Methanol resistance of *Burkholderia cepacia* lipase

Francesco Sasso[$], Antonino Natalello, Simone Castoldi[#], Marina Lotti, Carlo Santambrogio* and Rita Grandori*

Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 2, 20126 Milan, Italy

Francesco Sasso	f.sasso3@campus.unimib.it
Antonino Natalello	antonino.natalello@unimib.it
Simone Castoldi	simone.castoldi1@gmail.com
Marina Lotti	marina.lotti@unimib.it
Carlo Santambrogio	carlo.santambrogio@unimib.it
Rita Grandori	rita.grandori@unimib.it

$ Present address: *McCANN Medical Complete S.r.l., Via Valtellina 17, 20159 Milan, Italy*

Present address: *Unit of Clinical Pharmacology, Luigi Sacco University Hospital, Via Bassi 74, 20157, Milan, Italy*

*Corresponding authors

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Abbreviations: *Burkholderia cepacia* lipase (BCL), circular dichroism (CD), Fourier transform infrared spectroscopy (FTIR), *Burkholderia glumae* lipase (BGL), attenuated total reflection (ATR).

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Abstract

Lipases resistant to inhibition and denaturation by methanol are valuable tools for biotechnological applications, in particular for biofuel production. Microbial lipases have attracted a great deal of interest because of their stability at high concentrations of organic solvents. *Burkholderia cepacia* lipase (BCL) is tested here for robustness towards methanol in terms of conformational stability and catalytic activity in transesterification assays. This lipase turns out to be even more tolerant than the homologous and better characterized enzyme from *Burkholderia glumae*. BCL unfolding transition, as monitored by far-UV circular dichroism (CD) and intrinsic fluorescence, displays a T_m above 60 °C in the presence of 50% methanol. The protein unfolds at low pH, and the organic solvent affects the nature of the denatured state under acidic conditions. The protein performs well in transesterification assays upon prolonged incubations at high methanol concentrations. BCL is highly tolerant to methanol and displays particularly high conformational stability under conditions employed for transesterification reactions. These features depict BCL as a promising enzyme for biofuel industry.

Introduction

Lipases are important tools in biotechnology because of their robustness and versatility. These enzymes are used in a variety of bioconversions of industrial interest. Particularly attractive, nowadays, is the possibility to employ lipases for biodiesel production, by transesterification of waste oils with short-chain alcohols. Methanol is considered the alcohol of choice because of its low price. The concentrations of methanol used in transesterification reactions are quite high, ranging between ~30% and 100% (v/v) of the non-lipid phase. Usually, concentrations of 30-50% are used for the reactions performed by free enzymes [1-3], while 100% methanol as the non lipidic phase is usually applied to immobilized enzymes [4-6]. It is therefore important to characterize the effects of organic solvents in general, and of methanol in particular, on this class of enzymes. A great deal of data is reported in the literature, describing the effects of organic solvents on lipase activity, but limited information is available on their effects on protein stability and conformational properties. It's known that the shell of water surrounding the enzyme is important for its conformational stability [7, 8] and that organic solvents, like methanol, change the hydration state [9].

Burkholderia (previously *Pseudomonas*) lipases have attracted particular interest because of their high stability to heat and organic solvents [10-15]. *Burkholderia cepacia* lipase (BCL) is characterized by good performance in transesterification assays [2, 16-18] and good residual activity upon exposure to organic solvents [15, 19, 20]. Immobilized BCL has been successfully employed for biofuel production by oil methanolysis [21, 22]. The crystallographic structure has been solved by X-ray diffraction [23-25]. The protein displays the consensus α/β-hydrolase fold, with a mobile helical lid over the active-site cleft, as typical for lipases. Analyses by Fourier transform infrared spectroscopy (FTIR) of immobilized BCL has pointed out a small but general increase in β-structure content upon 2-h incubation at 20 °C in several different organic solvents,

however not including methanol [20]. No detailed investigation has been reported regarding methanol effects on BCL conformation, stability and activity.

The issue of BCL conformational stability is of particular relevance for industrial applications, considering that this enzyme, like *Burkholderia glumae* lipase (BGL), unfolds irreversibly in the absence of its steric chaperone LimA [26, 27]. In this work, we have undertaken structural and functional investigation of BCL in aqueous solutions in the presence of high methanol concentrations. The feasibility of enzyme recycling upon incubation in methanol has been tested by transesterification assays. The results indicate that BCL is one of the most methanol-tolerant structures among the lipases employed for biotechnological applications.

Materials and methods

The enzyme used in this study was the lipase from *Burkholderia cepacia*, from Sigma Aldrich (St. Louis, MO, USA). Triolein from Sigma- Aldrich (St. Louis, MO, USA) was ~65 % pure. An average molecular weight on 873 g/mol was assumed, on the basis of the free fatty acid composition provided by the producer. Methanol, *p*-nitrophenyl laurate, Triton X-100, ammonium acetate, methyl oleate, and oleic acid of analytical grade were from Sigma-Aldrich.

Activity assays

Lipase powder was dissolved in 10 mM sodium phosphate pH 7 and the solution was centrifuged 10 minutes at 4 °C and 10000 rpm to remove insoluble material. The supernatant was then purified by Zeba™ spin desalting columns (Thermo Fisher Scientific, Waltham, MA, USA), after 4 cycles of resin equilibration in the same buffer. The sample was used for enzyme assays either directly, or diluted with deionized water in the case of hydrolysis assays. One unit (IU) was defined as the amount of enzyme which releases 1 µmol of *p*-nitrophenol/min in the hydrolysis reaction described

below. Hydrolytic activity was determined by the increase in absorbance at 410 nm produced by the release of *p*-nitrophenol during the hydrolysis of 1 mM *p*-nitrophenyl laurate (dissolved in isopropanol) in 10 mM phosphate buffer pH 7 and 0.5 % Triton X-100 at room temperature. To start the reaction, 0.005 IU lipase was added to 1 ml of the reaction mixture. Hydrolysis was followed for 3 min. Measurements were performed in triplicate.

Reusability of BCL previously exposed to methanol was evaluated by preincubating the enzyme in aqueous solutions containing different methanol concentrations. In detail, the stock solutions contained 0.2 mg/ml lipase and 0, 50 and 73% (v/v) methanol. The incubation was performed for 18 h at 37 °C followed by centrifugation for 5 min at 11,200×*g* to separate soluble and insoluble protein fractions. The protein amount in the soluble fraction was determined by the Bradford assay. An equal volume, corresponding to 7 IU of enzyme in the control sample (0% methanol), was withdrawn from each supernatant and used to catalyze transesterification in a mixture of 1:5 triglyceride/methanol. Transesterification reaction was performed in 1.5 ml screw cap vials, mixing triolein (0.388 g), methanol (71.1 mg), deionized water up to a final volume of 550 µl and adding 7 IU of enzyme solution to start the reaction. The reaction mixture was incubated at 37 °C in a rotator shaker. The reaction was stopped separating organic and aqueous phases by centrifugation at 11,200×*g* for 5 min.

The lipase-catalyzed transesterification was assayed by FTIR spectroscopy, as previously described [18]. Briefly, 5 µl of the organic phase were deposed on the diamond element of the device for FTIR measurements in Attenuated Total Reflection (ATR, Golden Gate-Specac, USA). The ATR/FTIR spectra were collected by the Varian 670-IR spectrometer (Varian, Mulgrave, VIC, Australia), equipped by a nitrogen cooled mercury cadmium telluride detector, at 2 cm^{-1} of spectral resolution, 25 kHz of scan speed, 512 scan coadditions, and triangular apodization. The second derivatives of the measured absorption spectra were obtained after a Savitsky-Golay smoothing using the Resolutions-Pro software (Varian, Mulgrave, VIC, Australia). In a previous work [18], we

showed that the linear relationship between the methyl ester concentration and the ~1,435 cm^{-1} peak height in the second derivative spectra allows determining the methyl ester content in the samples without interference of free fatty acids, which, instead, affect the analyses of the absorption spectra in the same spectral region. Therefore, the concentration of the methyl esters in the reaction mixtures was determined by the intensity in the second derivative spectra of the peak around 1,435 cm^{-1}, assigned to the CH_3 deformation mode, using a straight-line calibration curve obtained with standard samples of known concentrations of the single components (Figure S1).

Spectroscopic measurements

Circular dichroism (CD) experiments in the far-UV (200-260 nm) were performed on a J-815 spectropolarimeter (Jasco, Tokyo, Japan) employing a quartz cell with 2 mm light path and 250 µl sample volume at 1.5 µM protein concentration. Thermal ramps were performed in the range 20-85 °C at a speed of 1 °C/min. The CD profiles at 222 nm were recorded with a data pitch of 0.2 °C. CD spectra were acquired with increments of 5 °C. A layer of 100 µl mineral oil was added on the top of the sample in order to avoid solvent evaporation. Deconvolution of CD spectra was performed by the CDpro software, employing the CDSSTR algorithm on a database of 43 soluble proteins [28].

Protein intrinsic fluorescence was analyzed by a Cary Eclipse spectrofluorimeter (Varian, Mulgrave, VIC, Australia) employing a quartz cell with 1 cm light path and 250 µl sample volume at 1.5 µM protein concentration. Fluorescence emission was measured in the range 310-450 nm upon excitation at 295 nm. Thermal ramps were performed in the range 20-85 °C with increments of 5 °C, allowing for 3-min equilibration at each temperature. A layer of 100 µl mineral oil was added on the top of the sample in order to avoid solvent evaporation.

Results

In order to investigate the effects of methanol on BCL structural properties, aqueous solutions containing 0 or 50% alcohol were tested. The latter condition represents an average solvent composition for the polar phase, in which lipases are dissolved for transesterification reactions.

Thermal unfolding

Far-UV CD was used to monitor protein secondary structure (Figure 1). The spectrum of the protein at room temperature and 0% methanol reveals the typical features of the α/β sandwich fold, with negative minima at 208 and 222 nm (Figure 1A). As temperature is increased, in the range 20-85 °C, the spectral features progressively change to those of a denatured protein, with a shoulder around 220 nm and a negative minimum at 205 nm. An isodichroic point at ~205 nm indicates an apparent two-state transition and is consistent with progressive accumulation of random-coil conformation. The ellipticity profile at 222 nm indicates that the process does not reach a plateau in the explored temperature range (Figure 1B), in agreement with the known thermal stability of this enzyme [29]. Although poorly estimated in the absence of a plateau, the T_m, calculated as the inflection point of the fitting curve, is ~76 °C.

In the presence of 50% methanol (Figure 1C), the pre- and post-transition spectra are similar to those in the absence of methanol but the transition is sharper and the T_m is shifted by more than 10 °C, to a value of ~61 °C (Figure 1B). Deconvolution of the CD spectra recorded at 20 °C indicates that methanol barely affects the secondary structure of BCL. Indeed, the measured α-helical and β-sheet amounts in the absence of methanol (39.3% and 29.9%, respectively) are almost unchanged in 50% methanol (38.8% and 28.8%), and are in close agreement with the ones calculated on the basis of X-ray crystallography (37.8% and 24.4%) [23, 24].

BCL is known to lose activity at acidic pH [20]. Thus, the effect of methanol on BCL conformation has also been tested as a function of pH. Figure 1B shows the thermal ramps by far-UV CD recorded at different pH values, at 0% and 50% methanol. The results indicate that methanol consistently lowers the T_m of the protein at each pH. No transition is observed at pH 2, since the protein is already unfolded at this pH, even at room temperature and in the absence methanol. The full spectra at pH 2 and pH 7, with and without methanol, are reported for better comparison in Figure 1D. The data in the absence of methanol indicate a dramatic loss of α-helical structure (from 39.3% to 22.1%) and accumulation of β-structure (from 29.9% to 39.5%) and random coil (from 30.8% to 37.5%) upon acidification. In the presence of methanol, instead, ordered secondary structure is remarkably conserved even at pH 2, with only a small decrease in α-helix (from 39.3% to 35.5%) in favor of β-structure (from 29.9% to 30.8%) and random coil (from 30.8% to 33.8%).

Thermal unfolding was also monitored probing tertiary structure by fluorescence spectroscopy, exploiting intrinsic Trp fluorescence (Figure 2). The emission spectra in the absence of methanol show the typical loss in quantum yield, along with a red shift, as temperature is increased. The T_m values calculated by signal intensity (~74 °C) and λmax (~76 °C) are in good agreement with each other and with the CD results. However, the transition profiles obtained by the two techniques are quite different, particularly in the absence of methanol, with the transition monitored by fluorescence being sharper. This behavior suggests that the loss of tertiary structure is more cooperative than the loss of secondary structure, when BCL is exposed to high temperatures. In the presence of 50% methanol, the trend is similar, but the T_m values drops to ~61 °C and ~62 °C, respectively, again in agreement with CD data. Thus, secondary and tertiary structure are lost concomitantly in a highly cooperative transition at elevated temperatures, while the protein seems to maintain an overall native-like conformation in the range 0-50% methanol and 20-50 °C.

The pre-transition baseline, too, displays some methanol effect. In the absence of methanol, there is a typical negative slope, which can be ascribed to temperature dependence of fluorescence

quenching. However, a pronounced positive slope is observed in the presence of 50% methanol. This result suggests that minor conformational rearrangements might take place in the presence of 50% methanol below 50 °C, affecting the structural environment of Trp sidechains. However, this effect involves only fluorescence intensity, and not emission wavelength.

Sustained performance

Transesterification reactions are typically carried out in biphasic aqueous/lipidic systems, where the enzyme is dissolved in the aqueous phase and catalyzes the reaction at the interface with an organic, lipidic phase. The incubation is generally performed over extended time at 37 °C. Thus, the recovery of soluble protein after 18-h incubations at variable methanol concentrations at 37 °C was tested by centrifugation and gel electrophoresis. The results are reported in Figure 3A. Almost all the protein is recovered in the soluble fraction at either 0% or 50% methanol, indicating that, at neutral pH, the presence of 50% methanol does not trigger protein aggregation even over extended incubation time. At 73% methanol, instead, a considerable amount of the protein is found in the pellet, consistent with limited BCL solubility under these conditions, likely triggered by partial denaturation and aggregation over time. The residual hydrolytic activity after the incubation is ~40% (Figure 3B), although quite large variations have been observed among samples deriving from different protein stocks (data not shown).

The enzyme performance in transesterification reactions was tested upon exposure at different methanol concentrations. BCL was pre-incubated for 18 h at 37 °C with 0, 50 or 73% (v/v) methanol, and the recovered soluble protein was assayed in transesterification reactions using triolein and methanol as substrates at a 1:5 molar ratio, previously identified as optimal for this enzyme [18]. Methyl ester formation was monitored by Fourier transform infrared spectroscopy (FTIR) in attenuated total reflection (ATR) mode, as previously described [18]. The measured

product concentrations, expressed as volume percentage in the organic phase of the reaction, are reported in Figure 4A, as a function of reaction time. A methyl ester content (v/v) of 10% corresponds to a conversion yield of ~9.5% under the here employed conditions. The maximum conversion yield reached by 24-h incubations was 40.3%. The activity data show that the conversion efficiency is almost unaffected by the pre-treatment in 50% methanol and slightly reduced upon incubation in 73% methanol, consistent with partial loss of the protein in the insoluble fraction. Activity values appear fairly constant upon normalization for protein concentration in the soluble fraction (Figure 4B). The data obtained without pre-incubation are comparable to those upon incubation at 0% methanol (data not shown).

Discussion

This study describes BCL response to high methanol concentrations. The conditions applied for this analysis reproduce the composition of the aqueous phase of transesterification reactors, in which the enzyme is dissolved for the production of methyl esters. The results point out an effect of methanol on structural thermostability, but indicate at the same time remarkable methanol tolerance of BCL in the temperature range 20-50 °C. Conformational stability in 50% methanol is even higher than previously reported for the paralogue from *Burkholderia glumae*, BGL [14]. In particular, BGL T_m in 50% methanol is between 52 and 55 °C, while BCL T_m under the same conditions and assessed by the same methods is between 61 and 62 °C. These data are consistent with the reported Tmax values for BCL activity, which are slightly above 50 °C [20, 30].

Measurements at variable pH and variable temperature indicate that methanol enhances BCL thermolability at acidic pH, too, but has unexpected effects on the nature of the denatured state at low pH. The protein conformation at pH 2 is dramatically altered by 50% methanol, leading to the accumulation of a non-native conformation rich in ordered secondary structure. The accumulation

of non-native secondary structure at acidic pH and 50% methanol could increase protein aggregation propensity under these conditions.

BCL displays good robustness towards prolonged incubations in 50% methanol at 37 °C, an attractive feature that could be exploited in the future for bioconversions based on enzyme recycling. Again, the performance is higher than observed for BGL, which precipitates in considerable amounts under the same conditions [14]. In the case of BCL, protein precipitation becomes significant only at higher methanol concentrations, as indicated by the data at 73% methanol, while the proteins remains fully soluble and fully active under transesterification conditions at 50% methanol. Altogether, these results hint at a remarkable resistance of this enzyme to high methanol concentrations and support feasibility of enzyme recycling for transesterification reactions. The conditions employed here were not optimized for conversion yield, in order to keep the system sensitive to minor losses in enzyme activity. It is well possible that higher conversion yields in transesterification reactions are achievable using higher enzyme concentrations and solvent optimization, even above 90% as reported for other catalysts [5, 17, 31-32].

Concluding remarks

The here presented results show that BCL retains native structure and activity upon prolonged incubations at high methanol concentrations at 37 °C, an interesting feature compared to other lipases, which are known to be inhibited by methanol [13, 15, 30, 33-34]. Altogether, these results point to this enzyme as an attractive biocatalyst for biofuel production.

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Conflict of interest statement

The authors declare no commercial or financial conflict of interest.

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Figure legends

Figure 1. *Thermal denaturation monitored by far-UV CD*. (A, C) Samples contain 1.5 μM BCL in 10 mM sodium phosphate, pH 7.0, and 0% methanol (A) or 50% methanol (C). The arrows indicate the direction of spectral changes as temperature is increased. (B) Thermal unfolding, monitored by ellipticity at 222 nm, at pH 7 (black lines), pH 5 (blue lines), pH 4 (green lines) and pH 2 (red lines), at 0% methanol (continuous lines) and 50% methanol (dashed lines). (D) CD spectra at 20 °C at pH 7 (black lines) and pH 2 (red lines) at 0% methanol (continuous lines) and 50% methanol (dashed lines).

Figure 2. *Thermal denaturation monitored by protein intrinsic fluorescence*. Samples contain 1.5 μM BCL in 10 mM sodium phosphate, pH 7.0, and 0% methanol (A-C) or 50% methanol (D-F). Raw spectra (A, D), intensity profiles (B, E) and emission wavelength profiles (C, F) are shown. The arrows indicate the direction of spectral changes as temperature is increased. The reported values and error bars correspond, respectively, to the average and the standard deviation over three independent measurements.

Figure 3. *Aggregation propensity monitored by denaturing polyacrylamide gel and activity assay*. (A) BCL distribution in the soluble (s) and insoluble (p) fraction after 18-hour incubation at 37 °C, in the presence of various concentrations of methanol. (B) hydrolytic activity measured on BCL samples preincubated for 18 h at 37 °C with 0%, 50%, and 73% methanol (v/v). The reported values are averaged over three independent experiments.

Figure 4. *Transesterification activity monitored by FTIR*. (A) Time course of transesterification reactions of BCL samples preincubated for 18 h with 0%, 50%, and 73% methanol (v/v). The recovered soluble enzyme was assayed in transesterification at 1:5 triglyceride:methanol ratio. (B) Transesterification activity after 24-h reaction upon 18-h incubation at variable methanol concentration.. Total activity (directly comparable to panel A) is shown by dashed bars. Specific activity, normalized for protein concentration in the soluble fraction, is shown by gray bars. Methyl ester content (v/v) was obtained from the 1,435 cm^{-1} peak intensity in the second-derivative spectra (Figure S1). The reported values are averaged over three independent experiments.

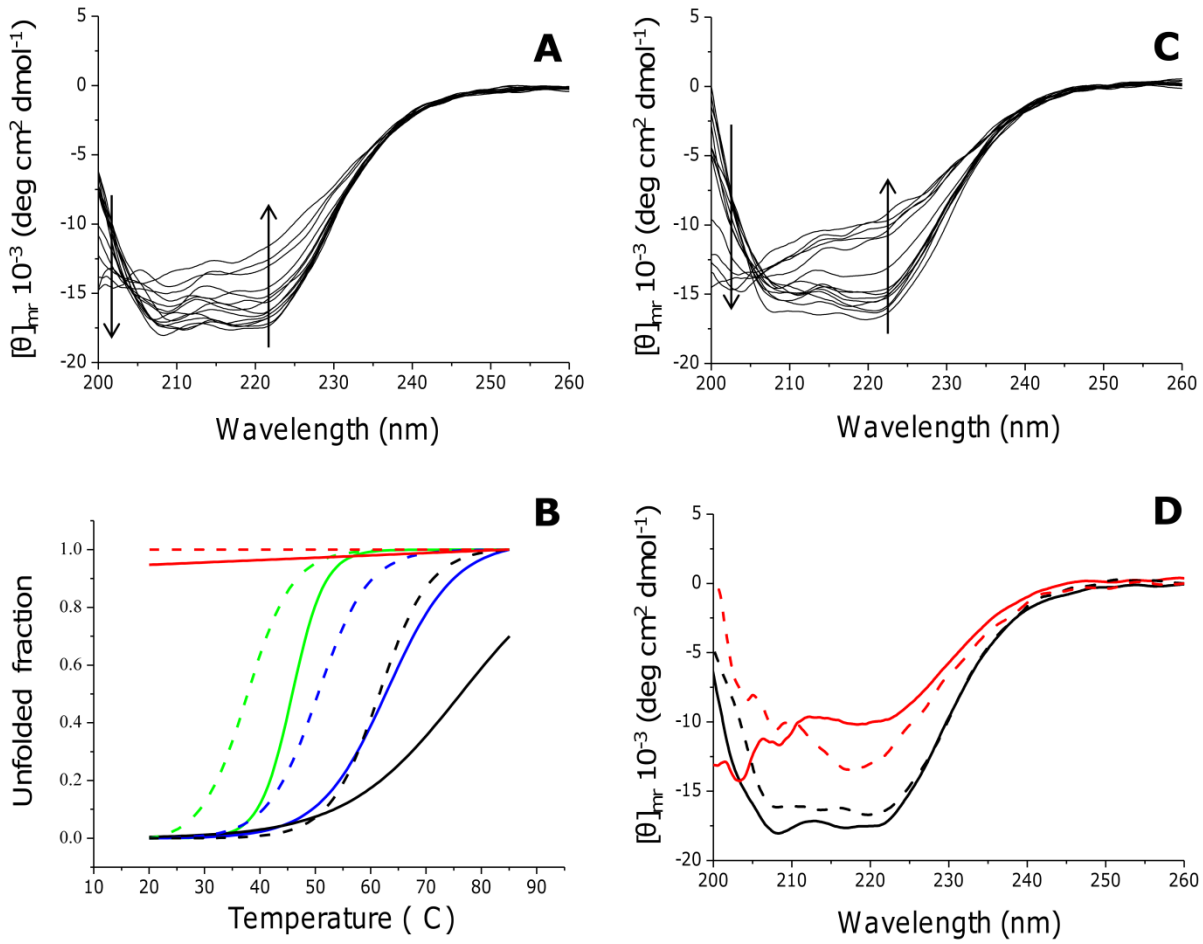
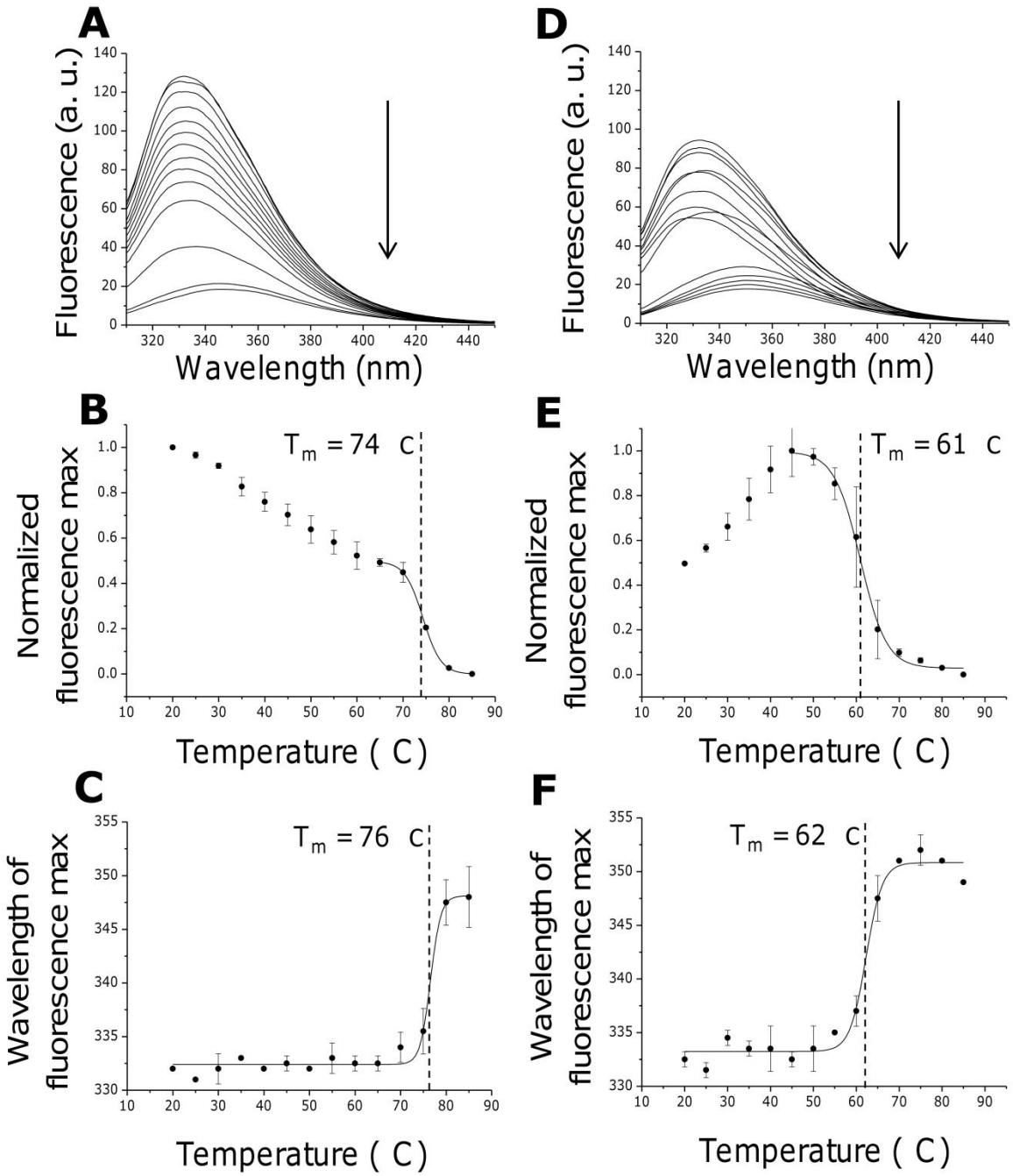


Figure 1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Normalized fluorescence max
T_m = 61 C
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Figure 2

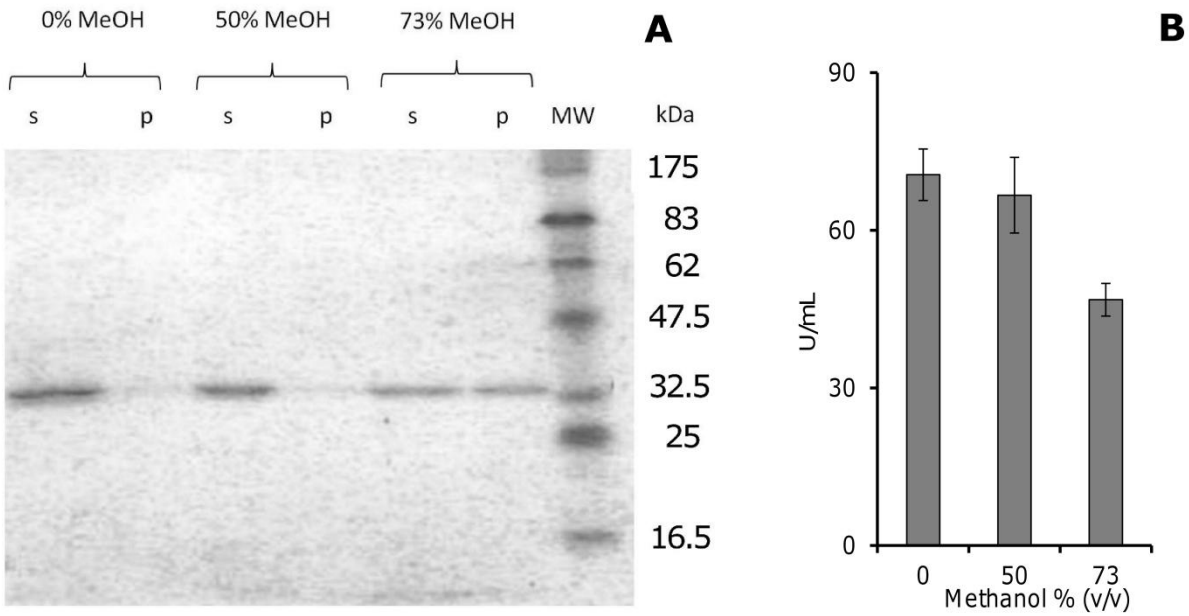


Figure 3

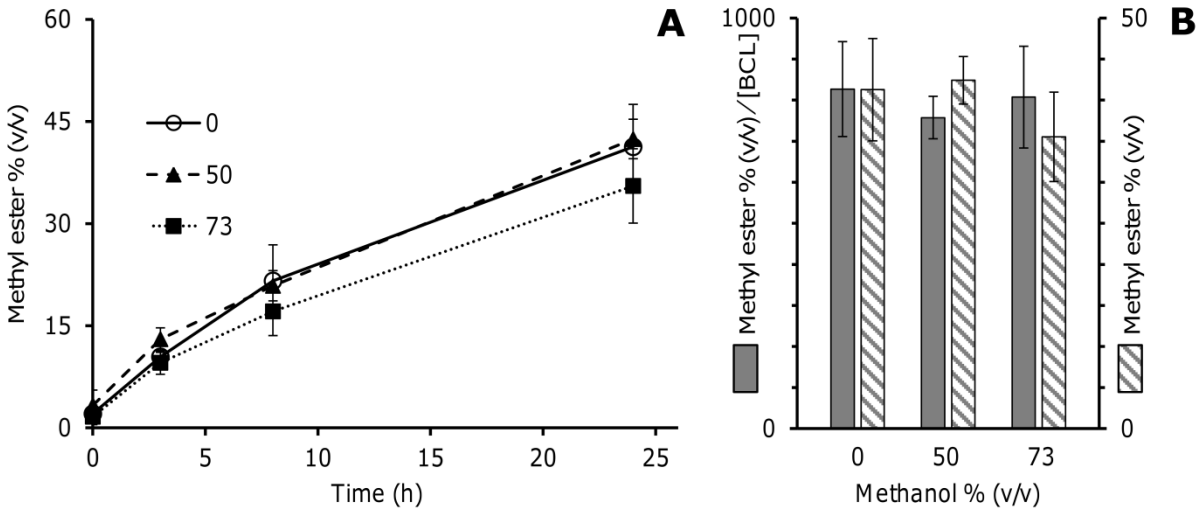
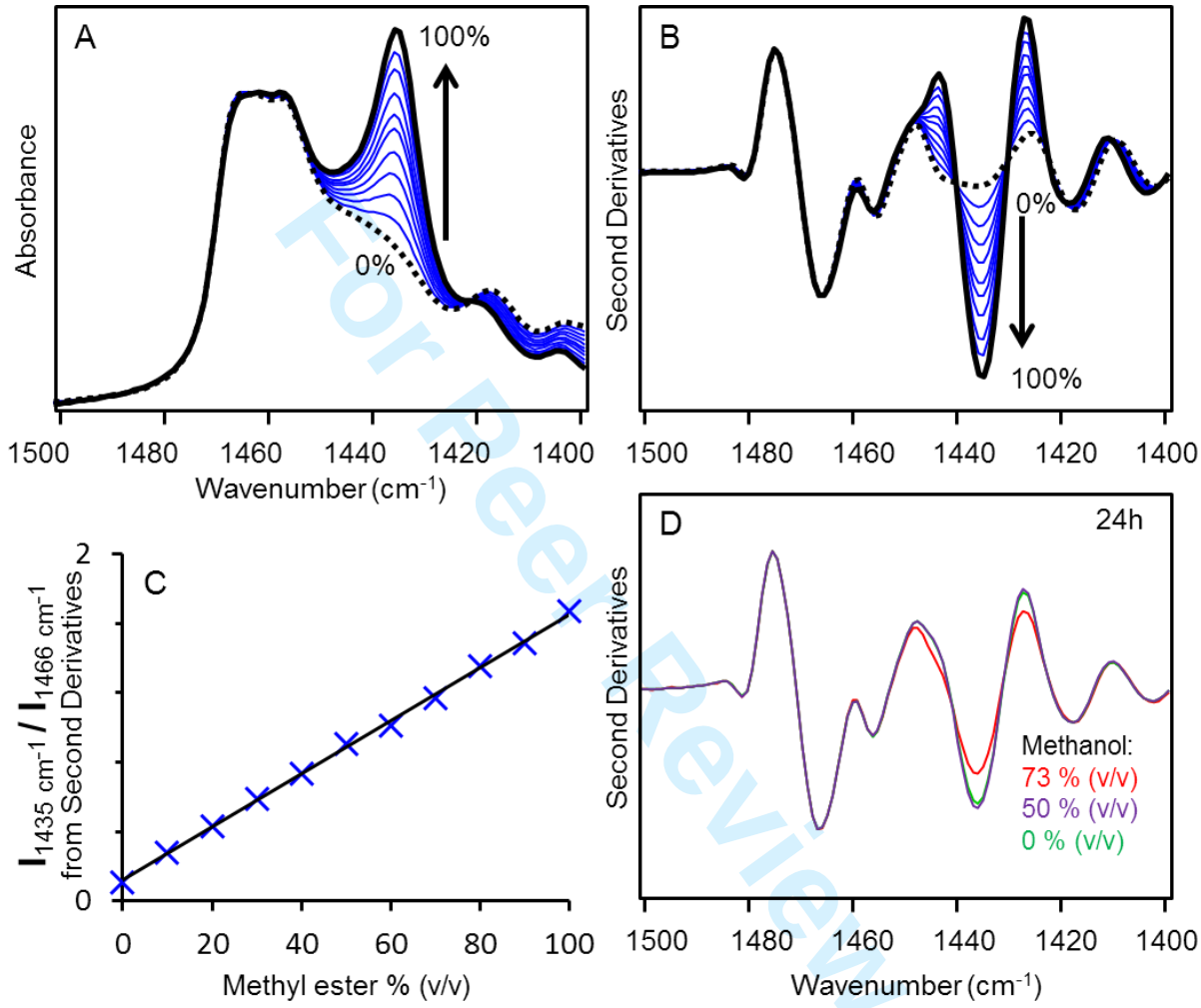


Figure 4

Supplementary materials

Methanol resistance of *Burkholderia cepacia* lipase

Francesco Sasso, Antonino Natalello, Simone Castoldi, Marina Lotti, Carlo Santambrogio, Rita Grandori



Supporting Figure 1. *Methyl ester determination by ATR/FTIR spectroscopy.* (A) Absorption spectra of standard samples at different methyl oleate concentrations, from 0% to 100% (v/v), reported in the 1,500-1,400 cm^{-1} spectral region. (B) Second derivatives of the absorption spectra in (A). (C) Linear regression calibration curve obtained by the intensity ratio at 1,435 cm^{-1} and at 1,466 cm^{-1} from the second-derivative spectra of (B). (D) Second derivatives of the absorption spectra of the reaction samples taken after 24 h of incubation under transesterification conditions. The BCL protein samples were preincubated for 18 h in methanol at 0%, 50%, and 73% (v/v).