was 36% higher for the truncated CALB enzyme than for the commercially available lipase (951 μg ethyl esters/mg truncated CALB versus 701 μg ethyl esters/mg commercial lipase: $[(951 - 701)/701] \times 100 = 36\%$). With soybean oil, the truncated CALB enzyme produced 50% more C16 plus C18 ethyl esters than the commercially available lipase (255 μg ethyl esters/mg truncated CALB versus 170 μg ethyl esters/mg commercial lipase). Both the truncated CALB (950 total from corn oil/254 total from soybean oil) and the commercial lipase (701 total from corn oil/170 total from soybean oil) gave a 4-fold greater yield of ethyl esters from corn oil than soybean oil.

3.3. Fatty acid ethyl ester production from ethanol and corn oil using resin-bound truncated CALB in conjunction with an ionic catalytic resin

When an ionic catalytic resin step was added before the CALB resin step, production of ethyl esters from corn oil was 53% greater than with CALB resin followed by ionic resin (Fig. 5) when enzyme was obtained from cultures in rich medium, 706 versus 462 μg ethyl esters/mg truncated CALB, respectively. Production of ethyl esters was similar regardless of the order of the resin steps using enzyme from cultures in selective medium. Compared to commercial lipase resin, the truncated CALB resin preceded by ionic resin produced 29% more ethyl ester/mg enzyme (data not shown).

3.4. Fatty acid ethyl ester production catalyzed by resin-bound truncated CALB using various ratios of ethanol to corn oil

Fatty acid ethyl ester production catalyzed by resin-bound truncated CALB using various ratios of ethanol to corn oil is shown in Fig. 6. Production of C16 and C18 fatty acid ethyl esters using a ratio of 5:1 (v/v) for ethanol to corn oil was 3.7-fold higher (2875 versus 774 μg ethyl esters/mg truncated CALB) than a ratio of 15:1 (v/v) and 8.4-fold higher (2875 versus 343 μg ethyl esters/mg truncated CALB) than a ratio of 30:1 (v/v) when the CALB resin was preceded by the ionic catalytic resin. The preference for the 5:1 ratio was similar for the reverse resin order.

3.5. Fatty acid alkyl ester production from corn oil using resin-bound truncated CALB with ethanol or n-butanol

Fatty acid alkyl ester production using resin-bound truncated CALB with ethanol or n-butanol and corn oil (5:1 (v/v) ratio of alcohol to corn oil) is presented in Table 2. Production of C18 plus C16 butyl esters was 56% higher than production of C16 plus C18

Table 1
Fatty acid ethyl ester production per mg enzyme from corn oil versus soybean oil using resin-bound truncated *Candida antarctica* lipase B was measured by gas chromatography. A commercially available lipase resin (Novozym 435) was used for comparison. The yield of C16 and C18 ethyl esters from supernatant per mg of CALB was 3.4- to 4.5-fold greater than the yield per mg of enzyme from the lysed pellet with either corn oil or soybean oil. Gas chromatography (GC) area is the mean of three replicates. A 1 μg ethyl ester (EE)/1 μL standard gave a GC area of 2,213,000.

Sample	GC average area	Standard deviation ($n = 3$)	Sample μg EE in 1 μL	mg Truncated CALB in 10 mL	μg EE/mg enzyme
Truncated CALB pellet SBO C16	13,149.78	5583.86	0.00594	4.85	12.25
Truncated CALB pellet SBO C18	47,891.33	26,258.05	0.02164	4.85	44.62
Truncated CALB supernatant SBO C16	14,526.12	6275.03	0.00656	1.5	43.76
Truncated CALB supernatant SBO C18	51,145.27	25,999.51	0.02311	1.5	154.08
Truncated CALB pellet CO C16	55,262.67	6435.3	0.02497	4.85	51.49
Truncated CALB pellet CO C18	155,098.16	28,527.32	0.07009	4.85	144.51
Truncated CALB supernatant CO C16	75,874.5	29,315.02	0.03429	1.5	228.57
Truncated CALB supernatant CO C18	174,581.24	85,062.33	0.07889	1.5	525.93
Novozyme 435 SBO C16	18,283.8	5427.27	0.00826	2.5	33.05
Novozyme 435 SBO C18	75,908.53	17,965.3	0.0343	2.5	137.2
Novozyme 435 CO C16	113,140.87	60,200.18	0.05113	2.5	204.5
Novozyme 435 CO C18	274,898.17	143,200.03	0.12422	2.5	496.88

Table 2
Fatty acid alkyl ester production from corn oil using resin-bound truncated *Candida antarctica* lipase B with absolute ethanol or n-butanol was determined using a 5:1 (v/v) ratio of alcohol to oil. Production of butyl esters was 56% higher than production of ethyl esters. Gas chromatography areas are the mean of three replicates.

Sample	GC average area	Standard deviation ($n=3$)	μg Ester in 1 μL	mg Truncated CALB in 10 mL	μg Ester/mg truncated CALB
C16 ethyl esters from CO and ethanol	228,121.50	93,606.09	0.1031	3.23	319.14
C18 ethyl esters from CO and ethanol	410,750.00	3288.05	0.1856	3.23	574.64
C16 butyl esters from CO and ethanol	254,097.50	2617.00	0.1148	3.23	355.48
C18 butyl esters from CO and ethanol	742,158.50	17,029.25	0.3354	3.23	1038.28

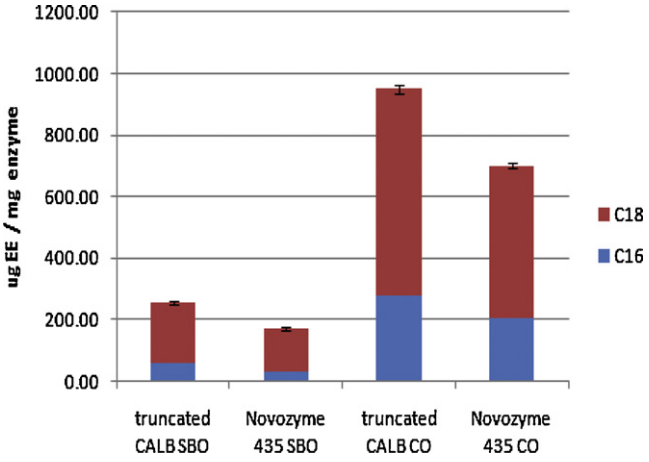

Fig. 4. The total fatty acid ethyl ester (EE) production (C16 plus C18) from corn oil (CO) versus soybean oil (SBO) using resin-bound truncated CALB was compared to that using a commercial lipase. C16 production is shown in blue; C18 in red. Production of ethyl esters from corn oil was 4-fold greater than from soybean oil using truncated synthetic CALB. In comparison to a commercially available resin-bound lipase, ethyl ester production from soybean oil was 50% higher and from corn oil was 36% higher using truncated CALB resin. Error bars are given at the top of each bar in the graph ($n=3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

ethyl esters with corn oil (1394 μg butyl esters versus 894 μg ethyl esters/mg truncated CALB). However, the truncated CALB enzyme appeared to yield approximately the same amount of C16 esters with either alcohol.

4. Discussion

A gene open reading frame for lipase B from *C. antarctica* containing nucleotides 178–1026 of the GenBank sequence for this lipase (accession number Z30645) was generated via PCR from a full-length synthetic CALB gene assembled previously that had mutations at positions 245 (C to T) and 986 (T to A) (Hughes et al., 2011). The objective was to determine the effect on the activity and expression of the enzyme if enzyme length is minimized by deleting the sequence not involved in the active site or the stereospecificity pocket. The amplified CALB ORF contained six different in-frame ATG start codons, the first artificially introduced and the second a mutation, capable of expressing six synthetic truncated CALB proteins containing amino acids 35–317, 57–317, 72–317, 83–317, 129–317, and 298–317, respectively, of the mature CALB enzyme (with T53M and F304Y). Prior to expression of the truncated CALB in galactose medium, the yeast engineered with the pYES2 DEST 52 plasmid was used for fermentation on glucose substrate in a Fernbach flask. The ethanol yield was more than 9 g/L (data not shown) similar to the yield for PJ69-4 yeast engineered with the plasmid containing the full-length CALB (Hughes et al., 2011). Thus, ethanol production was not affected adversely by the presence of the plasmid.

The pYES2 DEST 52 yeast transformants used as the expression system in this work would not be the system used in large-scale processing for production of lipases. Use of the pYES2 DEST 52 yeast was the most rapid way to demonstrate that an active synthetic CALB enzyme could be expressed from the truncated gene. Much more extensive work is needed to develop a stable strain for industrial use. Currently various strategies are being investigated to obtain a stable industrial *S. cerevisiae* strain for expression of the synthetic CALB gene. These include genetic factors, such as plasmid makeup, and environmental factors, such as bioreactor operation modes, that can be designed to overcome instability of the expression system (Zhang et al., 1996).

Western blot analysis detected five of the six different truncated CALB proteins expressed in galactose medium, suggesting multiple

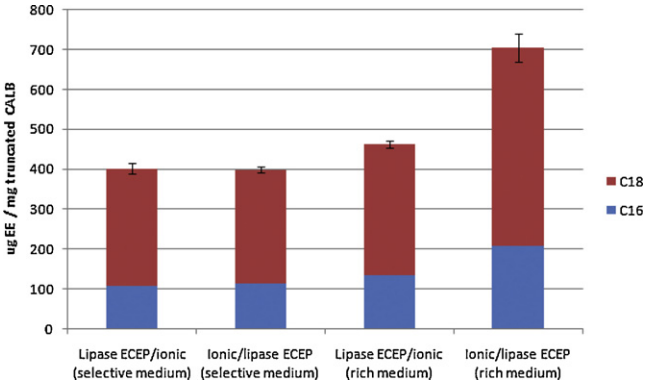

Fig. 5. Addition of an ionic catalytic resin step prior to the truncated CALB resin, increased yields of C16 (blue) plus C18 (red) fatty acid ethyl esters from corn oil by 53% compared to CALB resin followed by ionic resin. Error bars are given at the top of each bar in the graph ($n=3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

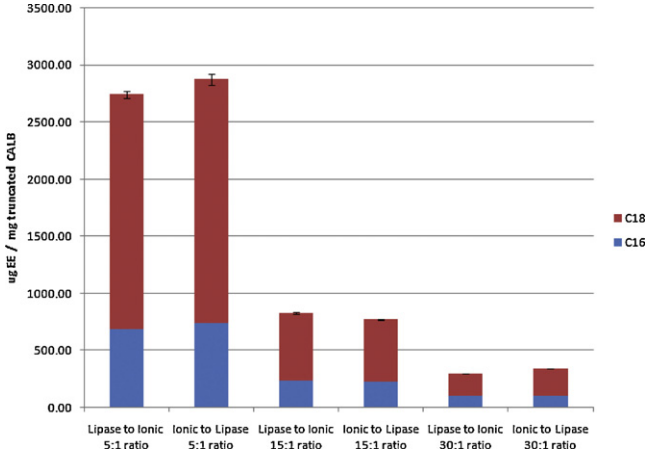

Fig. 6. Fatty acid ethyl ester production catalyzed by resin-bound truncated CALB was determined using various ratios of ethanol to corn oil. Production of C16 (blue) and C18 (red) fatty acid ethyl esters using a ratio of 5:1 (v/v) for ethanol to corn oil produced 3.7-fold and 8.4-fold greater yields than ratios of 15:1 and 30:1, respectively. Error bars are given at the top of each bar in the graph ($n=3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

translation starts from the six in-frame ATG codons. Four of these five truncated CALB proteins contained the complete catalytic triad consisting of Ser105, His224, and Asp187. Only the longest protein (33.8 kD) contained the oxyanion hole (Gly39 and Thr40) and the complete alcohol-binding pocket (Thr42, Ser47, and Trp104). The longest open reading frame, which corresponds to amino acids 35–317 of the mature enzyme (with mutations T53M and F304Y), appeared to be expressed in the greatest amount. It appears that elimination of the nucleotides coding for the amino acid residues prior to residue 35 in the sequence of the native enzyme and the introduction of an artificial ATG start codon contributes to high-level expression of the gene ORF for this truncated CALB variant. It might also be a function of the translation machinery in yeast. It has been shown that the yeast translation apparatus favors 5′ end-mediated translation over internal initiation (Thompson et al., 2001). The most likely reason for this is the synergy by which the 5′ terminal cap structure and the 3′ terminal polyadenosine sequences direct the binding of ribosomal subunits to the 5′ end of the mRNA (Preiss and Hentze, 1998). Thus, the translation machinery in *S. cerevisiae* does not seem to perform internal initiation readily in cells grown under normal laboratory conditions (Thompson et al., 2001). However, it is not clear what is driving the small amount of expression of the 28.4-kD protein in the glucose culture. Neither the pellet nor the supernatant from the glucose culture was assayed for ester production.

The truncated lipase enzymes immobilized on Sepabeads® EC-EP resin were active in catalyzing the production of fatty acid ethyl and butyl esters from corn oil and soybean oil suggesting that the removal of the initial 34 amino acids of the CALB sequence did not affect the catalytic activity adversely. Because the C16 and C18 fatty acid chains constitute essentially 100% of the composition by weight of both corn oil (99.8 wt%) and soybean oil (100 wt%) (Ma and Hanna, 1999), they were used for determining reaction progress. The ratio of C18 to C16 esters is a function of the substrate oil. As expected, the amount of C18 ethyl esters produced by the truncated CALB was greater than that of C16 esters, but the ratio (2.3–3.8 to 1) was less than the ratio in the substrate oils (7.5 to 1). This may be indicative of the preference that the truncated CALB has for the smaller substrate when using ethanol as the alcohol (Kato et al., 2007). The yield of C16 plus C18 ethyl esters from the supernatant per mg of enzyme was 3.4- to 4.5-fold greater than the yield from the pelleted lysed cells with either corn oil or soybean oil. This suggests that although levels of enzyme were very low in the supernatant, the enzyme was more active and thus the yields of esters per mg of enzyme were higher, possibly because fewer inhibiting materials, which would decrease enzyme coupling to the resin, were present in the supernatant than in the pellet or because contact between enzyme and substrate was enhanced in the more dilute supernatant solution where presumably the substrate is more accessible to the enzyme.

With corn oil, the total yield (pellet plus supernatant) of C16 plus C18 ethyl esters was 36% higher for the resin-bound truncated CALB enzyme compared to the commercially available lipase resin. With soybean oil, the truncated CALB enzyme produced 50% more total ethyl esters than the commercially available lipase resin. Both the truncated CALB and commercial enzymes gave a 4-fold greater yield of ethyl esters from corn oil than soybean oil possibly due to the purity of soybean oil, which has been shown to affect biodiesel production catalyzed by *C. antarctica* lipase (Watanabe et al., 2002). In comparison to the full-length enzyme, the total amount of truncated enzyme expressed was approximately 60-fold greater than the amount of full-length CALB expressed under the same conditions (Hughes et al., 2011). When used to catalyze the production of ethyl esters from soybean oil, the yield of ethyl esters per mg of enzyme was 6-fold greater for the truncated enzyme than for the full-length enzyme under the same conditions (Hughes et al., 2011).

When the transesterification process was run sequentially with an ionic catalytic resin followed by the lipase resin, truncated CALB produced 53% more ethyl esters from corn oil than when the lipase resin was followed by the ionic resin for enzyme from rich medium. The ionic resin may function to remove inhibiting material present in the corn oil. Enzyme from selective medium did not show as much improvement in yield. It is possible the rich medium allowed for increased nonspecific growth of cells without lipase expression. Rich medium more closely resembles the medium used in industrial biofuel production.

Fatty acid ethyl ester production catalyzed by resin-bound truncated CALB was determined using various ratios (v/v) of ethanol to corn oil. Production of C16 and C18 fatty acid ethyl esters using a ratio of 5:1 for ethanol to corn oil was 3.7-fold higher than a ratio of 15:1 and more than 8.4-fold higher than a ratio of 30:1 ethanol to corn oil. This indicates that even at the lowest ratio, alcohol availability is not limiting the reaction progress. Other researchers have determined that high concentrations of alcohol can adversely affect yield (Ranganathan et al., 2008). For example, *C. antarctica* lipase was shown to be irreversibly inactivated by contact with methanol (Shimada et al., 2002). Using the 5:1 ratio of alcohol to corn oil, differences in production activity of resin-bound truncated CALB in ethanol and butanol were studied. Butyl ester production was 56% higher than ethyl ester production. The ratio of C18 to C16 fatty acid esters produced was higher for butanol than for ethanol, 2.9 versus 1.8, respectively, suggesting that the enzyme preference for the C16 substrate is less when using butanol as the alcohol. The optimum ratio for alcohol to corn oil appears to be the one with the lowest amount of alcohol present.

Current efforts are directed toward solubilization of the truncated CALB enzyme and purification using immobilized metal affinity chromatography. Kinetic parameters, substrate preference, and optimal reaction conditions will be determined for the electrophoretically pure truncated CALB enzyme.

Although enzyme catalysts have many advantages over chemical catalysts in the production of biodiesel, a number of issues still remain to be addressed when using lipases as industrial biocatalysts, including high cost, loss of activity because of adsorption of formed glycerol, and reusability of the catalyst. Extensive research work will be required to demonstrate that the truncated CALB enzyme can be inexpensively produced, is active and stable under industrial conditions, and can be reused in consecutive reactions.

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