Supporting information

A flow cytometer based whole cell screening toolbox for directed hydrolase evolution through fluorescent hydrogels

*Nina Lülsdorf‡[a], Christian Pitzler‡[b], Michael Biggel[b], Ronny Martinez[c], Ljubica Vojcic[b], and Ulrich Schwaneberg[a,b]**

[a] *DWI-Leibniz Institut für Interaktive Materialien,*
Forckenbeckstraße 50, 52056 Aachen, Germany
[b] *Lehrstuhl für Biotechnologie, RWTH Aachen University,*
Worringerweg 3, 52074 Aachen, Germany
[c] *EW-Nutrition GmbH, Nattermannallee 1, 50829 Köln, Germany*
‡ joint first authors
*Address correspondence to: u.schwaneberg@biotec.rwth-aachen.de

Material and Methods

Chemicals were of analytical-reagent grade or higher quality and purchased from Carl Roth GmbH (Karlsruhe, Germany), Sigma-Aldrich (Hamburg, Germany) and AppliChem (Darmstadt, Germany) with the exception of Methacryloxyethylthiocarbamoyl-rhodamine B (Polyfluor 570), which was purchased from Polysciences Inc. (Warrington, PA, USA). Synthetic genes were ordered from GeneArt (Regensburg, Germany). All enzymes were purchased from New England Biolabs (Frankfurt, Germany). Oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany) in salt-free form. Plasmid isolation kit was purchased from Macherey-Nagel (Düren, Germany) and PCR purification kit from Qiagen GmbH (Hilden, Germany).

Cloning in expression vector

E. coli strains DH5α and BL21-Gold (DE3) were purchased from Agilent Technologies (Santa Clara, USA).

p-nitrobenzyl esterase from B. licheniformis (pNBEBL)

The gene *p*NBEBL (GenBank: AAU39577.1, [1]), was ordered as a synthetic gene and transformed into *E. coli* DH5α [2]. Plasmid, containing the synthetic gene, and the pET22b(+) expression vector were digested using *Xba*I (100 U) and *Eco*RI (100 U) restriction enzymes. After purification of the specific genes, the digested *p*NBEBL gene and vector pET22b(+) were ligated using T4 DNA ligase (5 U) resulting in pET22b(+)-*p*NBEBL. Plasmid construct was subsequently transformed into *E. coli* BL21-Gold (DE3) for expression experiments.

B. subtilis lipase A (BSLA)

BSLA (GenBank: NP_388152.1), as well as the expression vector pET22b(+) were double digested with *Nde*I and *Xho*I, the purified fragments were ligated with T4 DNA ligase (5 U). The resulted plasmid was transformed with *E. coli* BL21-Gold (DE3) for expression experiments.

Cellulase A2 (CelA2)

The gene CelA2 (GenBank: JF826524.1; [3]) was ordered as a synthetic gene with optimized codon usage for *E. coli*.

Diversity generation by random mutagenesis

Three sets of mutant libraries were generated by epPCR using pET22b(+)-*p*NBEBL pET22b(+)-BSLA, and pET28b(+)-CelA2 as template. The standard PCR was carried out using *Taq* polymerase (2.5 U), dNTP mix (0.2 mM each), DNA template (30 ng/µl) supplemented with different $MnCl_2$ concentrations (0.0, 0.1, 0.2 and 0.3 mM) (Table S1). Primers (0.4 pmol) were used according to the different templates (Table S2).

Table S1 Error prone PCR (epPCR) program for the following genes p*nbebl*, *bsla*, and *cela2*.

Step	Temperature [°C]	Time [sec]	Cycle [-]
Initial denaturation	94	60	1x
Denaturation	94	30	
Annealing		30	
p*nbebl*	58		
bsla	60		
cela2	60		25x
Elongation	72		
p*nbebl*		90	
bsla		120	
cela2		120	
Final elongation	72	300	1x

Table S2 Primers for epPCR (small letters indicates phosphorothioate nucleotides)

Primer name	Sequence 5'-3'
F_*p*NBEBL	cttgtcgacggaGCTCGAATTCTTATTA
R_*p*NBEBL	atacaactgtcgAAACACGCTTCGG
F_BSLA	cttgtcgacggaGCTCTCATTA
R_BSLA	ccggcgatggccATGGATATC
F_CelA2	cttgtcgacggaGCTCGAATTCTTATTA
R_CelA2	ccggcgatggccATGAGCGAAGAATGGCAGACCTATGAAAAAG

Amplification of the vector backbone was performed by using pET22b(+) as template containing a pelB leader sequence. PCR was carried out using *Pfu*S polymerase (Table S3) and specific primers were designed for each construct (Table S4). Resulting PCR products were digested with *Dpn*I (20 U) overnight at 37°C, and subsequently *Dpn*I was inactivated (80°C; 20 min). The PCR products were purified using QIAquick PCR Purification Kit. Cloning was performed by PLICing [4] and hybridized DNA fragments were transformed with competent *E. coli* BL21-Gold (DE3).

Table S3 PCR program for vector backbone

Step	Temperature [°C]	Time [sec]	Cycle [-]
Initial denaturation	98	60	1x
Denaturation	98	30	
Annealing		30	
p*nbebl*	54		
bsla	60		
cela2	60		25x
Elongation	72		
p*nbebl*		240	
bsla		180	
cela2		180	
Final elongation	72	300	1x

Table S4 Primers for vector backbone amplification (small letters indicates phosphorothioate nucleotides).

Primer name	Sequence 5'-3'
F_pET22b(+)-*p*NBEBL	cgacagttgtatCATACATCAT
R_pET22b(+)-*p*NBEBL	tccgtcgacaagCTTGC
F_pET22b(+)-BSLA	ggccatcgccggCTGGGCAGCGAGGAGCAGCAGACCAGCAGCAG
R_pET22b(+)-BSLA	tccgtcgacaagCTTGCGGCCGCACTCGAGC
F_pET22b(+)-CelA2	cgacagttgtatCATACATCAT
R_pET22b(+)-CelA2	tccgtcgacaagCTTGCGGCCG

Hydrogel Formation

For generating hydrogel around *E. coli* BL21-Gold (DE3) cells expressing active enzyme, pET22b(+) plasmids containing a *pelB* leader sequence were obtained. The *pelB* leader sequence leads to a periplasmic expression of the encoded genes.

A 10 ml preculture of *E. coli* BL21-Gold (DE3) cells producing either active hydrolases (pET22b(+)-*p*NBEBL, -BSLA, or -CelA2) or cells harboring empty pET22b(+) was grown overnight in 500 ml erlenmeyer flasks (LB media supplemented with 100 µg ampicillin /ml, 37°C, 250 rpm). 1 % of the precultures were used to inoculate a 20 ml production culture in a 500 ml erlenmeyer flask (LB media supplemented with 100 µg Ampicillin/ml, 37°C, 250 rpm). Upon induction with 0.1 mM isopropyl thio-β-D-galactoside (IPTG) at an optical densitiy (OD_{600}) of $\lambda_{600\ nm}$ = 0.6, enzyme was expressed (4 h, 30°C, 250 rpm). Cells were harvested by centrifugation (10 min, 4°C, 3220 g, Eppendorf centrifuge 5810 R) and cell pellet was washed two times with 10 ml PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 6.5). Cell density was adjusted to an OD_{600} of $\lambda_{600\ nm}$ = 2 using PBS. Hydrogel formation was initiated by mixing 960 µl cell suspension, 24.8 % Poly(ethylene glycol)$_{575}$ diacrylate (PEG_{575}-DA), 2.5 % 1-vinyl-2-pyrrolidone, 20 µl (0.5 % stock

concentration) Polyfluor 570, 2 µl (25 µM stock concentration) Fe_2SO_4*7H_2O dissolved in ddH_2O, 200 µl (200 mM stock concentration) respective substrate dissolved in PBS (cellobiose) or ddH_2O (β-D-(+)-glucose pentaacetate), 2 µl GOx type II dissolved in PBS (1.58 mM stock concentration) in a total reaction volume of 1.5 ml. Reaction was stopped by three times washing (1 min, 4°C, 3220 g) with 1 ml PBS. Prepared samples were used for confocal microscopy and flow cytometry analyses.

Confocal Microscopy

Confocal microscopy (Leica TCS SP8, Leica Microsystems) was performed using 63x oil immersion objective. A continuous wave laser (DPSS, 20 mW: 561 nm) was used for excitation and a highly sensitive prism spectral detector filter was used for emission (570 nm). Gain was set according to a minimal signal for the strains harboring an empty vector to 530.

Flow cytometry analyses and cell sorting

Flow cytometry was carried out as described previously [5]. Sorted cells were pelleted by centrifugation (1 min, 11000 rpm). Plasmid was isolated and transformed with competent *E. coli* Bl21-Gold (DE3) and plated on LB-agar plates supplemented with 100 mg ampicillin/l.

Protein expression in 96-well microtiter plates (MTP)

Colonies were transferred into 96-well flat bottom microtiter plates (MTP) containing 150 µl LB-medium supplemented with ampicillin (100 µg/ml). After overnight cultivation in a MTP shaker (37°C, 900 rpm, 70 % humidity), 150 µl main culture (LB-medium supplemented with 100 µg/ml ampicillin) were inoculated with 10 µl pre-culture (v-bottom MTP. The plates were cultured for 2.5 h (37°C, 900 rpm, 70 % humidity) until OD_{600} reached 0.6 and protein expression was induced by addition of IPTG (0.1 mM). After expression (4 h, 30°C, 900 rpm, 70 % humidity), plates were centrifuged (15 min, 4°C, 4000 g). Medium supernatant was removed and cell pellets were stored overnight at -20°C. Cell disruption was performed by resuspending the cell pellet in 150 µl PBS-buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) supplemented with lysozyme (1 mg/ml). After incubation (1 h, 37°C, 900 rpm, 70 % humidity), plates were centrifuged (15 min, 4°C, 4000 g). Supernatants of lysates were subsequently used in respective 96-well MTP screening systems.

Screening systems

Fluorometric assay for determining esterolytic activity of pNBEBL

The Amplite™ Red Kit (AAT Bioquest), usually obtained to quantify glucose, was further developed to detect esterolytic activity. In a coupled three enzyme reaction, the esterase converts β-D-(+)-glucose pentaacetate into glucose. Next, glucose is converted by a glucose oxidase (GOx) into glucono-δ-lactone and hydrogen peroxide. Released hydrogen peroxide is monitored by the conversion of a fluorogenic substrate (Amplite™) in the presence of horseradish peroxidase (HRP) into a fluorescent

product. 96-well MTP format was used to determine the activity of 25 µl cell lysate containing expressed *p*NBEBL, subsequently 25 µl glucose pentaacetate (stock concentration 60 mM) was added. Reaction was started by addition of 25 µl mastermix (430 µl assay buffer, 10 µl HRP, 10 µl GOx and 2 µl Amplite). Conversion of the fluorescent substrate was monitored at $\lambda_{540\ nm}/\lambda_{590\ nm}$ in a MTP reader (Tecan Infinite M1000 Pro).

Spectrophotometric assay for determining lipolytic activity of BSLA

The activity of BSLA was determined by using *p*-nitrophenyl acetate (*p*NPA). *p*NPA was dissolved in acetonitrile (stock concentration 100 mM) and subsequently diluted in triethanolamin buffer (TEA, 50 mM, pH 7.4) (stock concentration 1 mM). Reaction was initiated inside 96-well MTPs by mixing 90 µl of *p*NPA-TEA solution with 10 µl cell lysate containing BSLA. The release of *p*-nitrophenylate was monitored at 410 nm in a MTP reader (Tecan Sunrise).

Fluorometric assay for determining cellulolytic activity of CelA2

Activity of CelA2 was determined by using 4-methylumbelliferyl-β-D-cellobioside (4-MUC, [6, 7]) as described previously [3].

Purification

Enzyme production and purification

E. coli BL21-Gold (DE3) cells containing the plasmid pET22b(+)-*p*NBEBL, pET22b(+)-BSLA, pET22(b)-CelA2 or a corresponding variant were grown in shake flasks overnight (10 ml LB media supplemented with 100 µg/ml ampicillin, 37°C, 250 rpm) and used for inoculation of a production culture (100 ml LB media supplemented with 100 µg/ml ampicillin, 37°C, 250 rpm). Enzyme expression was induced by the addition of IPTG (0.1 mM final concentration) when the production culture reached an optical density (OD_{600}) of 0.6 and further cultivation was continued (30°C, 250 rpm, 4 h). *E. coli* cells were harvested by centrifugation (4°C, 10000 rpm, 20 min, Sorvall RC-6 Plus, Thermo Scientific) and frozen at -20°C overnight. Cell pellets were resuspended in 20 ml HEPES buffer (10 mM, pH 7.4). After cell disruption by sonification (3 times 30 s with 30 s cooling intervals, EmulsiFlex C3, Avestin Europe GmbH) on ice, cell debris was removed by centrifugation (4°C, 10000 rpm, 20 min) and supernatant was filtered (0.45 µm; Minisart RC 25 disposable syringe filter, Sartorius) and used for purification by cation exchange chromatography (BSLA) anion exchange chromatography (*p*NBEBL) or gel fitration (CelA2) with a protein purification system (ÄKTAprime plus, GE Healthcare Europe GmbHGE Life Sciences):

- 5 ml of *p*NBEBL samples were loaded Toyopearl Super Q 650c column and eluted with 19 % 1 M NaCl.
- 10 ml of BLSA-samples were loaded on a Toyopearl SP-650C column (Tosoh Bioscience) and eluted through an increasing gradient (0 % to 20 %) of sodium chloride solution (1 M).

- 1.2 ml of CelA2 samples were loaded on a HiPrep 16/60 Sephacryl S-400 HR (GE Healthcare Europe GmbHGE Life Sciences) gel filtration column.

Peak fractions were collected and analyzed for enzyme activity and by SDS PAGE. Fractions showing the highest enzyme activity were pooled and stored at 4°C until further use.

Determination of protein quantity and purity

Total protein concentration was determined using a BCA Protein Assay Kit (Pierce Chemicals) according to the manufacturer's protocol. Experion Automated Electrophoresis System (Bio-Rad) and the Pro260 Analysis Kit were used for purity determination by following the manufacturer's instructions.

Determination of enzyme kinetics for pNBEBL

Purified enzyme solutions were diluted to a concentration of 0.4 µg/µl. 25 µl were used for determination of V_{max} and K_m. For initiation of the reaction 25 µl of glucose pentaacetate in varied concentrations (0 mM – 50 mM in ddH_20) and 25 µl Amplite mastermix (see *Fluorometric assay for determining esterolytic activity of pNBEBL*) were combined. Conversion of the fluorescent substrate was monitored at $\lambda_{540\ nm}/\lambda_{590\ nm}$ in a MTP reader (Tecan Infinite M1000 Pro). Product formation rates were calculated using a standard curve obtained by measuring fluorescence values of a glucose dilution series. The determination of enzyme kinetics was performed with Prism 6 software (GraphPad) using a Michaelis-Menten kinetic derivation.

Determination of enzyme kinetics for BSLA

Purified enzyme solutions were diluted to a concentration of 71.8 µg/ml. For determination of kinetic parameters, 140 µl of *p*NPA solution in different concentrations (0 mM – 10 mM, in 1 % acetonitrile, 100 mM potassium phosphate buffer, pH 6.5) was added to 10 µl sample containing purified lipase A in 96-well MTP. The increase of absorption at 410 nm was recorded for 5 min at room temperature using a Tecan Infinite M1000 MTP reader (Tecan Group AG). The maximum linear rate was used to determine enzyme activity. Product formation rates were calculated using a standard curve obtained by measuring absorbance values of a *p*-nitrophenylate dilution series. The determination of enzyme kinetics was performed with GraphPad Prism 6 software using a Michaelis-Menten kinetic derivation.

Determination of enzyme kinetics for CelA2

Purified enzyme solutions were diluted to a concentration of 10 µg/ml. For determination of kinetic paprameters, 20 µl 4-MUC substrate solutions of different concentrations (0.0 µM – 1 µM, 40 µl 250 mM potassium phosphate buffer, pH 7.2) was added to 40 µl sample containing purified CelA2 in 96-well MTP. The increase in fluorescence at 450 nm was recorded for 20 min at 30°C using a Tecan

Infinite M1000 MTP reader (Tecan Group AG). Product formation rates were calculated using a standard curve obtained by measuring fluorescence values of a 4-Methylumbelliferone dilution series. The determination of enzyme kinetics was performed with GraphPad Prism 6 software using a Michaelis-Menten kinetic derivation.

Results:

Fig. S1 shows the increase in fluorescence or absorbance per second for two respective improved variants. Rescreening was performed for improved *p*NBEBL variants with Amplite™ assay (A), for improved BSLA variants with *p*NPA (B) and for improved CelA2 variants with 4-MUC assay (C).

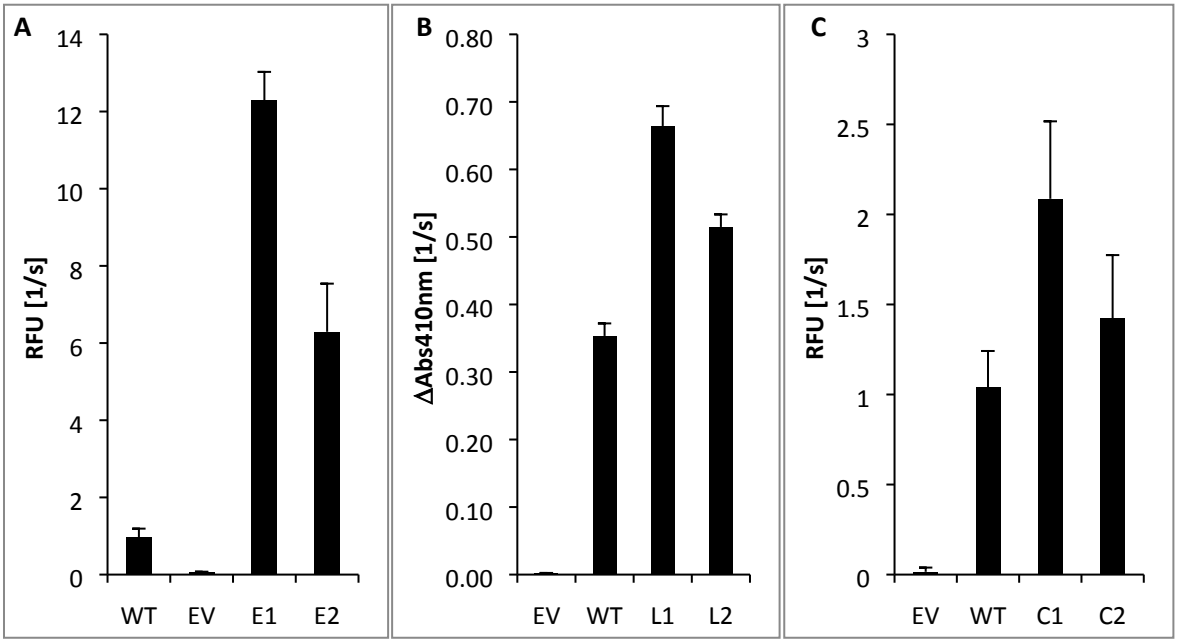
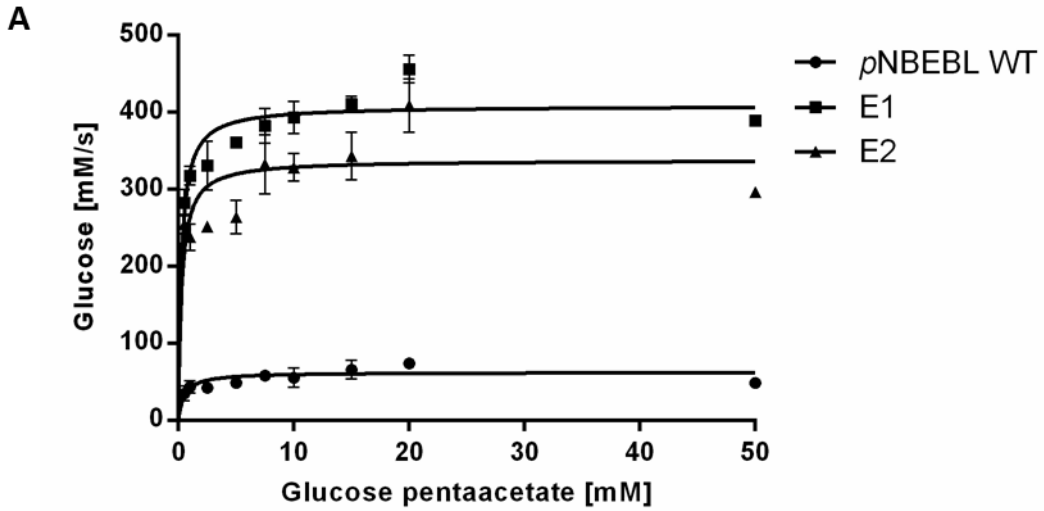


Fig. S1: Rescreening was performed for improved *p*NBEBL variants with Amplite™ assay (A), for improved BSLA variants with *p*NPA (B) and for improved CelA2 variants with 4-MUC assay (C).

Characterization of improved variants

In **Fig. S2** Michaelis-Menten kinetics for all purified enzymes, determined with the respective substrates are shown.



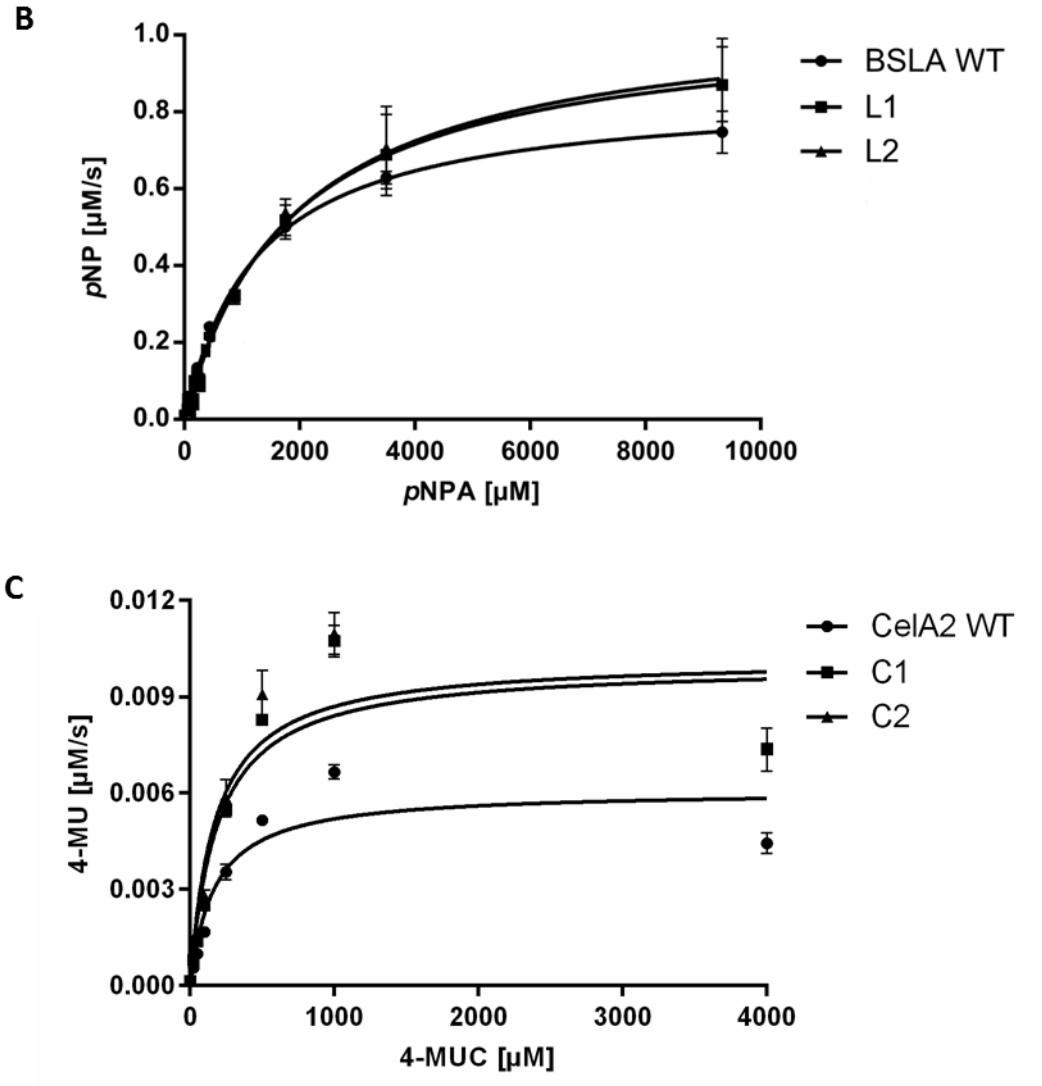


Fig. S2: Michaelis-Menten plots of improved hydrolase variants. After Screening and rescreening two improved variants for each enzyme as well as WT was purified and pure enzyme was used for detailed characterization. Michaelis-Menten kinetic was performed with the Amplite™ screening system for the esterase (A), the *p*NPA assay for the lipase (B) and the 4-MUC assay for the cellulose (C). The results were analyzed by GraphPad Prism 6 software [*p*NBEBL (WT, E1, E2), BSLA (WT, L1, L2) and CelA2 (WT, C1, C2)].

References

1. Veith, B., et al., *The complete genome sequence of Bacillus licheniformis DSM13, an organism with great industrial potential.* J Mol Microbiol Biotechnol, 2004. **7**(4): p. 204-11.
2. Inoue, H., H. Nojima, and H. Okayama, *High efficiency transformation of Escherichia coli with plasmids.* Gene, 1990. **96**(1): p. 23-8.
3. Lehmann, C., et al., *Reengineering CelA2 cellulase for hydrolysis in aqueous solutions of deep eutectic solvents and concentrated seawater.* Green Chemistry, 2012. **14**(10): p. 2719-2726.
4. Blanusa, M., et al., *Phosphorothioate-based ligase-independent gene cloning (PLICing): An enzyme-free and sequence-independent cloning method.* Anal Biochem, 2010. **406**(2): p. 141-6.
5. Pitzler, C., et al., *A fluorescent hydrogel-based flow cytometry high-throughput screening platform for hydrolytic enzymes.* Chem Biol, 2014. **21**(12): p. 1733-42.
6. Boschker, H.T. and T.E. Cappenberg, *A Sensitive Method Using 4-Methylumbelliferyl-beta-Cellobiose as a Substrate To Measure (1,4)-beta-Glucanase Activity in Sediments.* Appl Environ Microbiol, 1994. **60**(10): p. 3592-6.
7. Chernoglazov, V.M., A.N. Jafarova, and A.A. Klyosov, *Continuous photometric determination of endo-1,4-beta-D-glucanase (cellulase) activity using 4-methylumbelliferyl-beta-D-cellobioside as a substrate.* Anal Biochem, 1989. **179**(1): p. 186-9.