Enhancing the Methanol Tolerance of *Candida antarctica* Lipase B by Saturation Mutagenesis for Biodiesel Preparation

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Abstract

Methanol tolerance of a lipase is one of the important factors affecting its esterification ability in biodiesel preparation. By *B* factor indicated prediction of *Candida antarctica* lipase B (CalB) surface amino acids, 8 sites (Val¹³⁹, Ala¹⁴⁶, Leu¹⁴⁷, Pro²¹⁸, Val²⁸⁶, Ala²⁸⁷, Val³⁰⁶, and Gly³⁰⁷) with high *B* value indicating more flexibility were chosen to perform saturation mutagenesis. High-methanol-tolerant variants, CalB-P218W and -V306N, created larger haloes on emulsified tributyrin solid plate including 15% (v/v) methanol and showed 19% and 31% higher activity over CalB-WT (wild type), respectively. By modeling, a newly formed hydrogen bond in CalB-V306N and hydrophobic force in CalB-P218W contributing more stability in protein may have resulted in increased methanol tolerance. CalB-P218W and -V306N transesterified the soybean oil into biodiesel at 30 °C by 85% and 89% yield, respectively, over 82% by CalB-WT for 24 h reactions. These results may provide a basis for molecular engineering of CalB and expand its applications in fuel industries. The as-developed semi-rational method could be utilized to enhance the stabilities of many other industrial enzymes.

Introduction

Biodiesel (fatty acid methyl esters, FAMES) produced through transesterification of fats, vegetable or microalgae oils has received increasing interest in the last two decades as a renewable, biodegradable, and non-toxic fuel [1]. Compared with harsh acid or alkali conversion at high temperature, enzymatic transesterification has emerged as a more eco-friendly approach for biodiesel production with high outcomes [2].

Candida antarctica lipase B (CalB) is one of the most researched lipases for enzyme-catalyzed biodiesel preparation [3]. Mature CalB is composed of 317 amino acid residues, which constitute an α/β -hydrolase fold consisting of 10 α -helices and 9 β -strands in moisture surrounding with high stability [4]. A universal lipase always shows interfacial activation due to the existence of a lid, either a loop or a helix, shielding the active site (Ser-His-Asp catalytic triad, Ser¹⁰⁵-His²²⁴-Asp¹⁸⁷ in CalB) from the solvent and thus regulating the substrate accessibility to the catalytic cavity [5]. CalB has never shown any significant interfacial activation due to the absence of a traditional lid, but its α -helix 5 (works like a lipase lid) and α -helix 10 are shown at atomic resolution to be responsible for active site closing [4]. The reaction mixture for transesterification is hydrophobic and has a high concentration of methanol or ethanol to get a high rate of conversion of oil to FAMES. In such an environment, the biocatalyst's structure tends to denature [6]. For this reason, searching for high methanol tolerant lipases from the natural world [7, 8], or improving the existing lower-methanol-tolerant lipases into hyper-ones has become meaningful to achieve a competitive biodiesel and cost-effective production technology [9].

There are a few works conducted on improving lipase methanol stability through protein engineering. Directed evolution method using error-prone polymerase chain reaction (PCR) was performed to enhance methanol tolerance property of *Proteus vulgaris* lipase for biodiesel synthesis in harsh conditions [10]. By directed evolution using iterative saturation mutagenesis (ISM), methanol tolerance of *Thermomyces*

lanuginosus lipase has been enhanced for efficient biodiesel production from waste grease [11]. Saturation mutagenesis is a semi-rational approach to change the enzyme characteristics, such as activity [12], stability [13], specificity [14], and enantioconvergence [15].

As an important industrial enzyme, CalB was engineered with a few outstanding enzymatic properties [16]. CalB was divided into two regions, substrate-binding region and solvent-affecting region, and its variant (V139E, A92E) was yielded by integrating the activity and stability by multiple-site mutagenesis [17]. Following a structure-based engineering strategy, CalB active site was redesigned and constructed by saturation mutagenesis to improve the catalytic efficiency of poly(ϵ -caprolactone) synthesis and size of polymer products [18]. To date, different 3-D structures of CalB in different conformations [4] and at different resolutions (0.91 Å PDB code 5A71 [4], 1.5 Å PDB code 4K6G [19], 2.0 Å PDB code 4ZV7 [20], 2.5 Å PDB code 1TCC [21]) have been resolved. These should provide a unique opportunity to improve its capacity by in silico design. In this work, the methanol tolerance of CalB was enhanced by using a semi-rational approach of *B*-factor indicated prediction, saturation mutagenesis, and screening on tributyrin solid plate containing methanol. Application of high-methanol-tolerant variants of CalB for biodiesel preparation and molecular basis for methanol tolerance rising were also investigated.

Materials And Methods

Strains, Culture Media and Chemicals

The recombinant vector, pET28a-*CalB*, containing *CalB* gene (GenBank accession no. Z30645) was constructed and preserved in Jiangsu Provincial Engineering Laboratory for Biomass Conversion and Process Integration, Huaiyin Institute of Technology, China. The vector pET28a (Novagen, Madison, WI, USA) was used for expressions of gene *CalB* and its mutants in *Escherichia. coli* BL21(DE3), which was cultured in Luria-Bertani (LB) medium [22]. The Pfu DNA Polymerase and T4 DNA ligase were purchased from Sangon Biotech (Shanghai, China). All other chemicals of high grade were obtained from commercial sources.

Design of Saturation Mutagenesis

The crystal structure of lipase B from *Candida antarctica* (PDB code 5A71, chain B) was used as the original CalB 3-D structure [4], and visualized using the PyMOL software (<http://pymol.org>). The *B* factor values, namely atomic displacement parameters, of amino acids were calculated using the *B*-FITTER program [23].

Saturation Mutagenesis

By *B* factor indicated prediction of CalB surface amino acids, 8 sites (Val¹³⁹, Ala¹⁴⁶, Leu¹⁴⁷, Pro²¹⁸, Val²⁸⁶, Ala²⁸⁷, Val³⁰⁶, and Gly³⁰⁷) with high *B* value indicating being more flexible were chosen to perform saturation mutagenesis. Using pET28a-*CalB* as a template, full-length expression plasmid with a mutant *CalB* gene was amplified by long-distance inverse PCR (Fig. 1), in which a primer pair introduced an

amino acid changing to Val¹³⁹/Ala¹⁴⁶/Leu¹⁴⁷/Pro²¹⁸/Val²⁸⁶/Ala²⁸⁷/Val³⁰⁶/Gly³⁰⁷. The primer pairs, CalB-M1F and CalB-M1R, through to, CalB-M8F and CalB-M8R, were utilized for Val¹³⁹, Ala¹⁴⁶, Leu¹⁴⁷, Pro²¹⁸, Val²⁸⁶, Ala²⁸⁷, Val³⁰⁶, and Gly³⁰⁷ saturation mutagenesis, respectively. The primer pairs are shown in Table 1. Conditions for PCR amplification of *CalB* mutants were as follows: denaturation for 10 min at 94 °C; 30 cycles for 30 s at 94 °C, for 30 s at 58 °C, and for 6 min at 72 °C; and a final elongation for 10 min at 72 °C.

Table 1 Primers for PCR amplification of mutant *CalB* genes.

Primer name	Primer sequence (5'→3') ^a	Size (bp)
CalB-M1F	CTCGCCGGCCCTCTCGATG	19
CalB-M1R	NNNGGTGCCCTTGTAGTCG	19
CalB-M2F	CTCGCGGTTAGTGCACCC	18
CalB-M2R	NNNATCGAGAGGGCCGGCG	19
CalB-M3F	GCGGTTAGTGCACCCTCC	18
CalB-M3R	NNNTGCATCGAGAGGGCC	18
CalB-M4F	CTGTTCGTCATCGACCATG	19
CalB-M4R	NNNCCCACACACGGCCTG	18
CalB-M5F	GCGGGTCCAAAGCAGAAC	18
CalB-M5R	NNNGATGGCTGCAGCTGC	18
CalB-M6F	GGTCCAAAGCAGAACTGC	18
CalB-M6R	NNNCACGATGGCTGCAGC	18
CalB-M7F	GGCAAAAGGACCTGCTCC	18
CalB-M7R	NNNTGCAAAGGGGCGGGC	18
CalB-M8F	AAAAGGACCTGCTCCGGC	18
CalB-M8R	NNNTACTGCAAAGGGGCG	18

^a The saturation mutagenesis codons are boxed.

Screening of Variants

The full length PCR amplification DNA fragments with two blunt ends fused automatically (cyclized) from the fore-end to the tail-end by catalysing T4 DNA ligase (Fig. 1) and therefore transformed into *E. coli* BL21(DE3). The transformants growing on LB solid plate were picked into liquid LB, cultivated, and induced by isopropyl-β-D-thiogalactopyranoside (IPTG) to produce recombinant CalB-WT (wild type) or

CalB variant. The crude lipases were obtained by ultrasonic treatment of the cultivated cells following centrifugation. The crude CalB mutants were screened preliminarily on a tributyrin (Sangon, Shanghai, China) -methanol solid plate for acquiring high-methanol-tolerant variants. The screening plate was prepared according to the method reported previously with slight modifications as follows: 3.75 g agar, 7.5 mL tributyrin, 19.0 mL 3% (w/v) polyvinyl alcohol, and 221 mL 50 mM KH_2PO_4 - Na_2HPO_4 buffer (pH 7.0) were added into a 500-mL flask and blended under microwave heating for homogeneity [24]. The mixture was cooled to $\sim 50^\circ\text{C}$, then added 15% (v/v) methanol, immediately poured into $\phi 12$ -cm plates and cooled down at room temperature. The $\phi 0.6$ -cm filter paper rounds were put upon the tributyrin-methanol solid plate, and each 5.0 μL CalB sample was added on each filter paper round, followed by incubating at 35°C for 24 h. The heat inactivated CalB sample was used as the control. The methanol tolerance changing of the CalB variant was estimated by the difference value of the halo diameter (clear zone encircled the filter paper round) minus CalB-WT. There is a linear relationship between the logarithm of enzymatic activity and the diameter of halo [25].

Purification of CalB-WT and CalB Variants

After inducing with 0.25 mM IPTG at 25°C for 8 h, the transformant *E. coli* BL21(DE3) cells were harvested and suspended in the same volume of 50 mM KH_2PO_4 - Na_2HPO_4 buffer (pH 7.0) and followed by ultrasonic processing. The resulting supernatant was concentrated by ultrafiltration and purified using a Ni^{2+} -chelating agent (Novagen, Madison, WI, USA) [26]. Imidazole at low concentration (20 mM in this research) was used in the binding and wash buffer to minimize the binding of unwanted host cell proteins. Imidazole at optimized high concentration (100 mM in this research) was applied to elute the enzyme (target CalB protein). The purified CalB was concentrated in an aqueous solution and quantified with a BCA protein assay kit (Real-Times, Beijing, China).

Partial Enzymatic Characteristics Analysis

The activities of high-methanol-tolerant CalB variants were quantified according to the method described previously with slight modifications [27]. Briefly, 1 vol of tributyrin and 7 vol of 1.0% (w/v) polyvinyl alcohol in 50 mM KH_2PO_4 - Na_2HPO_4 buffer (pH 6.5) were blended (10,000 rpm) till homogeneity was achieved. Then, 9 mL of the emulsified tributyrin and 1 mL of the suitably diluted enzyme were mixed, incubated at 30°C for 10 min, and terminated by adding 15 mL of ethanol. The heat inactivated CalB instead of the active one was used as the control. The amount of free butyric acid released from tributyrin was measured by titration using 50 mM NaOH. One unit (U) of lipase activity corresponds to the amount of enzyme able to release 1 mmol/min of fatty acid under the standard assay conditions (at pH 6.5 and 30°C for 10 min). Corresponding to U/mg, it was defined as the active lipase amount (U) per milligram of purified CalB protein. All data of enzyme activities or relative ones were expressed as the mean $\pm$ standard deviation from three independent experiments or parallel measurements. Statistical comparison was made using Student's *t*-test. The level of statistical significance was defined as $P \leq 0.05$ or $P \leq 0.01$.

Determination of temperature and pH optimum, and stability of CalB was performed according to the method described previously with modifications [27]. The temperature optimum of CalB was determined at a temperature range of 20–50 °C under the standard conditions for lipase activity assay. To measure its temperature stability, CalB aliquots were incubated at different temperatures of 20–50 °C for 1.0 h before evaluating the retained activity of CalB under the standard assay conditions. In this work, the thermostability was defined as the temperature at or below which the residual activity of CalB was over 85% of its initial activity. The pH optimum of CalB was estimated using the emulsified tributyrin prepared with 50 mM KH_2PO_4 - Na_2HPO_4 buffer (pH 5.0–8.0), at the optimum temperature for 10 min. For its pH stability estimating, CalB aliquots were incubated at 30 °C and at different pH values of 50 mM KH_2PO_4 - Na_2HPO_4 buffer (pH 4.5–8.5) for 1.0 h before assessing the retained activity of CalB under the standard assay conditions. The pH range over which the residual activity of CalB was more than 85% of its initial activity was defined as the range of its pH stability.

Methanol Tolerant Assay

Aliquots of purified CalB were diluted in different concentrations of methanol-buffer (50 mM KH_2PO_4 - Na_2HPO_4 , pH 6.5) solution (0–35% of methanol), and incubated at 30 °C for 24 h on a rotary incubator (100 rpm). The residual activity was measured under the standard assay conditions. CalB diluted in 50 mM KH_2PO_4 - Na_2HPO_4 (pH 6.5) without methanol was used as a control.

Biodiesel Preparation

CalB-WT and high-methanol-tolerant variants (CalB-P218W and -V306N) were used for biodiesel preparation. To prepare biodiesel, 20.0 g soybean oil, 3.7 mL methanol (kept 4:1 molar ratio of methanol to oil), 1 mL water (5% of oil weight), and 100 mg CalB were added to a 250-mL stoppered flask. The transesterification reactions were catalyzed by CalB at 30 °C for 24 h on a rotary incubator (200 rpm). Each 100 μL sample was withdrawn from the reaction mixture at specified time points and centrifuged to collect the upper phase of FAMES for quantitative analysis [28].

Biodiesel Analysis by Gas Chromatography

GC-2030 gas chromatograph (Shimadzu Corp., Kyoto, Japan) equipped with a capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm , INNOWAX, Angellym, USA) and a flame ionization detector was used for quantitative analysis of FAMES. Each 1.0 μL sample was injected into the GC using a split mode with a split ratio of 1:30 for analysis. High purity nitrogen was used as carrier gas at a flow rate of 1.0 mL/min. The temperature of the column was raised from 200 to 240 °C at a rate of 4 °C/min, and then kept at 240 °C for 5 min [29]. The temperatures of the injector and detector were set at 220 and 270 °C, respectively [30].

Analysis of Methanol Stable Variants

The crystal structure of lipase B from *Candida antarctica* (PDB code 5A71) was used as the modeling template for 3-D structure predictions of the CalB-P218W and -V306N. The 3-D structures of CalB-P218W and -V306N were homologically modeled using the MODELLER 9.9 program (<http://salilab.org/modeller/>) and visualized by using the PyMOL software. The alignment of 3-D structures was carried out by using the PyMOL software.

Results And Discussion

Saturation Mutagenesis

The amino acid residues of CalB with top-16 high *B* factor values are shown in Table 2. The candidate sites for saturation mutagenesis should have high *B* factor values and be on the molecular surface and not at the strand ends or in the catalytic cavity. Amino acid residues at the strand end usually have high *B* factor values for their easily and freely wiggling though the residue distributions around the end limiting the end's moving [31]. Changing amino acid residues in or adjacent to the catalytic cavity may decrease the enzyme activity [32]. Finally, eight sites (Val¹³⁹, Ala¹⁴⁶, Leu¹⁴⁷, Pro²¹⁸, Val²⁸⁶, Ala²⁸⁷, Val³⁰⁶, and Gly³⁰⁷) with high *B* factor values on CalB molecular surface indicating more flexibility were chosen to perform saturation mutagenesis, as shown in Fig. 2.

Table 2 The amino acid residues of CalB with high *B* factor values.

Amino acid	Site	<i>B</i> factor value	On surface (T/F) ^a	Special reason ^b	Chosen (Y/N) ^c
Pro	317	68.31	T	At strand end	N
Ala	146	66.67	T		Y
Asp	145	65.67	F	Essential for interfacial activation [4]	N
Ile	285	61.67	T	In catalytic cavity	N
Gly	288	61.41	F		N
Ala	287	61.16	T		Y
Gly	307	60.61	T		Y
Thr	316	60.36	T	At strand end	N
Val	139	59.67	T		Y
Pro	218	59.59	T		Y
Val	306	57.64	T		Y
Val	286	54.52	T		Y
Leu	147	52.84	T		Y
Ala	141	52.59	F	In catalytic cavity	N
Leu	144	50.33	F	In catalytic cavity	N
Gly	41	50.22	F		N

^a T, on the surface; F, not on the surface.

^b Special reason for not chosen for saturation mutagenesis.

^c Y, chosen for saturation mutagenesis; N, not chosen.

Screening of High-methanol-tolerant Variants

For screening of target variants after saturation mutagenesis for each position, 94 colonies, in theory, should be picked for 95% coverage [33]. After saturation mutagenesis, mutants on the plate were picked, cultivated, induced by IPTG, treated by ultrasonic, and finally screened by adding crude CalB variants to filter paper rounds on emulsified tributyrin solid plate including 15% (v/v) methanol. Each library has gotten more than 100 variants for screening on an agar plate; most of them showed lacklustre methanol stability performance compare to CalB-WT. Among nearly 900 variants of 8 libraries, 11 variants showed remarkable methanol stabilities and two outstanding variants that finally confirmed CalB-P218W and -

V306N, created larger haloes, and showed 19% and 31% higher activity over CalB-WT, respectively. By one-site saturation mutagenesis at one amino acid site, it yields only one site mutant and usually a limited low level changing of methanol tolerance. It is worthy of integrating different low-level positive mutants at different positions into one CalB molecule to obtain high-methanol-tolerant variants by site-directed mutagenesis or iterative saturation mutagenesis (ISM). As a result of applying ISM, a pronounced increase in thermostability of maize endosperm ADP-glucose pyrophosphorylase has been reported [34].

Partial Enzymatic Properties

The methanol-tolerant variants (CalB-P218W and -V306N) and original lipase (CalB-WT) were purified by Ni^{2+} -chelating affinity chromatography for analyzing their enzymatic properties. The specific activity of purified CalB-P218W and -V306N towards tributyrin under the standard assay conditions were 81.5 ± 2.6 and 86.7 ± 3.2 U/mg, respectively. They were like the specific activity of CalB-WT (82.1 ± 2.3 U/mg) and slightly more than the specific activity of CalB reported previously (~ 30 U/mg) [35].

The temperature optima (100% relative activity) of CalB-P218W and -V306N at pH 6.5 were all 30 °C same as CalB-WT (Fig. 3a). After incubating at different temperatures for 1.0 h, the CalB-WT, -P218W, and -V306N retained over 85% of their original activities at 35 °C or below but declined rapidly over 35 °C (Fig. 3b). The purified CalB-WT, -P218W, and -V306N exhibited higher relative activities over a pH range of 5.5–8.0 (measured at 30 °C). Their pH optima (100% relative activity) were at pH 6.5 (Fig. 4a). Relative activities or relative residual activities of CalB-WT and its variants at the same temperature are not statistically different ($P > 0.05$). Incubated at different pH values (4.5–8.5) for 1.0 h, CalB-WT, -P218W, and -V306N were highly stable (more than 85% of their original activities) over a wide pH range of 5.0–8.0 (Fig. 4b). Relative activities or relative residual activities of CalB-WT and its variants at the same pH are not statistically different ($P > 0.05$), except relative activities at pH 8 are statistically different ($P < 0.01$). CalBs expressed in different host cells, including *Escherichia coli* [36], *Pichia pastoris* [35], and *Aspergillus oryzae* (CalB on the resin of Novozym 435) [37] exhibit similar effects by temperature and pH. As an important biocatalyst for transesterification, CalB could act in mild conditions of almost neutral pH and room temperature that reveals more eco-friendly and cost-saving biodiesel production.

Methanol Tolerance Analysis

With an increase of methanol concentration, the stabilities of CalB-WT, -P218W, and -V306N were decreased (Fig. 5). After 24-h incubation at different methanol concentrations, the CalB-V306N retained over 85% of its original activity in 20% of methanol or below, but declined rapidly over 25% of methanol (Fig. 5). Relative residual activities of CalB-WT and its variants at the same methanol concentration are not statistically different ($P > 0.05$) when methanol concentration is below 5%, and are statistically different ($P < 0.01$) when methanol concentration is between 10%–35%. The CalB-P218W retained over 85% of its original activity in 15% of methanol or below but declined rapidly over 20% of methanol. However, CalB-WT was stable in 10% of methanol or below, quickly declined over 15% of methanol. CalB-

V306N showed more stability than CalB-WT and -P218W in the presence of methanol. For methanol concentrations above 10% (vol/vol), the catalytic activity of CalB in the methanol-toluene mixture was at 30% of the maximum activity [38].

Biodiesel Preparation

By a quantitative analysis of FAMEs collected at specified reaction time points, biodiesel yields increased along with the extension of reaction time (Fig. 6). FAME yields of CalB-WT and -P218 at the same reaction time are not statistically different ($P > 0.05$). FAME yields of CalB-WT and -V306N at the same reaction time are not statistically different ($P > 0.05$) when reaction time is below 4 h and are statistically different ($P < 0.05$) when reaction time is between 18–24 h. CalB-P218W and -V306N transesterified the soybean oil into biodiesel by 85% and 89% yield, respectively, over 82% by CalB-WT for 24 h reactions. Biodiesel preparations by CalBs at different statuses have been reported. CalB in an *Aspergillus oryzae* whole-cell biocatalyst afforded a methyl ester content of more than 90% after 6 h with the addition of 1.5 M equivalents of methanol [39]. Covalently immobilized CalB on carboxylated single-walled carbon nanotubes using 3.7 wt% of the enzyme to sunflower oil converted the oil in 83.4% yield after 4 h at 35 °C [40]. Immobilized CalB (Novozym 435) using 4 wt% of the enzyme to soybean oil and methanol, preincubated in methyl oleate for 0.5 h, could transesterify soybean oil into biodiesel in 97% yield within 3.5 h by a stepwise addition of 0.33 molar equivalent of methanol at 0.25–0.4 h intervals [41]. To avoid the negative effect of a high concentration of methanol on lipase, adding methanol to the reaction mixture at intervals is a useful strategy for biodiesel production [42]. CalB co-immobilized with *Thermomyces lanuginose* lipase (TLL) (CalB : TLL ratio 2.1) converted palm oil to FAMEs with 94% yield within 24 h, in which 0.15% weight of enzyme to oil emerged synergistic effect between CalB and TLL [43]. There is a high expectancy of CalB-P218W and -V306N to convert oil into biodiesel in a high yield by an integrated strategy of immobilizing lipase, pretreating lipase, adding methanol at intervals though the lipases are high-methanol-tolerant, varying molar ratio of methanol to oil, and synergistic effect with other lipases [44].

Molecular Basis for Methanol Tolerance Rising

The open and closed conformations of CalB variants were modeled using chain A (open conformation) and chain B (closed conformation) of CalB-WT crystal structure (PDB code 5A71) as templates, respectively. By 3-D structure alignment, the difference between the open and closed conformations of CalB-WT or modeled CalB variant (CalB-P218W or -V306N) mainly existed in a state of α -helix 5 and 10. Either for open or closed conformations between CalB variant and CalB-WT, the difference mainly existed in the site of the mutant amino acid (P218W or V306N), and its surrounding. In modeled CalB-P218W, the side chain of Trp²¹⁸ oriented to Val¹⁹⁴, leading to drastically shrinking of the shortest distance between site 218 and Val¹⁹⁴ from 7.75 to 3.42 Å, where the final distance was still larger than the maximum one for hydrogen bond formation (Fig. 7a, b). Hydrogen bonds make favorable contribution to protein stability, and their lengths are usually 2.5–3.3 Å [45]. Whereas the indole ring of Trp²¹⁸ brought an enhanced hydrophobic force and may result in a CalB-P218W methanol tolerance increase. In modeled

CalB-V306N, the side chain of Asn³⁰⁶ was much longer and oriented to Thr³¹⁶, which made the distance between site 306 and 316 shorten to 2.88 Å and become more beneficial for hydrogen bond formation (Fig. 7c, d). It also promoted β -sheet folding in strands consisted of Gly³⁰⁷ through to Val³¹⁵, making the local molecular surface more rigid. In the biodiesel producing system, the oil-methanol mixture reduced environmental dielectric [46]. The introduced polar interactions or hydrophobic forces should be responsible for the stability elevation of lipases [47], as well as CalB variant (CalB-V306N or -P218W) in methanol solution.

Conclusions

In this work, 8 amino acid sites on the molecular surface of *Candida antarctica* lipase B (CalB) with high *B* values indicating being more flexible were chosen to perform saturation mutagenesis. High-methanol-tolerant variants, CalB-P218W and -V306N, created larger haloes on emulsified tributyrin solid plate including 15% (v/v) methanol and showed 19% and 31% higher activity over CalB-WT, respectively. A newly formed hydrogen bond in CalB-V306N and hydrophobic force in CalB-P218W might have enhanced their methanol tolerance. CalB-P218W and -V306N transesterified the soybean oil into biodiesel by 85% and 89% yield, respectively, over 82% by CalB-WT for 24 h reactions.

Declarations

Author Contribution Z. Tan performed the experiments, wrote the original manuscript, and reviewed the manuscript. X. Li checked the original data. H. Shi performed the data analysis. X. Yin conceived and designed the experiments. X. Zhu contributed analysis tools. M. Bilal reviewed the manuscript. M. M. Onchari reviewed the manuscript. All authors read and approved the final manuscript.

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Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest All authors declare no competing interests.

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Figures

Figure 1

A schematic representation for saturation mutagenesis of CalB gene on a plasmid. By long-distance inverse PCR, a primer pair introduced an amino acid changing to Val139/Ala146/Leu147/Pro218/Val286/Ala287/Val306/Gly307 of CalB.

Figure 2

The candidate sites for saturation mutagenesis on the molecular surface of CalB (PDB code 5A71, chain B) [4]. Note: Eight candidate sites (Val139, Ala146, Leu147, Pro218, Val286, Ala287, Val306, and Gly307) with high B factor values on CalB molecular surface were in red.

Figure 3

Effects of temperature on CalB-WT and its variants; a), activities of CalB-WT and its variants at different temperatures, and b), thermostabilities of CalB-WT and its variants. Reaction conditions: for a) 9 mL of the emulsified tributyrin (pH 6.5) and 1 mL of suitably diluted CalB, at 20–50 °C for 10 min, and for b) 9 mL of the emulsified tributyrin (pH 6.5) and 1 mL of suitably diluted CalB, at 20–50 °C for 1 h, followed by at 30 °C for 10 min.

Figure 4

Effects of pH on CalB-WT and its variants; a), activities of CalB-WT and its variants in different pH buffer, and b), pH stabilities of CalB-WT and its variants. Reaction conditions: for a) 9 mL of the emulsified tributyrin (pH 5–8) and 1 mL of suitably diluted CalB, at 30 °C for 10 min, and for b) 1 mL of suitably diluted CalB (pH 4.5–8.5), at 30 °C for 1 h, followed by adding to 9 mL of the emulsified tributyrin (pH 6.5), at 30 °C for 10 min.

Figure 5

Methanol tolerance of CalB-WT and its variants. Reaction conditions: CalB diluted in methanol-buffer (pH 6.5) solution (0–35% of methanol), at 30 °C for 24 h, followed by 9 mL of the emulsified tributyrin (pH 6.5) and 1 mL of above-mentioned diluted CalB, at 30 °C for 10 min.

Figure 6

Biodiesel preparation by CalB-WT and its variants. Reaction conditions: 20.0 g soybean oil, 3.7 mL methanol, 1 mL water, and 100 mg CalB in a 250-mL stoppered flask, 30 °C, and 200 rpm.

Figure 7

Close-up of structural mutation sites in CalB variants; a), CalB-WT P218, b), CalB-P218W, c), CalB-WT V306, and d), CalB-V306N. In modeled CalB-P218W, the side chain of Trp²¹⁸ oriented to Val¹⁹⁴, leading to drastically shrinking of the shortest distance between site 218 and Val¹⁹⁴ from 7.75 to 3.42 Å where it enhanced hydrophobic force. While in modeled CalB-V306N, the side chain of Asn³⁰⁶ was much longer and oriented to Thr³¹⁶, which made the distance between site 306 and 316 shorten to 2.88 Å in which it became more beneficial for hydrogen bond formation and also promoted β -sheet folding in strand consisted of Gly³⁰⁷ through to Val³¹⁵.