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Integration of bioreduction and product isolation: Selection of one co-solvent for *in situ* substrate supply and extractive product isolation

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Kurzfassung

Bei der Reaktion von *o*-Chloroacetophenon zu dem entsprechenden *S*-Alkohol mit rekombinantem *E.coli* kommt es zu einer raschen Deaktivierung des Katalysators durch Substrat und Produkt. Das Einsetzen einer zweiten wasserunlöslichen Phase ist eine gängige Lösung dieses Problems. Dadurch kommt es zu einer Verlängerung der Lebenszeit des Katalysators und einer gesteigerten Ausbeute. Hexan und Hexanol wurden in der vorliegenden Arbeit aufgrund ihrer Eigenschaften wie Umweltverträglichkeit, mehr Extraktionsvermögen und Handhabung anderen getesteten Lösungsmitteln vorgezogen. Der Einsatz einer zweiten Phase führte in einigen Fällen zu einer Umsatzsteigerung auf das 9-fache. Ein Scale-Up der Reaktion auf 0,5 L wurde in einem Bioreaktor mit Temperatur und pH-Kontrolle realisiert. Reaktionsgemische mit 20 oder 50 % v/v organischer Phase und 40 g_{CDW}/L bildeten nach 24 Stunden Reaktionszeit stabile, milchige Emulsionen aus. Die höchste Produktkonzentration (291 mM) wurde mit 20 % v/v Hexan und 300 mM *o*-Chloroacetophenon erreicht. Die Produktisolierung erforderte die Abtrennung der organischen Phase von der restlichen Reaktionsmischung. Die Biomasse ließ sich durch Filtration nicht abtrennen. Eine Absetzzeit von 24 Stunden ergab keine Abtrennung organischer Phase. Eine Zentrifugation von 30 Minuten bei 3220 g führte zu fast vollständiger Separation der wässrigen Phase und teilweiser Abtrennung der organischen Phase. Der Großteil der Biomasse sammelte sich in einer Mulmschicht an der Phasengrenze. Unter dem Mikroskop wies die Emulsion Biomasse stabilisierte Lösungsmitteltöpfchen in einer kontinuierlichen wässrigen Phase auf. Der Einfluss von Temperatur, pH Wert, Ionenstärke und Tensidzugabe auf das Trennverhalten der Emulsion wurde untersucht. Durch Zugabe von NaOH auf einen pH-Wert von 13,5 gefolgt von einer dreistufigen Extraktion (gesamt 1,2 v/v Hexan) konnten 87 % des gewonnen Produkts isoliert werden. Dies stellt eine Halbierung der Lösungsmittelmenge im Vergleich zu früheren Arbeiten dar.

Abstract

The bioreduction of *o*-chloroacetophenone to the *S*-alcohol with *E. coli* provides a case of fast catalyst deactivation by substrate and product. The introduction of a second phase is a common solution to prolong catalyst lifetime and intensify processes in the biotransformation of hydrophobic substrates. Hexane and hexanol were chosen as most suitable second phases amongst the tested solvents in terms of eco-compatibility, extraction capability and handling. In the present case addition of co-solvent improved bioreduction productivity up to 9-fold. Scale-up of the reaction with pH and temperature control was implemented in a 0.5 L stirred tank reactor. Reaction mixtures containing 20 or 50 % v/v co-solvent and 40 g_{CDW}/L formed milk-like emulsions after 24 h. The highest conversion of 97 % was obtained with 20 % v/v hexane in the bioreduction of 300 mM *o*-chloroacetophenone. The biomass was not separable by filtration and no spontaneous separation of organic phases after 24 h of batch settling was observed. Centrifugation (3220 g, 30 min) led to nearly full separation of the aqueous phase but only partial separation of the organic phase. Most biomass was concentrated in a compacted crud phase at the organic-aqueous interphase. Light microscopy of the emulsion showed solvent droplets coated with biomass in an aqueous, continuous phase. Change in temperature, pH, ionic strength and addition of surfactants were investigated as methods to improve phase separation. Addition of NaOH to a pH value of 13.5 and three extraction steps with a total of 1.2 v/v hexane led to an isolated yield of 87 %. Solvent consumption was halved compared to product isolation from an untreated reaction mixture.

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Integration of bioreduction and product isolation: Selection of one co-solvent for *in situ* substrate supply and extractive product isolation

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1. Introduction

Whole cells constitute the preferred form of the biocatalyst when enzyme protection and coenzyme recycling is required. The most often exploited whole cell biotransformation is hence the reduction of a hydrophobic ketone precursor to the corresponding optically pure alcohol (Pollard and Woodley, 2007). Protection of enzymes from high concentrations of substrates and products by the cell wall, leads, however, to a reaction that is slowed down by mass transfer. Compensation of low activities by a high loading of the low-cost catalyst causes severe problems in product isolation. Therefore, the two main bottlenecks, low catalyst activity and solids separation from the liquid product phase, are affected differently by changes in the catalyst loading. Optimization of process conditions for unstable biocatalysts is a challenging task that necessitates integration of biotransformation and product isolation. The reduction of *o*-chloroacetophenone to (*S*)-1-(2-chlorophenyl)ethanol by *Candida tenuis* xylose reductase (*Ct*XR) provides a case of ketone reduction with fast enzyme deactivation by substrate and product. A recombinant *E. coli* co-expressing *Ct*XR and an enzyme for NADH-recycling was used to stabilize the catalyst (Kratzer et al., 2011). However, the whole cell biocatalyst was disintegrated within hours under reaction conditions, which still limited productivity. Further separation of the catalyst-containing, aqueous phase from product and substrate was accomplished by addition of a water immiscible solvent. Full conversion of 300 mM *o*-chloroacetophenone required a catalyst concentration of 50 g_{CDW}/L and 20 % v/v hexane. The high catalyst loading led to an emulsion-like reaction mixture. Three consecutive extraction steps with an excess of organic solvent (in total 2.4 v/v hexane) and phase separation by centrifugation was required to isolate 86 % of the product (Eixelsberger et al., 2013). Process analysis showed that isolation of 20 g (*S*)-1-(2-chlorophenyl)ethanol

consumed approximately 30 % of the total production cost. The problem of emulsion formation is common to whole cell catalyzed reactions. However, research on downstream processing of biocatalytic reactions where emulsion formation requires additional downstream processing steps is currently underrepresented (Brandenbusch et al., 2010; Cuellar et al., 2015; Furtado et al., 2015). In this work one co-solvent for the two-phase bioreduction and the subsequent product extraction was selected. Selection criteria were based on the solvents' chemical and physical properties, extractive capabilities for substrate and product, suitability during biotransformations and downstream processing. Special focus was put on product isolation from the Pickering emulsion-like reaction mixture. Separation behavior of the reaction mixture was investigated during filtration, gravity settling and centrifugation. Phase separation was optimized with regard to variation in pH and temperature, addition of salts, chaotrops or surfactants. Finally a strategy to select one co-solvent for bioreduction and product extraction guided by bioreaction suitability and separation behavior could can be provided.

2. Materials and methods

2.1. Chemicals and strain

Ammonium sulfate, ampicillin, D-xylose, guanidine hydrochloride (guanidine HCl), hexane (≥ 99 %), NAD^+ (≥ 97.5 % pure), Triton X-100, cetyltrimethylammonium chloride (CTABr) were purchased at Roth (Karlsruhe, Germany). Chloramphenicol, dipropylether (≥ 99 %), hexanol (98 %), kanamycin, *o*-chloroacetophenone, polymyxin B sulfate and sodium formate were obtained from Sigma-Aldrich (Vienna, Austria). Celite® 545 (particle size 20 - 45 µm) was from Fluka (Buchs, Switzerland). Sodium dodecyl sulfate (SDS) was purchased at Serva (Heidelberg, Germany). B-Per® Reagent was from Pierce (Rockford, IL, USA) and 1-(2-chlorophenyl)ethanol from Alfa Aesar (Karlsruhe, Germany). All other chemicals were from Sigma–Aldrich/Fluka or Roth, and were of the highest purity available. 15 and 50 mL tubes were purchased at Sarstedt (Wr. Neudorf, Austria); 1.5 mL tubes at Eppendorf (Wien, Austria). The strain used was an *E. coli* Rosetta2 carrying pETDuet_XR_FDH and an additional pRSF-1b plasmid encoding for *Candida boidinii* formate dehydrogenase (Mädje et al., 2012). Cultivation was done in shaken flasks as described earlier (Schmölzer et al., 2012). Bioreactor cultivation was optimized by Eixelsberger et al. (2013) and is summarized in the Supporting Information.

2.2. Partition of substrate and product between buffer and co-solvents

The organic phases used were hexane, hexanol, heptanol, octanol and dipropylether, the aqueous phase was 100 mM potassium phosphate buffer, pH 6.2. Mixtures contained 50 % v/v organic solvent in a total volume of 10 mL. 5 mM of *o*-chloroacetophenone was dissolved in the organic phase and 5 mM 1-(2-chlorophenyl)ethanol in the aqueous phase. The phases were combined, filled into 10

mL glass tubes (screw capped from Pyrex) and mixed at an end-over-end rotator (SB3 from Stuart) at 30 rpm and room temperature. Ketone and alcohol concentrations were measured in both phases after 2 h of stirring to ensure equilibrium conditions. HPLC analysis was performed as described previously (Schmölzer et al., 2012).

2.3. Biotransformations

A 100 mM potassium phosphate buffer, pH 6.2, was supplemented with NAD^+, polymyxin B sulfate and sodium formate prior to suspension of freeze dried cells. The suspension was gently mixed at room temperature on a magnetic stirrer for one hour to allow for cell rehydration and permeabilization. The liquid substrate *o*-chloroacetophenone was pre-dissolved in the co-solvent. The formate concentration in the reaction mixtures always exceeded the *o*-chloroacetophenone concentration by 50 mM.

2.3.1. Eppendorf tube scale.

Reductions were performed in a total volume of 1 mL incubated in 1.5 mL Eppendorf tubes. Experiments were carried out on an end-over-end rotator (SB3 from Stuart) at 30 rpm and room temperature. Phase compositions of bioreductions in 1 mL scale are listed in the Supporting Information Table S4.

2.3.2. Bioreactor scale.

Total reaction volumes were 0.5 L. Reductions were performed in a Labfors (Infors HT) with a working volume of 1 L, a vessel diameter of 11.25 cm, equipped with a twin 6-blade disc turbine with a stirrer diameter of 4.5 cm. pH was controlled by addition of 1 M H_3PO_4 and 2 M KOH, temperature was kept at 25°C (double jacket vessel) for 24 h (Eixelsberger et al., 2013). Phase compositions of bioreductions in 0.5 L scale are

listed in the Supporting Information Table S5. HPLC analysis was performed as described previously (Schmölzer et al., 2012).

2.3.3. Product isolation at bioreactor scale.

The reaction mixture contained 300 mM *o*-chloroacetophenone with 20 % v/v hexane as co-solvent. Further 200 mL of hexane were added and the pH was adjusted to 13.5. The mixture was incubated at 25°C and 500 rpm in the bioreactor for 30 minutes. Centrifugation was done using a Sorvall RC-5B centrifuge for 30 minutes at 9000 rpm (4400 g). The separated organic phase was collected by pipetting. Two further extraction steps were carried out using 200 mL of hexane each time with subsequent stirring and centrifugation. Solvent was removed using a rotary evaporator (Laborota 4000, Heidolph, Schwabach, Germany) at 40°C and 100 mbar.

2.4. Filtration

A Schott DURAN Büchner filtering funnel with grade 4 frit (10-16 µm nominal max. pore size, Supporting Information Figure S1), 50 mL capacity and 3.5 cm disc diameter was used. A vacuum of 10 mbar was applied using a vacuum pump (model MZ 2 CNT, ultimate vacuum 7 mbar, from Vacuubrand, Wertheim, Germany), equipped with a fine vacuum gauge (range 150-10-3 mbar) from ILMVAC (Ilmenau, Germany). For high filter aid loading 6 g Celite were suspended in 50 mL of water to precoat the filter. The suspension was subjected to filtration; the filtrate was collected and five times re-filtered. 10 g of Celite were mixed with 30 mL reaction mixture prior to filtration. For low filter aid loading 4 g Celite were used as precoat, and 2 g were suspended in 30 mL reaction mixture prior to filtration.

2.5. Settling and centrifugation

2.5.1. Settling cell

The settling cell consisted of a glass cylinder with two agitator shafts each equipped with 4 pitch 4-blade impellers and temperature control (double jacket vessel with a total volume of 1L) (Henschke et al., 2002). A mechanical overhead stirrer (Heidolph RZR2021, Schwabach, Germany) with hand wheel for speed adjustment was used. Coaxial mixing with counter rotating mixing elements prevented spinning of the liquid after mixing was stopped. (A photo of the cell (Figure S2) and the cleaning procedure are given in the Supporting Information.) The solvent mixture was filled into the cell and mixed for 5 min to saturate each phase with the other. For the separation experiments liquid components were mixed (800 rpm, 30 s, 30°C) and the time noted until complete phase separation occurred. This was repeated five times for each solvent mixture and the mean values were calculated.

2.5.2. Settling in 50 mL tubes.

For batch settling experiments 50 mL, sterile, polypropylene tubes from Sarstedt (diameter 28 mm, length 115 mm) were used as settling cells. Reaction mixtures were gently mixed and distributed to tubes with 30 mL per each tube. (The increase in aqueous phase caused by the automated pH correction with acid and base during the reaction was compensated by the addition of the respective amount of co-solvent.) After addition of additives (Triton X-100, CTABr, SDS, NaCl, ammonium sulfate, guanidine HCl), adjusting of pH (2, 5, 7, 10, 13.5) tubes were incubated at different temperatures (room temperature, -18°C, 4°C, 30°C) for 24 h. All settling conditions are listed in Table 1. Photos of the incubated tubes were taken at specified time points in order to illustrate phase separation behavior over time. After 24 h the tubes were

subjected to centrifugation at 4000 rpm (3220 g). After 10 min, photos were taken, the tubes were further centrifuged for 20 min and final photos were taken.

2.6. Light microscopy

Samples were investigated under a light microscope equipped with a camera (Leica Microsystems DM LS2 and DM LB2; camera CDF350 FX, Vienna, Austria). 5 µL of each sample were transferred onto an object plate and fixed with a coverglass (both from Carl Roth, Karlsruhe, Germany). Photos were taken with 10-fold and 40-fold enlargement.

3. Results

Water immiscible co-solvents are used to extract the toxic substrate from the catalyst-containing, aqueous phase. The substrate concentration in the aqueous phase remains low and converted substrate is replenished by partition from the co-solvent (Collins et al., 2015). Hydrophobic products are likewise extracted from the aqueous into the organic phase. *o*-Chloroacetophenone and the corresponding alcohol turned out as highly toxic to the whole cells. The presence of the product, 1-(2-chlorophenyl)ethanol, had an especially negative impact on catalyst lifetime (Schmölzer et al., 2012). Therefore a second phase that shows a high affinity to the product was searched.

3.1. Selection of the second phase

Choice of the most appropriate second phase is critical as organic solvents themselves display toxicity towards biocatalysts. The primary effect of solvents is on the cell membrane of microorganisms: hydrophobic molecules partition into cytoplasmic membranes, expand membranes and thereby permeabilize cells (Daugelavičius et al., 2000). Accumulation within membranes depends on the hydrophobicity of the lipophilic compound (log*P* value, logarithmized partition coefficient between *n*-octanol and water) and its structure. Solvent toxicities generally decrease with increasing log*P* values. Previously co-solvents covering a range of log*P* values from 0.7 to 6.8 were tested as second phases in whole cell reductions of *o*-chloroacetophenone (Kratzer et al., 2011; Schmölzer et al., 2012). Hexane (log*P* 3.8) turned out as the most suitable second phase among ethyl acetate (log*P* 0.68), butyl acetate (log*P* 1.7), heptane (log*P* 4.3), dodecane (log*P* 6.8) and the highly hydrophobic ionic liquid BMIMPF$_6$ (Kratzer et al., 2011; Schmölzer et al., 2012). The ideal second phase would provide a sufficient

high substrate concentration, a complete *in situ* removal of the product and low intrinsic bio-toxicity. Hexane, however, displayed higher affinity for the substrate as compared to the product. The objective of the present study was therefore to select a co-solvent that shows differential affinity for *o*-chloroacetophenone and 1-(2-chlorophenyl)ethanol. Co-solvents that resembled on the one hand hexane structurally and on the other hand the product with regard to its hydroxy group were searched. The solvents hexanol (log*P* 1.86), heptanol (log*P* 2.14) and octanol (log*P* 2.92) are built up by an aliphatic alkyl chain and a terminal hydroxy group. Dipropylether has the same amount of carbon atoms as hexane, interrupted by an oxygen atom.

3.2. Partition

Partition of *o*-chloroacetophenone and 1-(2-chlorophenyl)ethanol between the organic phases hexane, hexanol, heptanol, octanol, dipropylether and buffer was determined under equilibrium conditions. Highest *o*-chloroacetophenone and 1-(2-chlorophenyl)ethanol concentrations in the aqueous phase were measured when hexane served as second phase. The lower affinity of hexane for the alcohol was reflected by a 10-fold higher concentration of 1-(2-chlorophenyl)ethanol in the aqueous phase. Partition coefficients ($P_{L_O L_W} = \frac{c_{L_O}}{c_{L_W}}$, Table 2) of ketone and alcohol for hexane were calculated to 87 and 10, respectively. Dipropylether displayed similar affinities towards ketone and alcohol with $P_{L_O L_W}$ values of 270 and 231. Hexanol, heptanol and octanol showed an approximately 2-fold preference for the alcohol with $P_{L_O L_W}$ values of ~500.

3.3. Biotransformation

The co-solvents hexanol, heptanol, octanol and dipropylether were investigated as second phases. Substrate concentrations of 100, 300 and 500 mM were tested with 20, 30, 50 % v/v co-solvent in 1 mL scale experiments. Results were referenced to bioreductions with hexane (as optimized by Schmölzer et al., 2012) and to bioreductions without a second phase. Goal of the study was full conversion (>95 %) of the substrate with a catalyst loading of 40 g_{CDW}/L.

3.3.1. Small scale experiments with 100 mM substrate and 20 % v/v co-solvent.

Co-solvents were compared in whole cell reductions of 100 mM *o*-chloroacetophenone. Conversions of bioreductions with reaction times of 8 or 45 h are shown in Table 3. After 8 h no product was formed in reactions without co-solvent. Presence of 20 % v/v co-solvent increased conversions to 8 - 14 %. An increased reaction time of 45 h led to a conversion of 7 % without a second phase and to conversions of 32 - 52 % with co-solvents. Polymyxin B sulfate has previously shown to accelerate the reaction by increasing mass transfer over the cell membrane (Schmölzer et al., 2012). Catalyst permeabilization by prior incubation of cells with the cell perforating antibiotic Polymyxin B sulfate boosted conversions to 74 - 95 % after only 8 h reaction time. Prolonged reaction time of 45 h led to conversions of ≥ 93 % with all second phases. The aliphatic alcohols tested showed highly similar behavior (Table 3). The 19 and 38°C higher boiling points of heptanol and octanol as compared to hexanol (157°C) led to exclusion of heptanol and octanol in further experiments. Cell permeabilization by Polymyxin B sulfate was used in all further experiments.

3.3.2. Effect of increased substrate concentration at 20 % v/v co-solvent in small scale experiments.

The use of 300 and 500 mM substrate led to higher absolute product concentrations as compared to 100 mM substrate (Table 4). Conversions were 79 - 95 % for 300 mM substrate and 55 - 64 % for 500 mM. An increase in reaction time from 24 to 45 h did not increase conversions. Low conversions at high substrate concentrations suggest deactivation of the whole cell catalyst. Experiments with higher amounts of co-solvent were carried out to decrease substrate and product concentrations in the aqueous phase.

3.3.3. Effect of increased co-solvent amount in small scale experiments.

Use of 30 and 50 % v/v co-solvent with substrate concentrations of 300 and 500 mM decreased conversions significantly (Table 4). Hexanol and dipropylether showed in general similar results compared to hexane. Hexane and hexanol were further used for large scale experiments (0.5 L) while dipropylether was excluded due to its tendency to form explosive organic peroxides over storage. A reaction time of 24 h was chosen for the bioreactor scale experiments.

3.3.4. Bioreactor scale experiments.

Heterogeneous reaction mixtures obtained from 1 mL scale bioreductions did not provide enough material to retrieve representative samples of the aqueous and organic phase during the reaction. Therefore a stirred tank reactor with the additional benefit of pH control was used. The obtained reaction mixtures were used in follow-up experiments to study separation behavior and optimize product isolation. 20 and 50 % v/v hexane and hexanol were tested as second phases in bioreductions of 300 mM *o*-chloroacetophenone (Table 5). Reaction mixtures with 50 % v/v co-solvent were prepared despite lower conversions because phase separation is more easily detected

in 50/50 as compared to 20/80 mixtures. A conversion of 97 % obtained with 20 % v/v hexane was consistent with small scale experiments and previous results (Table 5; Figure 1A; Schmölzer et al., 2012; Eixelsberger et al., 2013). Analysis of the aqueous phase showed a substrate concentration starting from 0.05 mM at the beginning of the reaction and increasing to 0.5 mM after 24 h. Product concentration was 8 mM after one hour and reached a stable concentration of 10 mM after 3 hours (Figure 1A). Bioreductions with 20 % v/v hexanol led to a conversion of 71 % (Table 5; Figure 1B). The substrate concentration in the aqueous phase was 0.02 mM in the beginning and 0.2 mM after 24 h (Figure 1B). The product concentration in the aqueous phase was 11 mM after one hour and steadily decreased to 4 mM after 24 h. Substrate concentrations in the aqueous phase were 2.5-times lower when hexanol was used instead of hexane. Initial rates of product formation were calculated to 70 and 50 mM/h for reactions with hexane and hexanol, respectively (0-3 h, Figure 1A&1B). Use of 50 % v/v co-solvent led to 29 and 17 % lower conversion for hexane and hexanol, respectively, as compared to reductions with 20 % v/v co-solvent. After 24 h the reaction mixture was a homogenous, viscous, milk-like emulsion. A reaction mixture with 50 % v/v hexane but without substrate was treated exactly as a bioreduction to study effects of substrate and product on emulsion properties. Filtration of the biomass, batch settling and centrifugation was investigated in order to reduce the amount of extraction solvent as reported by Eixelsberger et al., 2013.

3.4. Filtration

Filtration was previously used to separate cells from whole cell bioreduction mixtures (Gröger et al., 2006). The filter aid Celite (Kieselghur) was applied as a precoat to protect the filter and as an additive to the reaction mixture to increase cake permeability. The reaction mixture containing 20 % v/v hexane was used in filtration

experiments (Supporting Information, Figure S1). The ~10 and 5 mL of filtrate obtained with high and low filter aid loading, respectively, consisted solely of the aqueous phase. After 2 - 3 min the filter blocked in both experiments and filtration stopped. Fast filter blocking due to a high biomass concentration excluded filtration as a method to separate phases.

3.5. Phase separation in batch settling and centrifugation

Sedimentation and coalescence of the reaction mixture in comparison to the plain buffer/solvent mixture were investigated in batch settling experiments.

3.5.1. Settling cell.

Phase separation times for the co-solvent water systems (phase ratios 20/80) with hexane, hexanol, heptanol and octanol were between 23 and 41 s (Supporting Information, Figure S2, Table S6, Henschke et al., 2002). The reaction mixture (20 % v/v hexane), however, showed only minor changes during settling in the first hour. Long separation times of reaction mixtures containing biomass rendered utilization of a settling cell that was constructed for fast separation, excessive. For this reason transparent 50 mL tubes without internal mixing for settling experiments of reaction mixtures were used in all further experiments.

3.5.2. Batch settling in 50 mL tubes.

Settling behavior of the obtained emulsions was investigated focusing on the separation of the product-containing, organic phase. Main factors determining the emulsion stability are the presence of surfactants or solid particles at the interface, surface charges of droplets and particles and viscosities of the continuous phases (Tadros, 2013). General methods to break emulsions are change in temperature or pH, increase in ionic strength and addition of surfactants. Higher temperatures tend to

destabilize emulsions by lowering the viscosities of the liquid phases (Chen and Tao, 2005; Rayner et al. 2014). The effect of temperature on phase separation was investigated by incubating reaction mixtures at -18°C, 4°C, 22°C and 37°C. High volatility of hexane prevents the use of higher temperatures during settling. Displacement of cells or surface active chemicals from the interface by surfactants was investigated by the addition of nonionic Triton X-100, anionic SDS or cationic CTABr. Surface charges play a key role in emulsion-stability; pH and ionic strength of the aqueous phase are hence main factors determining emulsion stability (Li and Stöver, 2008). The effect of 1 M NaCl, 2 M NaCl, 1 M guanidine hydrochloride, 0.8 M $(NH_4)_3SO_4$ on separation behavior was investigated. Chaotropic salts (guanidine hydrochloride, $(NH_4)_3SO_4$) might have the additional effect of aggregating extracellular proteins that otherwise could act as emulsion stabilizing surfactant. (All settling conditions are listed in Table 1.) Tubes were incubated and photos were taken over time to visually assess separation behavior.

3.5.3. Batch settling of reaction mixture containing 50 % v/v hexane.

Gravity settling of the untreated reaction mixture (22°C) led to the formation of an aqueous bottom phase after ~2 h that gradually increased to 28 % v/v (of the total reaction volume) after 24 h. No organic phase formation was observed within 24 h (Figure 2A). Centrifugation at 3220 g for 30 min led to 45 % v/v aqueous phase, 17 % crud phase and 5 % pellet at a constant volume of the organic phase (Figure 2A). Variations in pH (2, 5, 7, 10) or temperature (-18°C, 4°C, 37°C), addition of NaCl, ammonium sulfate, guanidine-HCl, SDS, CTABr or Triton X-100 did not result in the separation of a hexane phase by gravity settling. Only NaOH addition to a pH of 13.5 led to the spontaneous separation of an organic phase after 30 min that increased to 23 % v/v after 24 h. Subsequent centrifugation improved hexane separation to 36 %

v/v (Figure 3A; time course of phase separation is shown in Figure 3B). Centrifugation after freezing at -18°C for 24 h resulted in an organic phase separation of 37 % v/v. All further conditions showed formation of an organic phase after centrifugation that was below 36 % v/v. An especially low volume of the separated organic phase was seen in the tubes containing guanidine HCl (Figure 2A). The reaction mixture without substrate was furthermore investigated (Figure 2B, Figure 3C). No hexane phase formed under any condition during 24 h of gravity settling. After additional centrifugation for 30 min best organic phase separation (33 % v/v) was obtained for samples that were incubated at 37°C. Especially low or even no organic phase was obtained in settling and centrifugation experiments of reaction mixtures containing NaCl, guanidine HCl and ammonium sulfate. Interestingly, formation of a second, gelatinous, crud phase was observed under most conditions (Figure 3C) except for reaction mixtures that were adjusted to pH 2 (Figure 2B).

3.5.4. Batch settling of reaction mixtures containing 20 % v/v hexane.

Phase separation behavior of the reaction mixture containing 20 % v/v hexane was analyzed at 22°C, at pH 13.5 and at 37°C and pH 13.5. No organic phase separated during gravity settling for 24 h. After 30 min of centrifugation 12 % v/v of hexane separated from the reaction mixture (22°C) and 15 % from the reaction mixture that was adjusted to pH 13.5. These values reflect approximately the lower phase ratio of hexane. No additive effect was seen when the reaction mixture was incubated at 37°C and pH 13.5.

3.5.5. Batch settling of reaction mixtures containing hexanol.

No separation of hexanol was obtained during gravity settling of reaction mixtures containing 50 % v/v hexanol (Figure 2D). Centrifugation (30 min) of the untreated reaction mixture improved separation to 33 % v/v organic solvent. 40 % v/v of organic

phase separated in the reaction mixture containing SDS. All other conditions showed less or no separation. Especially low or even no organic phase was obtained in settling and centrifugation experiments of reaction mixtures containing hexanol and salts as additives. A maximum amount of 3 % v/v organic phase formed upon centrifugation when 20 % v/v hexanol was used as co-solvent (data not shown).

3.6. Bioreactor scale bioreduction and product extraction

20 % v/v hexane and 300 mM *o*-chloroacetophenone were used in a bioreduction with subsequent product extraction. A conversion of 97 % was reached after 24 h. Addition of base to a pH of 13.5 prior to product extraction led to an isolated yield of 87 % after three extraction steps with 1.2 v/v hexane.

4. Discussion

4.1. Solvent selection

Our strategy to select a co-solvent for bioreduction and subsequent product extraction followed the criteria listed in Table 6. Starting from a list of the most commonly used water immiscible co-solvents non-toxic solvents with low explosion risk that do not react with substrate or product under reaction conditions were searched. Boiling points below 150°C were preferred. Previous studies have shown that co-solvents with log*P* values ≤1.7 were not suitable as co-solvents for the investigated whole cell bioreduction (Kratzer et al., 2011) and best results were obtained with hexane.

4.2. Partition and bioreductions

Partition of 1-(2-chlorophenyl)ethanol and *o*-chloroacetophenone between buffer and organic solvents showed that 1-(2-chlorophenyl)ethanol extraction was favored with all new solvents as compared to hexane. The aliphatic alcohols even showed a two-fold preference for the product 1-(2-chlorophenyl)ethanol. However, also the *o*-chloroacetophenone concentration in the aqueous phase was lower when hexanol, heptanol, octanol or dipropylether was used (Table 2). The concentration of the product found in the aqueous phases was, despite differing partition coefficients for hexanol or hexane and buffer, relatively similar for these co-solvents during the reaction (Figure 1) suggesting that the biomass influenced the equilibrium and/or slowed down mass transfer between phases. Low substrate concentrations found in the aqueous phases suggest furthermore a substrate limitation in the initial phase of the reaction (0 - 3h). Substrate concentrations in the aqueous phases measured after 24 h (i.e. at low reaction rates, Figure 1) were lower than expected. The toxicity of organic solvents on whole cells decreases as their log*P* value increases (de Bont, 1998). It is therefore

interesting that bioreductions with hexanol and hexane showed similar results despite their corresponding log*P* values are 1.8 and 3.8, respectively. Hornby and Cullis (1981) previously found that *n*-alkans, compared to the corresponding *n*-alkanols, were more potent in destabilizing lipid bilayer structures due to their higher similarity to phospholipids that build up membranes (Weber and de Bont, 1996). This might compensate for the lower log*P* values of the used alkanols.

4.2.1. Recoveries.

Total recoveries of substrate and product in small scale bioreductions were between 72 and 86 % per measuring series (Table 3, Table 4). Inaccurate mass balances might stem from handling problems of small reaction volumes, evaporation of the organic solvent during the reaction and/or analytes that remain in the cell sludge despite dissolution of the reaction mixture into a 10-fold excess of ethanol (see Materials and methods section). The notice that higher recoveries were obtained with higher substrate concentrations, longer reaction times and additional cell permeabilization (Table 3, Table 4) suggests improved analyte dissolution from the biomass after harsh reaction conditions. Similarly, recoveries increased over time in 0.5 L scale bioreductions with 20 % v/v hexane or hexanol from ~90 % after 1 h to 95 % after 24 h (data not shown).

4.3. Phase separation

The enormous increase in phase separation time between plain buffer/organic solvent mixtures and reaction mixtures was ascribed to the presence of biomass. Emulsions that are stabilized by microorganisms have been previously classified as Pickering-like emulsions (e.g Dorobantu et al., 2004). The classical Pickering emulsion represents an oil-in-water emulsion stabilized by solid particles adsorbed to the oil droplet surface

(Figure 4A). The formation of a Pickering emulsion depends mainly on the ability of the solid particle to form adhesive forces to both phases (intermediate wettability) (Pickering, 1907). The phase that shows higher affinity to the particle is the continuous phase (Chevalier and Bolzinger, 2013). Light microscope images of reaction mixtures showed hexane droplets surrounded by continuous water phases containing biomass (Figure 5A).

4.3.1. Hexane versus hexanol.

The separation of an organic phase AND a cell pellet during centrifugation of reaction mixtures suggests a relatively low affinity of the cells towards the organic phases (Figure 2). Comparison of reaction mixtures showed that the presence of hexane further promotes phase separation compared to reaction mixtures containing hexanol. Centrifugation led to the separation of a hexane phase under all reaction conditions (Figure 2A, 2C); with hexanol an organic phase was only obtained under certain conditions with generally less pelleted cells (Figure 2D). Adhesive forces between biomass and organic phase are hence stronger with the less hydrophobic hexanol compared to hexane as indicated in Figure 4B and 4C.

4.3.2. Effect of substrate and product.

The effect of substrate and product was studied by comparing the settling behavior of the reaction mixture containing 50 % v/v hexane to a control experiment lacking substrate. The absence of substrate and product caused the formation of a further, translucent and gelatinous, phase situated above the crud phase (Figure 3C). The additional phase captured between 3.5 and 100 % of the hexane phase and was found under all conditions except pH 2 (Figure 2B). The appearance of the phase suggested that it consists of partially precipitated proteins that were entirely precipitated in the presence of substrate and product.

4.3.3. Co-solvent phase ratio.

Reduction of hexane from 50 to 20 % v/v led to 5 - 6 % v/v less crud phase at a comparable ratio of separated hexane. Reaction mixtures with hexanol showed organic phase separation primarily at a 50/50 phase ratio (Figure 2D). Interestingly, these reaction mixtures tended to form bicontinuous structures as seen in the light microscope image in Figure 5B. The ability of reaction mixtures with hexanol to form bicontinuous structures is again explicable by the high affinity of hexanol to the biomass which was further favored by the 50/50 phase ratio (Tavacoli et al., 2015).

4.3.4. Effect of pH and salt.

The reaction mixture with 50 % v/v hexane and the pH value adjusted after the reaction to 13.5 was the only settling condition that showed spontaneous separation of the organic phase (Figure 2A). Interestingly, reaction mixtures containing 50 % v/v hexanol showed better separation without pH adjustment. Therefore, a positive effect by mere cell disruption at extreme pH values was excluded. Compaction of the crud phase of Pickering emulsions by added salt due to a weakened electrostatic repulsive force has been previously described (Chevalier and Bolzinger, 2013) but was not seen in the present study. An especially negative effect was observed when hexanol served as a second phase (Figure 2D). The opposite effect of salt (including adjustment of different pH values) on separation behavior is explicable by differing hydrophobicities of hexane and hexanol. Adsorption of *E. coli* cells at the organic solvent-water interface requires partial wetting of the biomass by the two liquids i.e. adsorption of the *E. coli* cells to buffer and the organic solvents. High emulsion stability is obtained when the solid particles show intermediate wettability (Hunter et al., 2008). When hexane is used as a second phase, the *E. coli* shows much higher affinity to the buffer and the resulting emulsion is less stable compared to hexanol. Furthermore, the clear preference of the

E .coli cells leads to a relatively stable system i.e. perpetuations in ionic strength do not alter the separation behavior significantly (Figure 4B). The pronounced negative effect of salt addition on the separation of hexanol is explicable by the relatively high affinity of *E. coli* cells to hexanol. Addition of salt and the accompanied increase in ionic strength further enhances the affinity of *E. coli* towards hexanol previously called 'salting out' effect (Lindahl et al., 1981) (Figure 4C).

4.3.5. Effect of surfactants.

Addition of Triton X-100, CTABr and SDS had no or negative effect on phase separation when hexane was used as second phase during the biotransformation. Remarkably, addition of SDS led to separation of 80 % of organic phase (40 % v/v of total volume) when 50 % v/v hexanol was used as second phase, which was the highest amount of organic phase obtained after centrifugation in this study. SDS seems to increase the already (predominantly) negative surface charge of bacteria. The increased cell hydrophilicity and thereby higher affinity to the buffer phase might have led to the observed improved organic phase separation (Figure 4C).

4.3.6. Effect of settling temperature.

An increase in temperature from 22°C to 37°C in settling experiments improved organic phase separation from 21 to 33 % v/v in the control experiment using 50 % v/v hexane but lacking substrate. The reaction mixture with 50 % v/v hexane showed highest organic phase separation after freezing at -18 °C for 24 h. A general effect that higher temperatures favor droplet collision and coalescence (Chen and Tao, 2005) was not observed.

We conclude a general emulsion destabilizing effect under harsh conditions leading to cell disintegration (extreme pH values, toxic substrates and products) as previously reported by Collins et al. (2015).

5. Conclusion

Hexanol, heptanol, octanol and dipropylether showed higher affinity to (*S*)-1-(2-chlorophenyl)ethanol and *o*-chloroacetophenone compared to previously used hexane (Table 2). Actual alcohol and ketone concentrations in the biocatalyst containing aqueous phase were hence lower when hexanol was used as second solvent instead of hexane (Figure 1). Low substrate concentrations with hexanol as second phase lowered reduction yields. An increase in substrate concentration did not lead to higher conversion (Table 4). Product solutions were classified as oil-in-water emulsions stabilized by *E. coli* cells that were adsorbed to the aqueous-oil interface (Figure 5). Filtration of the Pickering emulsion failed due to immediate filter blocking (Supporting Information, Figure S1). The presence of biomass led to an enormous increase in separation time during batch settling. No spontaneous separation of the organic phase from the untreated reaction mixture was observed when hexane or hexanol served as second phases. Changes in temperature, pH, ionic strength and addition of surfactants were investigated as methods to improve phase separation. Adjustment of the reaction mixture containing 50 % v/v hexane to pH 13.5 was the only condition resulting in spontaneous separation of organic phase. During centrifugation hexane was superior in terms of organic phase separation and crud compaction under most conditions. Only addition of SDS led to a higher recovery of hexanol after centrifugation. In general, reaction mixtures with hexanol showed more stable emulsions. Our results suggested a concentration of 20 % v/v hexane during the bioreduction of 300 mM *o*-chloroacetophenone and addition of base to a pH value of 13.5 after the reaction and repeated extraction using low v/v ratios of hexane. A conversion of 97 % in the bioreduction with 87 % of isolated yield was reached. The previously used amount of extractive solvent was halved at constant isolated yield (Eixelsberger et al., 2013). Harsh reaction conditions that deactivate the *E. coli* cells (Schmölzer et al., 2012) and repeated extraction render biocatalyst recycling infeasible.

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8. Tables

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Table 1. Reaction mixtures from 0.5 L scale were distributed to settling tubes to test the effect of temperature, pH, salts and surfactants on settling behavior. Conditions and concentrations of settling experiments are listed.

		Hexane			Hexanol	
Parameter	Conditions	Reaction w. 50 % v/v	Reaction w. 20 % v/v	Blank 50 % v/v	Reaction w. 50 % v/v	Reaction w. 20 % v/v
Temperature	22°C	x	x	x	x	x
	4°C	x		x	x	
	-18°C	x		x	x	x (pH 13.5)
	37°C	x	x (pH 13.5)	x	x	x (pH 6.2, 13.5)
pH	7	x		x	x	
	5	x		x	x	
	2	x		x	x	
	10	x		x	x	
	13.5	x	x	x	x	x
Salts	NaCl 1 M	x		x	x	
	NaCl 2 M	x		x	x	
	Guanidine HCl 1 M	x		x	x	
	$(NH_4)_3SO_4$ 0.8 M	x		x	x	
Surfactants	Triton X-100 0.2 mM	x		x	x	x
	CTABr 0.9 mM	x		x	x	
	SDS 8.2 mM	x		x	x	x

Table 2. Partition coefficients $P_{L_O L_W}$ of o-chloroacetophenone and 1-(2-chlorophenyl)ethanol between various solvents and buffer.

	o-chloroacetophenone $P_{L_O L_W}$		1-(2-chlorophenyl)ethanol $P_{L_O L_W}$	
System	Plain buffer/solvent	Reaction mixture[1]	Plain buffer/solvent	Reaction mixture[1]
Hexane	87	135	9.7	109
Hexanol	277	1695	494	250
Heptanol	233	n.d.	499	n.d.
Octanol	210	n.d.	509	n.d.
Dipropylether	270	n.d.	231	n.d.

[1]After a reaction time of 24 h. Mass balances for plain buffer/solvent mixtures showed recoveries of 99.9 ± 9.6%. n.d. not determined.

Table 3. Effect of polymyxin B sulfate[1] and reaction time on bioreductions[2] of 100 mM o-chloroacetophenone using 20 % v/v co-solvents. Results in % conversion.

Polymyxin B sulfate	No[3]		36 µM[4]	
Reaction time	8 h	45 h	8 h	45 h
No second phase	0	7	74	52
Hexane 20 % v/v	8	47	95	93
Hexanol 20 % v/v	9	35	90	99
Dipropylether 20 % v/v	14	32	88	98
Heptanol 20 % v/v	13	52	86	100
Octanol 20 % v/v	13	51	91	100
Recoveries (Average ± Stdev)	72 ± 4	79 ± 8	80 ± 4	78 ± 2

[1]With 36 µM polymyxin B sulfate (concentration referred to total reaction volume).
[2]1 mL scale.
[3]Experiments were done in duplicate.
[4]Experiments were done in quadruplicate.

Table 4. Bioreductions[1,2] of o-chloroacetophenone with 20, 30 and 50 % v/v co-solvents. Results in % conversion[3].

Co-solvent	(% v/v)	Substrate 300 mM		Substrate 500 mM	
		24h	45h	24h	45h
None	-	11	14	11	8
Hexane	20	95	96	64	63
	30	81	77	46	25
	50	39	35	17	20
Hexanol	20	79	81	56	50
	30	69	71	30	26
	50	36	30	14	12
Dipropylether	20	85	79	55	55
	30	67	67	32	29
	50	29	26	14	7

[1]With 36 µM polymyxin B sulfate (concentration referred to total reaction volume). [2]1 mL scale. [3]Experiments were done in duplicates, recoveries are 86 ± 5 %.

Table 5. Comparison of co-solvents in bioreductions[1,2] of 300 mM o-chloroacetophenone using phase ratios of 20/80 and 50/50 (co-solvent/buffer). Results in % conversion.

Co-solvent	(% v/v)	*o*-chloroacetophenone (mM)	Conversion (%)
Hexane	20	300	97
	50	300	68
Hexanol	20	300	71
	50	300	54
Hexane	50	0	No reaction

[1]With 36 µM polymyxin B sulfate (concentration referred to total reaction volume); reaction time was 24 h.
[2]Bioreactor scale.

Table 6. Strategy for co-solvent selection in whole cell biocatalysis with integration of biotransformation and product isolation (hydrophobic substrate and product is assumed).

Criteria	Characteristic
Safety	Non-toxic Low explosion risk
Reactivity	No reaction with substrate or product
Boiling point	<150°C, (preferable <100°C)
Hydrophobicity	Low water solubility (log*P* >1.7)
Extraction capability	Affinity for substrate and product
Bioreaction suitability	Conversion in 2-phasic bioreaction
Phase separation	Settling behavior of organic phase

9. Figures

Figure 1 Whole cell reduction of *o*-chloroacetophenone in reaction mixtures containing 20 % v/v hexane (A) or 20 % v/v hexanol (B). Time course of conversions (solid black lines); product (grey bars) and substrate (black bars) concentrations in the aqueous phase are shown……43

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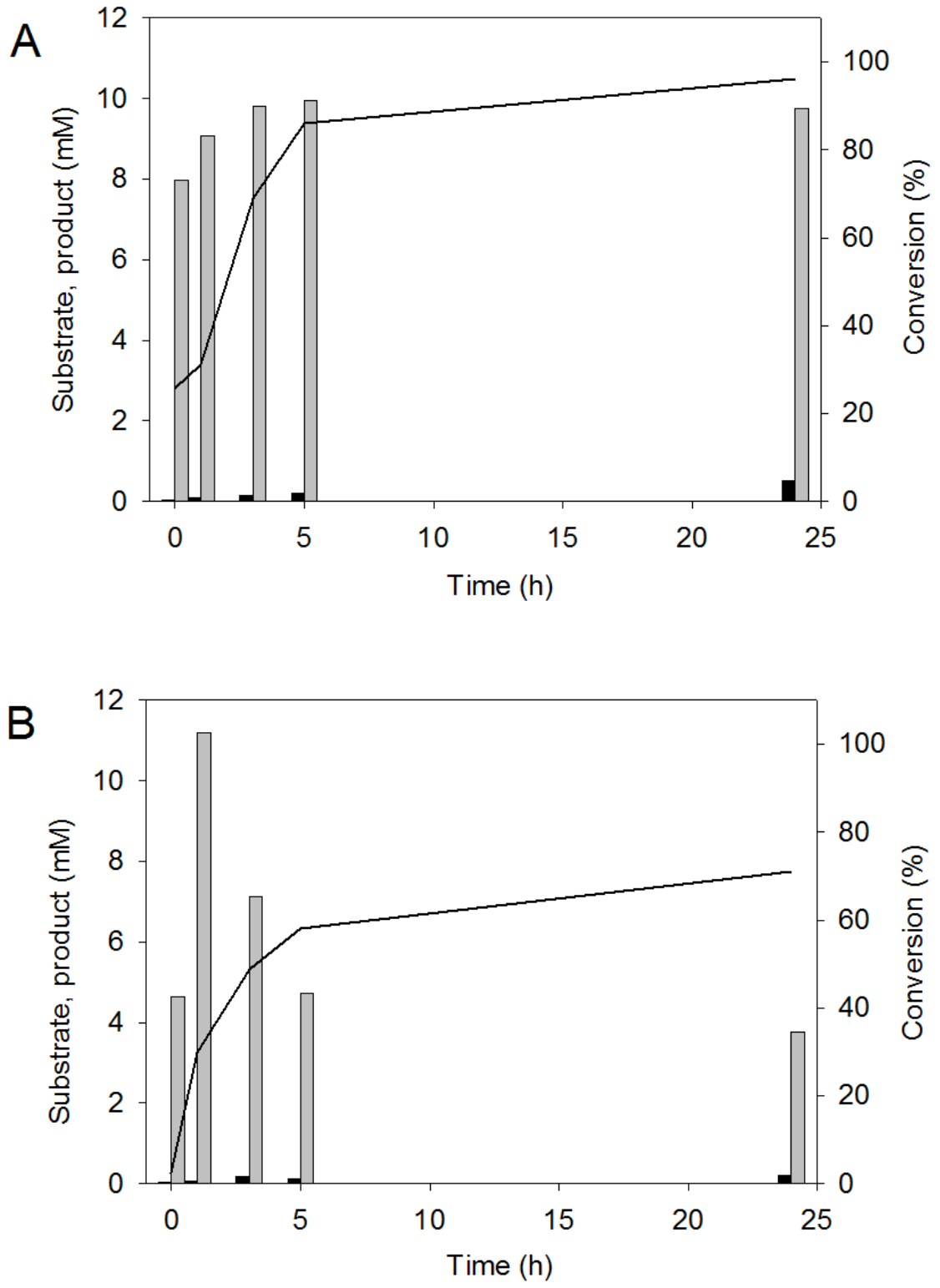
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Conversion (%)
Time (h)
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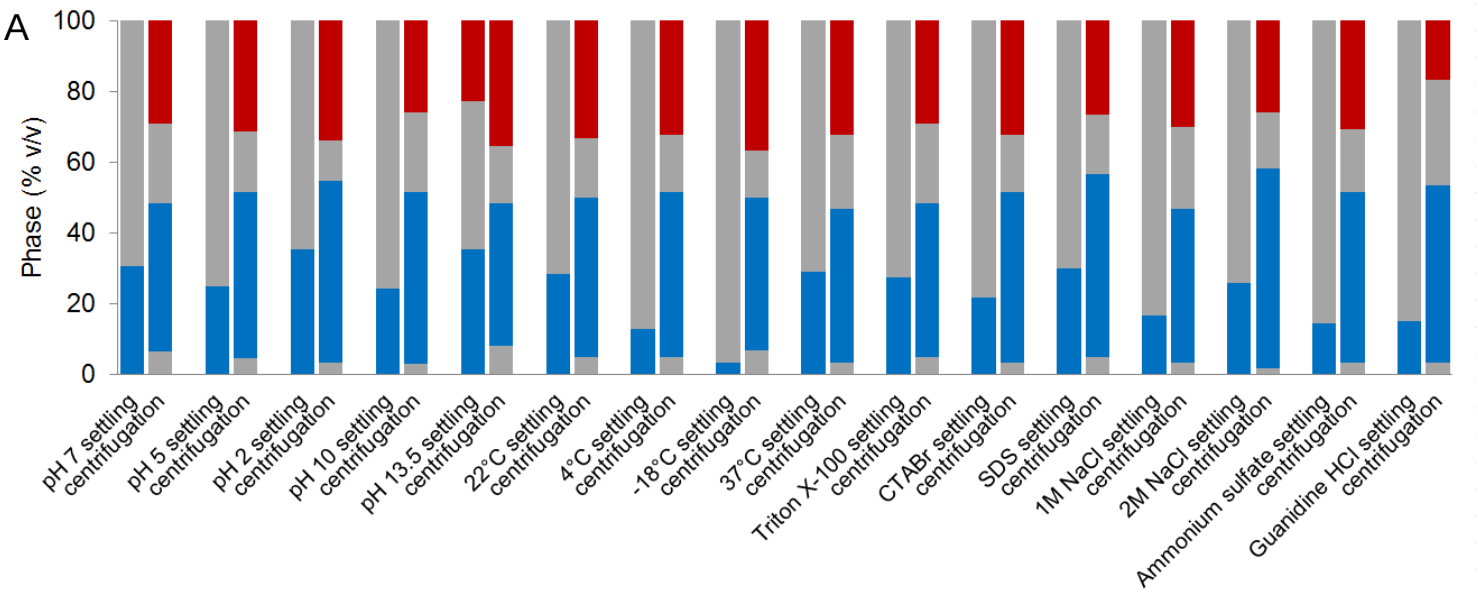
2
4
6
8
10
12
0
20
40
60
80
100
0
5
10
15
20
25
B
Substrate, product (mM)
Conversion (%)
Time (h)

Figure 1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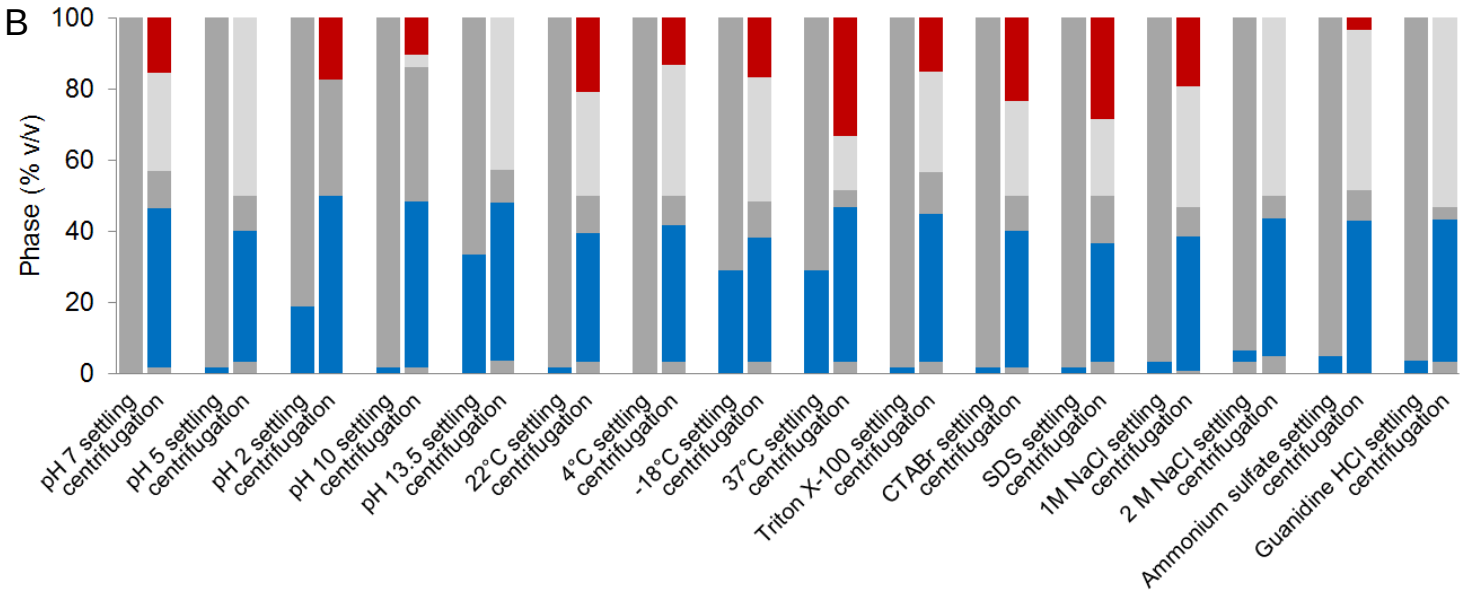
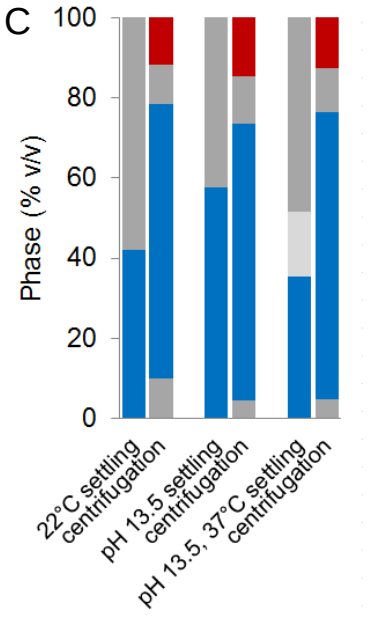
pH 5 settling centrifugation
pH 2 settling centrifugation
pH 10 settling centrifugation
pH 13.5 settling centrifugation
22°C settling centrifugation
4°C settling centrifugation
-18°C settling centrifugation
37°C settling centrifugation
Triton X-100 settling centrifugation
CTABr settling centrifugation
SDS settling centrifugation
1M NaCl settling centrifugation
2M NaCl settling centrifugation
Ammonium sulfate settling centrifugation
Guanidine HCl settling centrifugation


B
Phase (% v/v)
100
80
60
40
20
0
pH 7 settling centrifugation
pH 5 settling centrifugation
pH 2 settling centrifugation
pH 10 settling centrifugation
pH 13.5 settling centrifugation
22°C settling centrifugation
4°C settling centrifugation
-18°C settling centrifugation
37°C settling centrifugation
Triton X-100 settling centrifugation
CTABr settling centrifugation
SDS settling centrifugation
1M NaCl settling centrifugation
2 M NaCl settling centrifugation
Ammonium sulfate settling centrifugation
Guanidine HCl settling centrifugation


C
Phase (% v/v)
100
80
60
40
20
0
22°C settling centrifugation
pH 13.5 settling centrifugation
pH 13.5, 37°C settling centrifugation

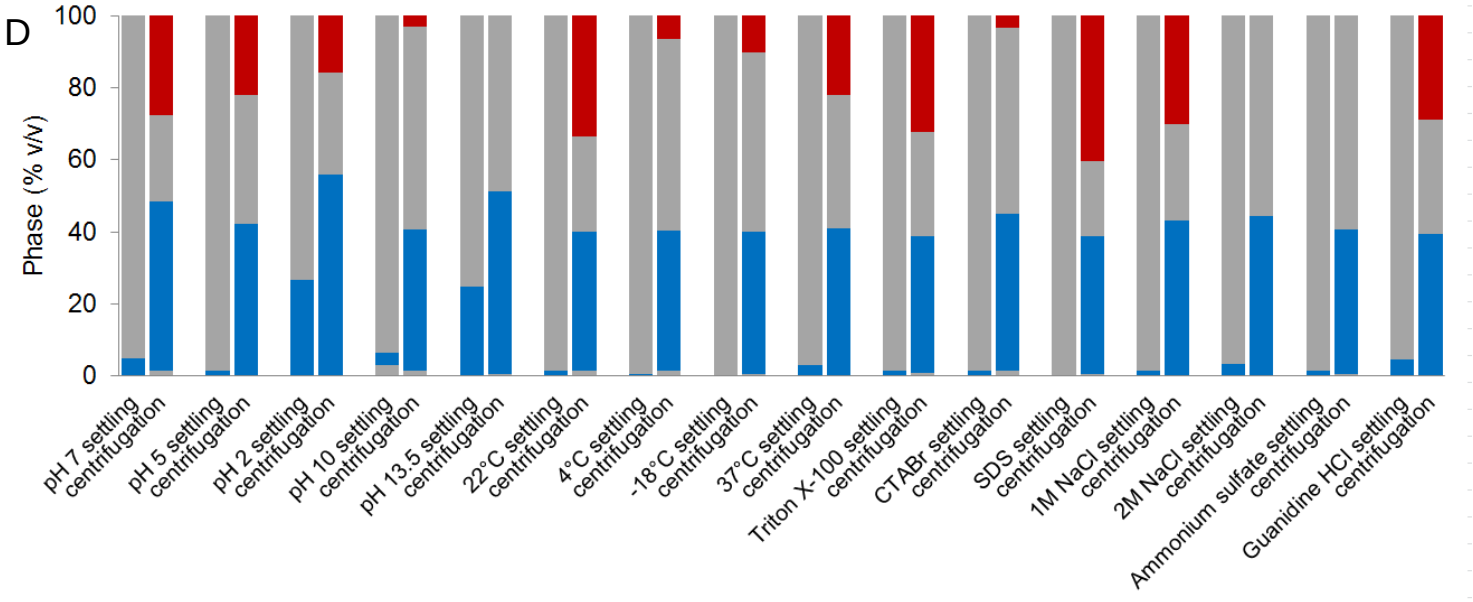

D
Phase (% v/v)
100
80
60
40
20
0
pH 7 settling centrifugation
pH 5 settling centrifugation
pH 2 settling centrifugation
pH 10 settling centrifugation
pH 13.5 settling centrifugation
22°C settling centrifugation
4°C settling centrifugation
-18°C settling centrifugation
37°C settling centrifugation
Triton X-100 settling centrifugation
CTABr settling centrifugation
SDS settling centrifugation
1M NaCl settling centrifugation
2M NaCl settling centrifugation
Ammonium sulfate settling centrifugation
Guanidine HCl settling centrifugation

Figure 2

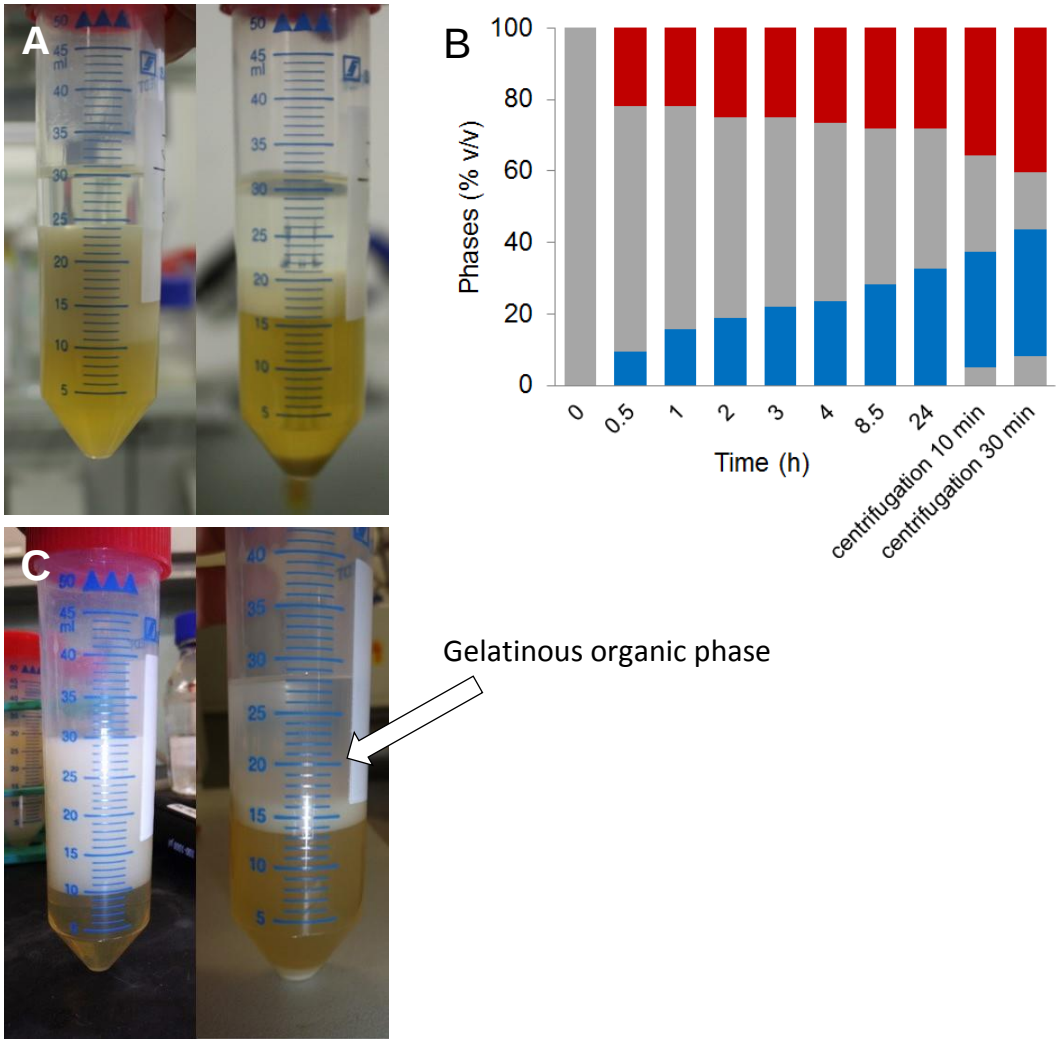

A
B
Phases (% v/v)
100
80
60
40
20
0
0
0.5
1
2
3
4
8.5
24
centrifugation 10 min
centrifugation 30 min
Time (h)
C
Gelatinous organic phase

Figure 3

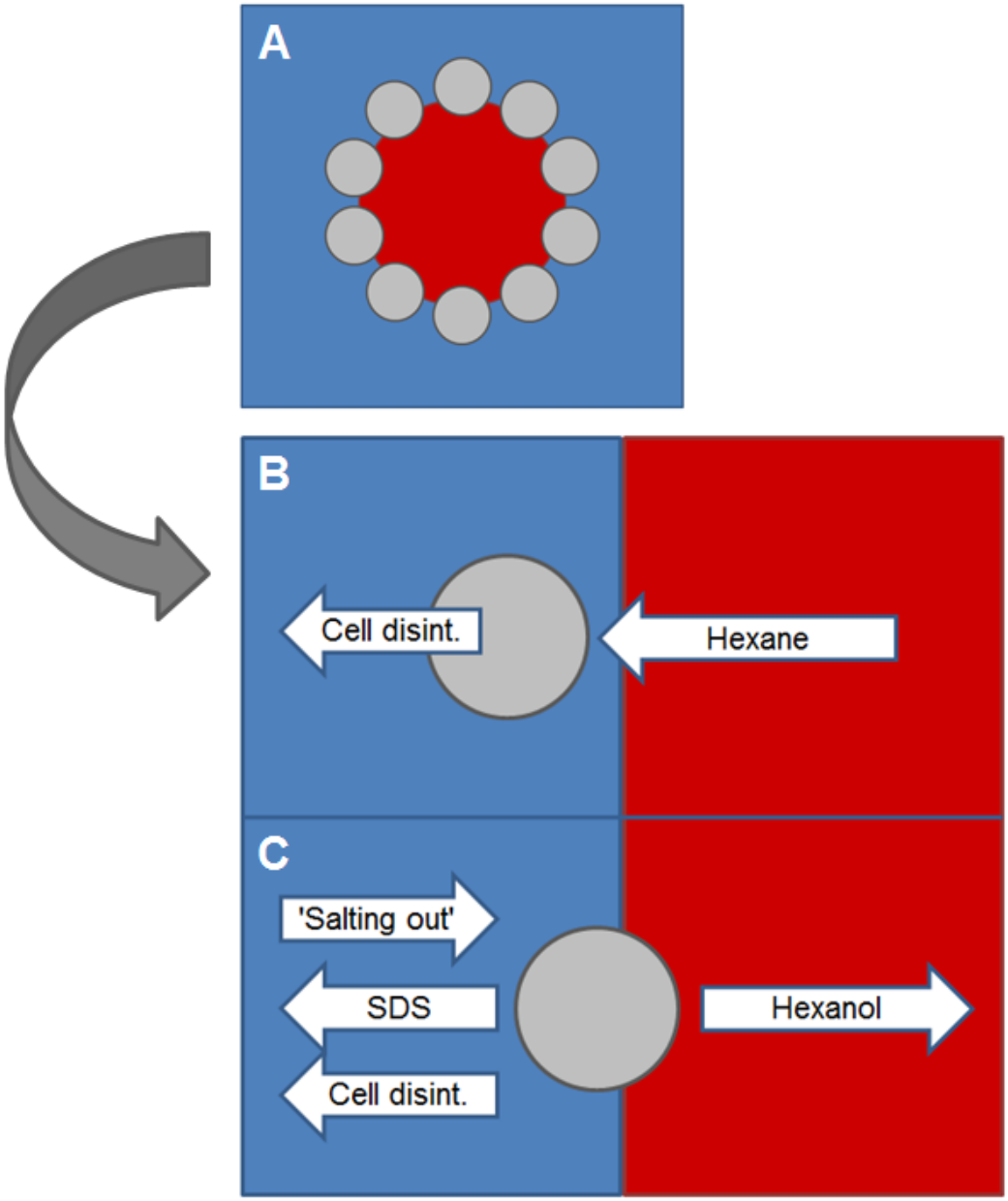


Figure 4

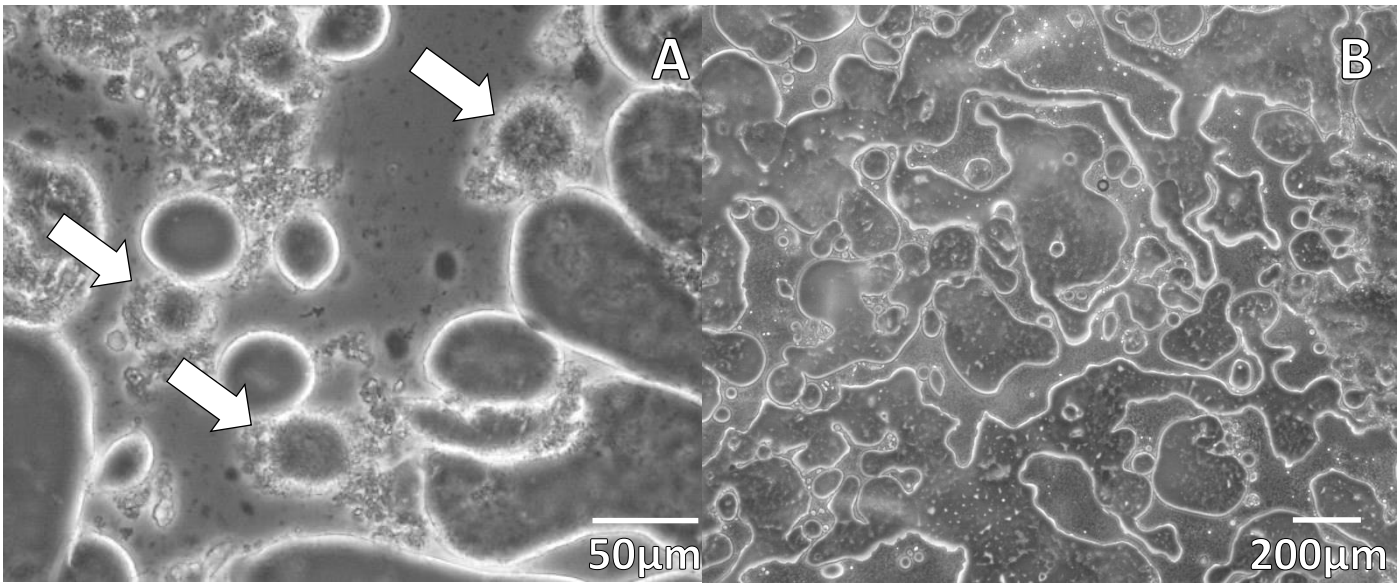


Figure 5

10. Supporting Information

1. *E. coli* cultivation

Table S1. Preculture growth medium.

Table S2. Bioreactor growth medium.

Table S3. Trace element solution.

Bioreactor cultivation of whole cell catalyst.

2. Biotransformation

Table S4. Bioreduction conditions for small scale experiments.

Table S5. Bioreduction conditions for large scale experiments.

3. Filtration

Figure S1. Visual documentation of filtration experiments. High filter aid loading (A), low filter aid loading (B), blocking and foaming during filtration (C).

4. Settling experiments

Figure S2. Settling cell.

Cleaning procedure.

Table S6. Coalescence times for aqueous and organic liquid mixtures without biomass.

Table S1. Preculture growth medium.

Part	Component	Concentration (g/L)
A	Glucose·H_2O	5.5
B	Peptone from casein	10
	Yeast extract	5.0
	NaCl	5.0
	NH_4Cl	1.0
	$MgSO_4$·7 H_2O	0.25
	Trace element solution	1.0*
	Polypropylene glycol	0.1*
C	K_2HPO_4	3.0
	KH_2PO_4	6.0

*mL/L

Table S2. Bioreactor growth medium.

Part	Component	Concentration (g/L)
A	K_2HPO_4	4.0
	KH_2PO_4	4.0
	$Na_3C_6H_5O_7$·$2H_2O$	2.67
	Na_2HPO_4	2.8
	Polypropylene glycol	0.1*
B	$(NH_4)_2SO_4$	3.5
	NH_4Cl	3.0
	$MgCl_2$·$6H_2O$	0.83
C	$CaCl_2$·$2H_2O$	0.15
D	Glucose·H_2O	20
E	Trace element solution	1.0*

*mL/L

Table S3. Trace element solution.

Component*	Concentration (g/L)
$FeSO_4$·$7H_2O$	4
$MnSO_4$·H_2O	1
$AlCl_3$·$6H_2O$	0.55
$CoCl_2$	0.4
H_3BO_3	0.1
$CuSO_4$·$5H_2O$	0.15
$ZnSO_4$·$7H_2O$	0.2
Na_2MoO_4·$2H_2O$	0.2

*All compounds were dissolved in 5 M HCl.

10.1.1. Bioreactor cultivation of whole cell catalyst

Bioreactor cultivation was performed in a Biostat CT 6.9 Liter BioReactor (B. Braun Biotech International GmbH, Melsungen, Germany) with a working volume of 5 L, a vessel height to vessel diameter ratio of 2, equipped with a twin 6-blade disc turbine stirrer (stirrer diameter to vessel diameter ratio of 0.4). pH was maintained at 7.0 by automated addition of 1 M H3PO4 and 2 M KOH, temperature was kept at 37°C and 18°C (double jacket vessel). The inoculum (OD_{600} 8) was prepared in two 1000 mL baffled shaken flasks using 250 mL of complex medium supplemented with 115 mg/L ampicillin, 50 mg/L kanamycin, 34 mg/L chloramphenicol (Table S1, S3). The bioreactor medium was a minimal medium (20 g/L glucose) with a C/N ratio of 4.8, supplemented with 115 mg/L ampicillin, 50 mg/L kanamycin, 34 mg/L chloramphenicol (Table S2, S3). Start OD_{600} was 0.5, cultivation temperature 37°C, air saturation was kept at 45 % by an agitation and airflow cascade. At an OD_{600} of 2.0, 1 mM IPTG and 115 mg/L ampicillin was added and the temperature was decreased to 18°C. After 24 h of cultivation, 115 mg/L ampicillin was again added. Cells were harvested after 48 h by centrifugation. Cell activities were measured in the cell-free extracts. The B-Per® cell lysis reagent was used for protein extraction prior to enzyme activity measurements. Reductase and dehydrogenase activities were assayed spectrophotometrically as described previously (Schmölzer et al., 2012). Measurements were performed in 100 mM potassium phosphate buffer, pH 6.2. Biomass was freeze-dried (Christ α 1-4 lyophilizer, Braun Biotech International) and stored at -20°C.

Table S4. Bioreduction conditions for small scale experiments. Concentrations refer to total reaction volumes of 1 mL.

	Buffer (% v/v)	Co-solvent[2] (% v/v)	Cells (g_{CDW}/L)	*o*-Chloroacetophenone (mM)	HCOONa (mM)	NAD^+ (mM)	PM B (µM)	Reaction time (h)
Buffer[1]	100	0	40	100/300/500	150/350/550	0.5	wo/36	8/45
2-phasic	80	20	40	100/300/500	150/350/550	0.5	wo/36	8/45
2-phasic	70	30	40	100/300/500	150/350/550	0.5	36	8/45
2-phasic	50	50	40	100/300/500	150/350/550	0.5	36	8/45

[1]*o*-Chloroacetophenone was predissolved in ethanol to a final ethanol concentration of 5 % v/v of the total reaction volume.
[2]Organic phase constituents are indicated by gray background.

Table S5. Bioreduction conditions for large scale experiments. Concentrations refer to total reaction volumes of 0.5 L.

	Buffer (% v/v)	Co-solvent[1] (% v/v)	Cells (g_{CDW}/L)	Ketone (mM)	HCOONa (mM)	NAD^+ (mM)	PM B (µM)
Hexane 20	80	20	40	300	350	0.5	36
Hexane 50	50	50	40	300	350	0.5	36
Hexane 50	50	50	40	300	350	0.5	36
Blank hexane 50	50	50	40	300	350	0.5	36
Hexanol 50	50	50	40	300	350	0.5	36

[1]Organic phase constituents are indicated by gray background.

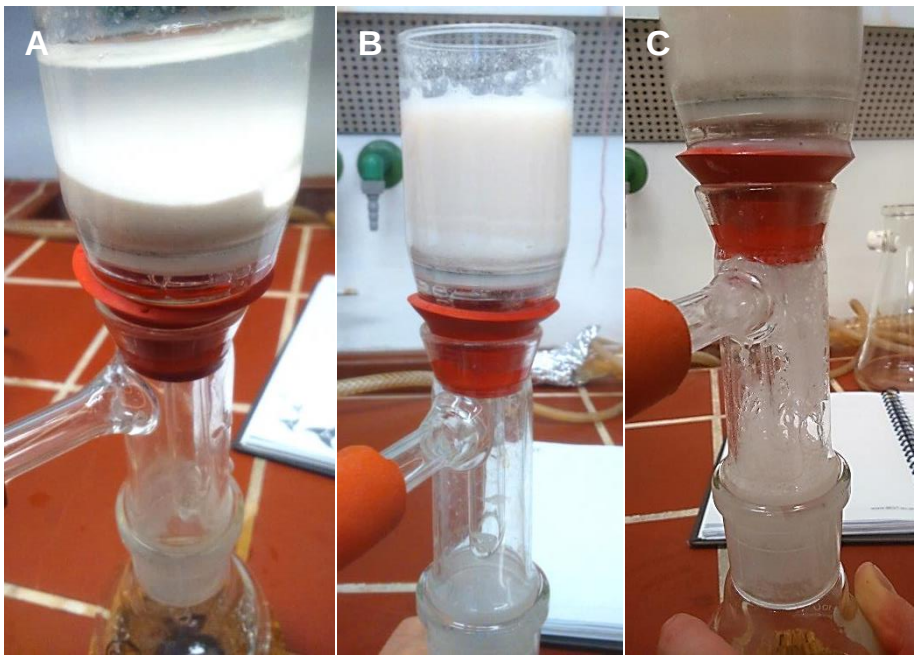


Figure S1. Visual documentation of filtration experiments. High filter aid loading (A), low filter aid loading (B), blocking and foaming during filtration (C).

Figure S2. Settling cell (Henschke et al., 2002).

10.1.2. Cleaning procedure

The cell was dismantled and all parts were cleaned prior to the experiments. The metal and Teflon parts were incubated in 5 % H_2SO_4 for 24 h and then rinsed several times with distilled water. The glass cell was filled with 20 % H_2SO_4, incubated for 45 min, rinsed four times with distilled water; the process was repeated three times. Between experiments with identical solvents the cell was rinsed with acetone and distilled water.

Table S6. Coalescence times for aqueous and organic liquid mixtures without biomass.

Organic / aqueous phase ratio (v/v)	Separation time (s)
Hexane / water (20/80)	41 ± 1
Hexanol / water (20/80)	23 ± 0.4
Heptanol / water (20/80)	33 ± 1
Octanol / water (20/80)	32 ± 1

11. Appendix

Table A1. Activity of cells grown in shaking flask cultivation

	XR [U/L]	XR [U/g]	FDH [U/L]	FDH [U/g]
28.03.2014	30236	1326	3925	172
04.07.2014	105704	6274	7816	464
31.07.2014	66932	5673	3863	327
21.08.2014	3715	n.d.	394	n.d.
22.08.2015	13594	n.d.	394	n.d.

Table A2. Activity of cells grown in bioreactor (5 L) cultivation

	XR [U/L]	XR [U/g]	FDH [U/L]	FDH[U/g]
Thomas E.1	11079	597	1321	71
Thomas E.2	12312	742	1676	101
Jän.15	9497	863	852	77
13.03.2015	81730	1587	7932	154
21.03.2015	137828	2190	6560	104
Aug.15	85980	1864	7036	153
09.12.2015	86215	1693	5270	104

Table A3. Two-phasic bioreductions[1] of 300 mM *o*-chloroacetophenone with 20, 30 and 50 % v/v constant aqueous phase composition. Results in % conversion (*S*-1-(2-chlorophenyl)ethanol in mM).

Co-solvent (% v/v)		8 h	45 h
None		60 (147)	67 (138)
Hexane	20	82 (173)	94 (167)
	30	72 (128)	90 (142)
	50	70 (87)	79 (110)
Hexanol	20	73 (148)	98 (171)
	30	67 (106)	96 (151)
	50	44 (71)	95 (117)
Dipropylether	20	81 (142)	93 (151)
	30	60 (115)	84 (138)
	50	35 (62)	76 (100)

[1]With 36 µM polymyxin B sulfate (concentration referred to total reaction volume).

11.1. Settling curves

Phase volume ratios of settling experiments performed in 50 mL tubes after 24 h gravity settling and after additional 10 min and 30 minutes of centrifugation. Red bars indicate the organic phases, blue bars indicate aqueous phases, grey bars indicate pelleted biomass and crud phases.

11.1.1. Reaction mixtures with 50 % v/v hexane

pH Adjustments……..…….57-58

Temperature……..……59

Surfactants…….….60

Salts…….61

11.1.2. Reaction mixtures with 50 % v/v hexane without substrate

pH Adjustments……..…….62-63

Temperature……..……64

Surfactants…….….65

Salts…….66

11.1.3. Reaction mixtures with 20 % v/v hexane……..…..……67

11.1.4. Reaction mixtures with 50 % v/v hexanol

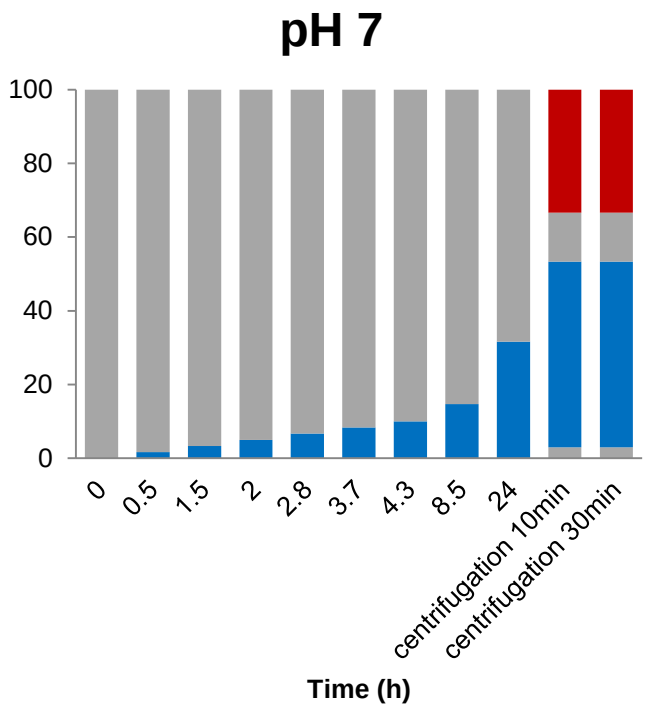
pH Adjustments……..…….68-69

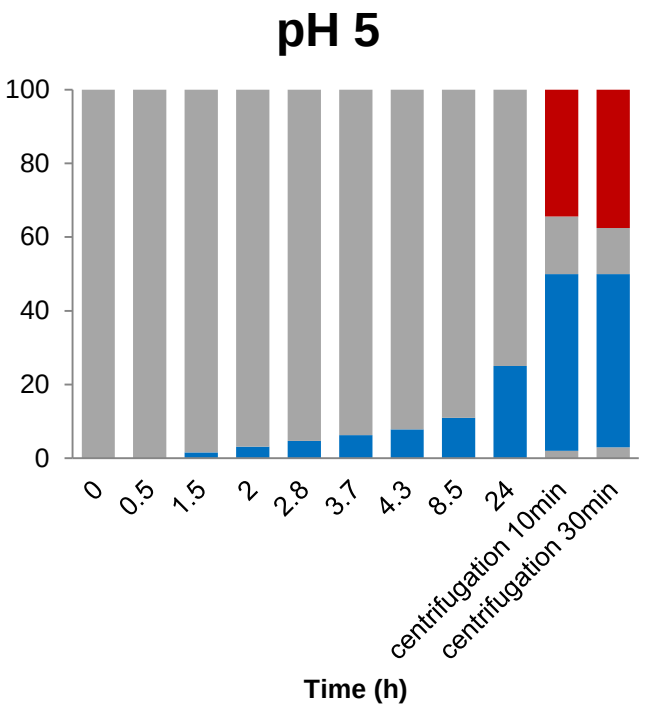
Temperature……..……70

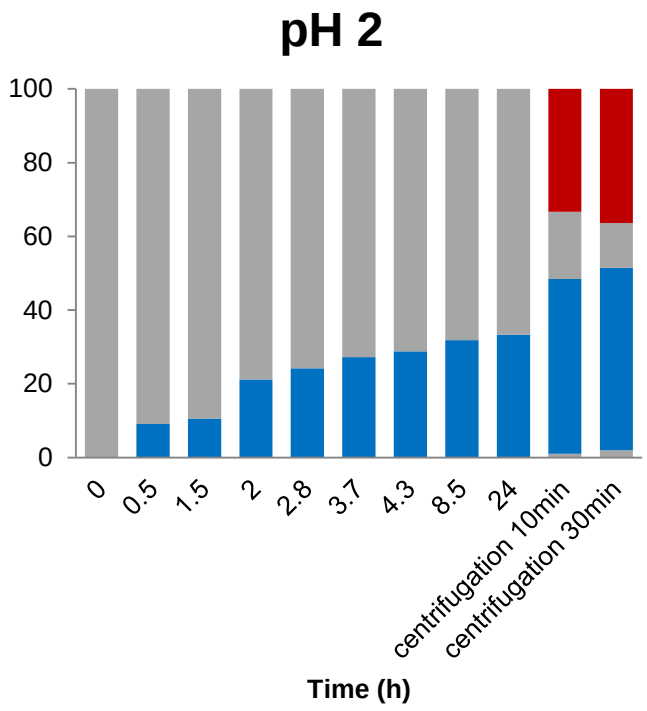
Surfactants…….….71

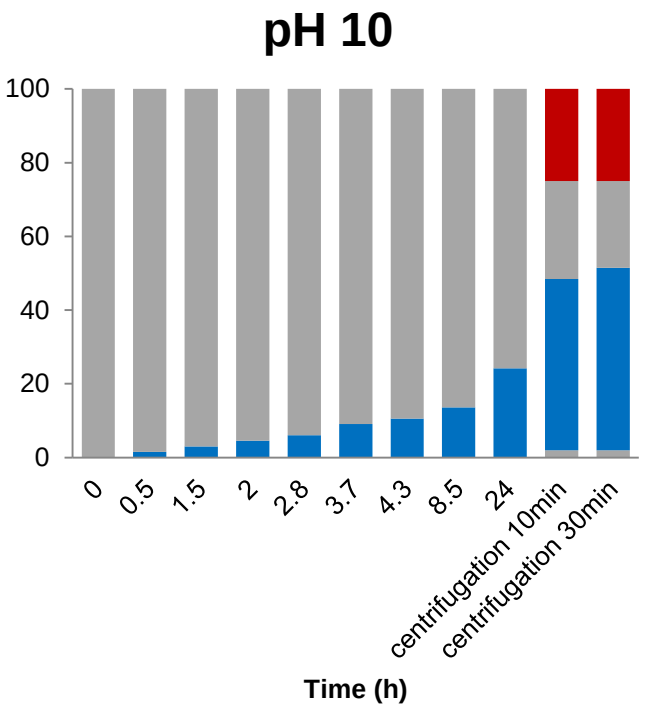
Salts…….72

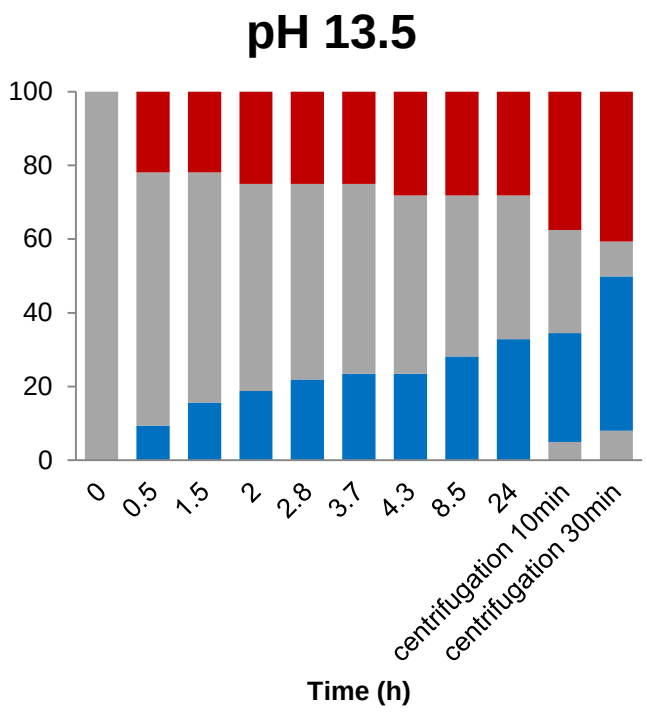
11.1.5. Reaction mixtures with 20 % v/v hexanol…….……73-74

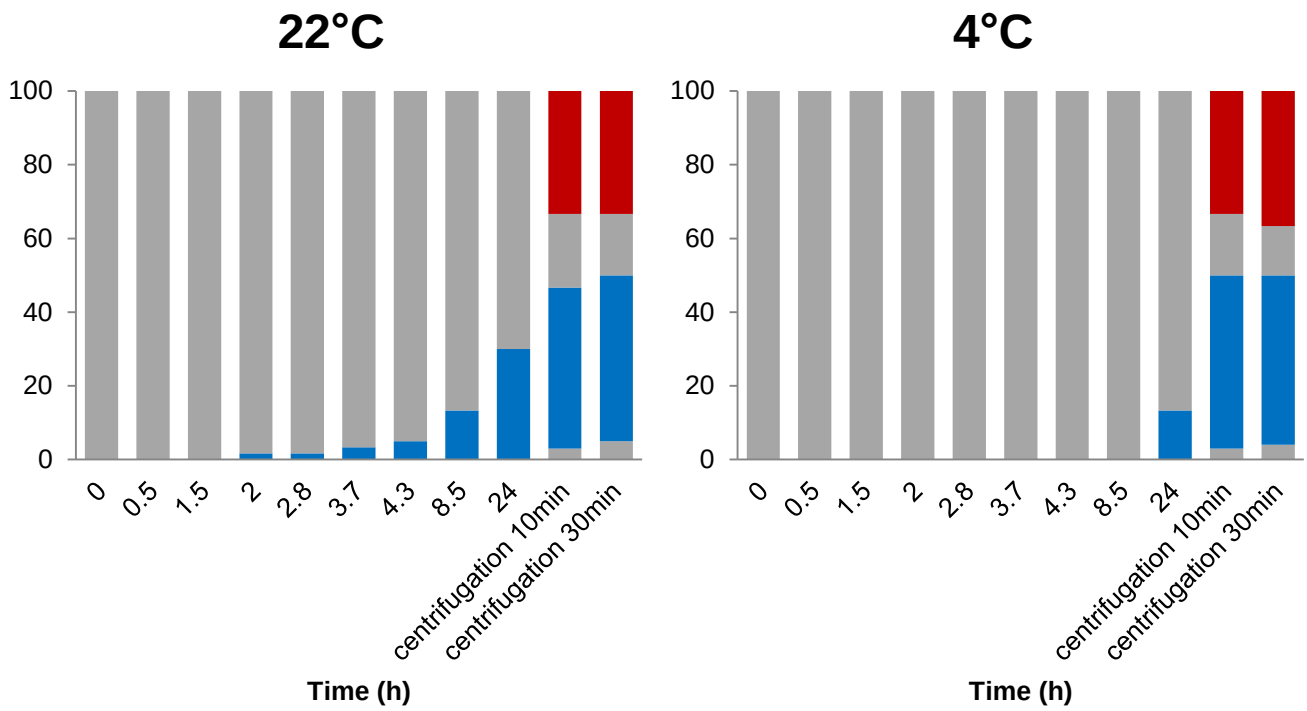

pH 7
100
80
60
40
20
0
0
0.5
1.5
2
2.8
3.7
4.3
8.5
24
centrifugation 10min
centrifugation 30min
Time (h)


pH 5
100
80
60
40
20
0
0
0.5
1.5
2
2.8
3.7
4.3
8.5
24
centrifugation 10min
centrifugation 30min
Time (h)

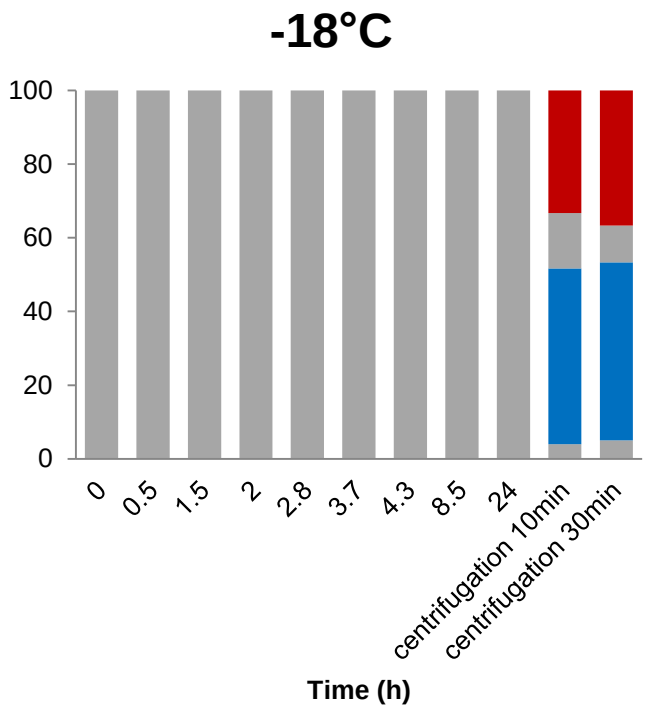

pH 2
100
80
60
40
20
0
0
0.5
1.5
2
2.8
3.7
4.3
8.5
24
centrifugation 10min
centrifugation 30min
Time (h)


pH 10
100
80
60
40
20
0
0
0.5
1.5
2
2.8
3.7
4.3
8.5
24
centrifugation 10min
centrifugation 30min
Time (h)


pH 13.5
100
80
60
40
20
0
0
0.5
1.5
2
2.8
3.7
4.3
8.5
24
centrifugation 10min
centrifugation 30min
Time (h)


22°C
100
80
60
40
20
0
0
0.5
1.5
2
2.8
3.7
4.3
8.5
24
centrifugation 10min
centrifugation 30min
Time (h)

4°C
100
80
60
40
20
0
0
0.5
1.5
2
2.8
3.7
4.3
8.5
24
centrifugation 10min
centrifugation 30min
Time (h)


-18°C
100
80
60
40
20
0
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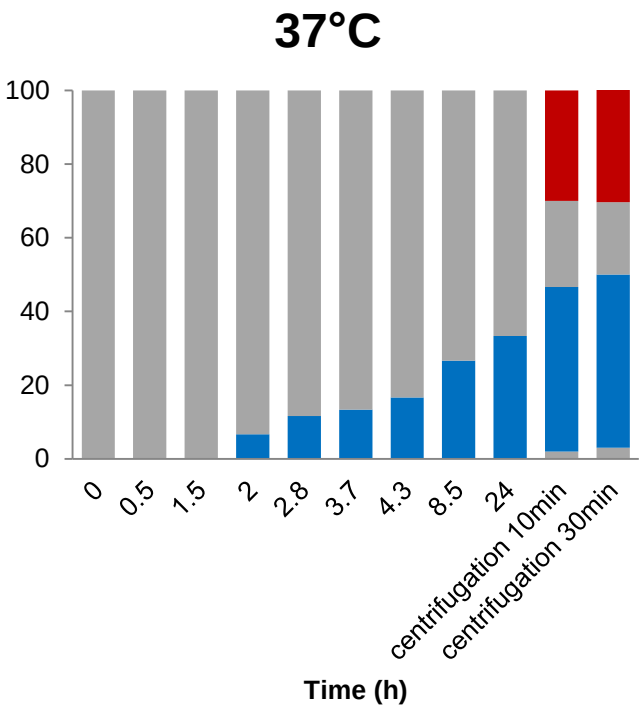
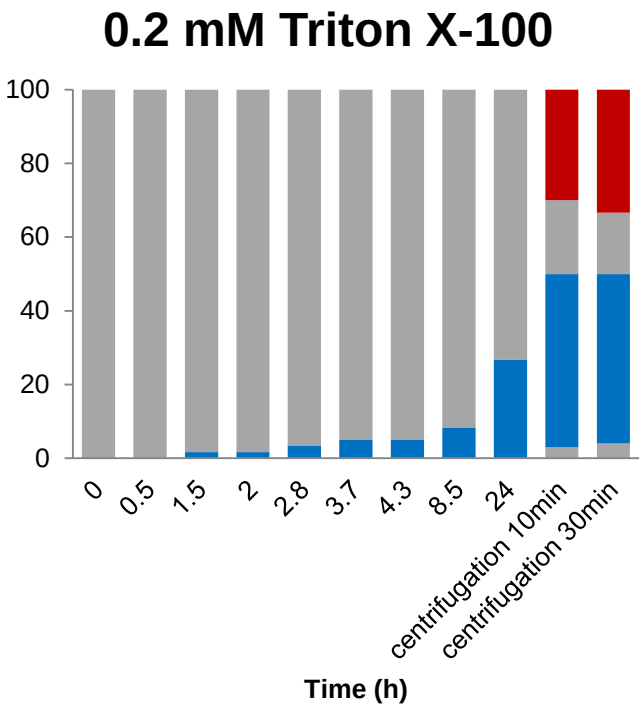
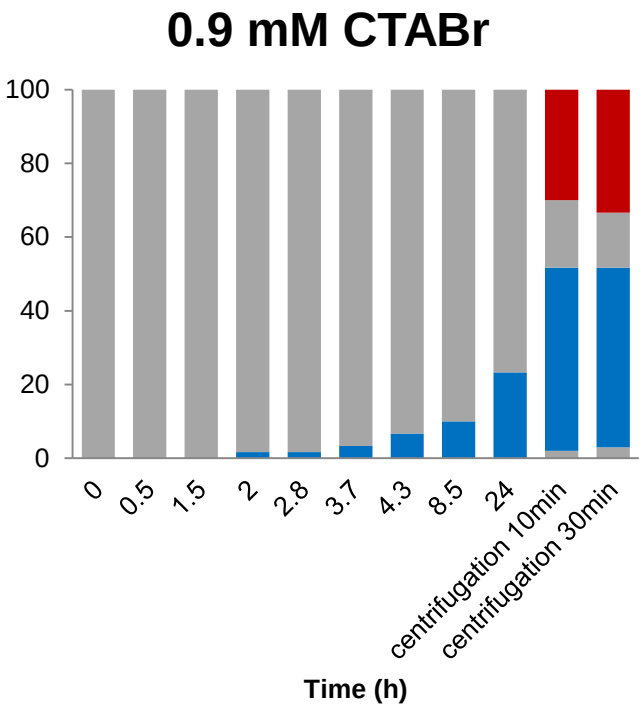
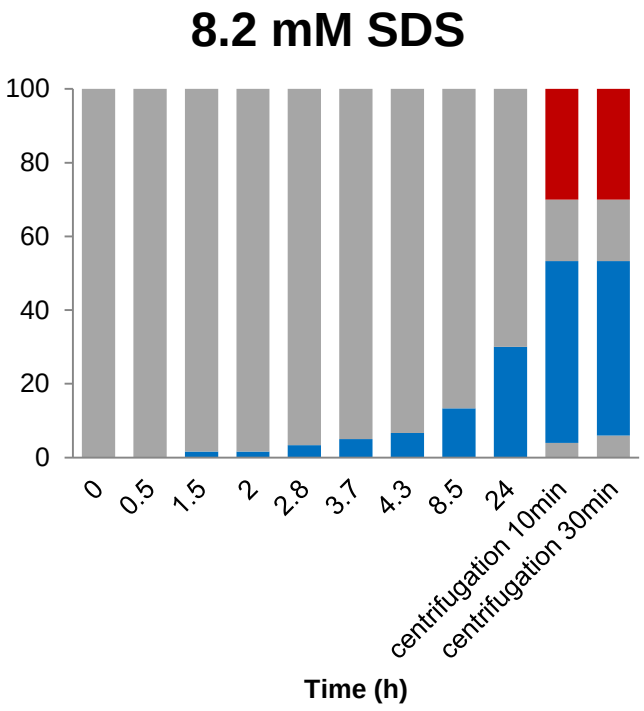
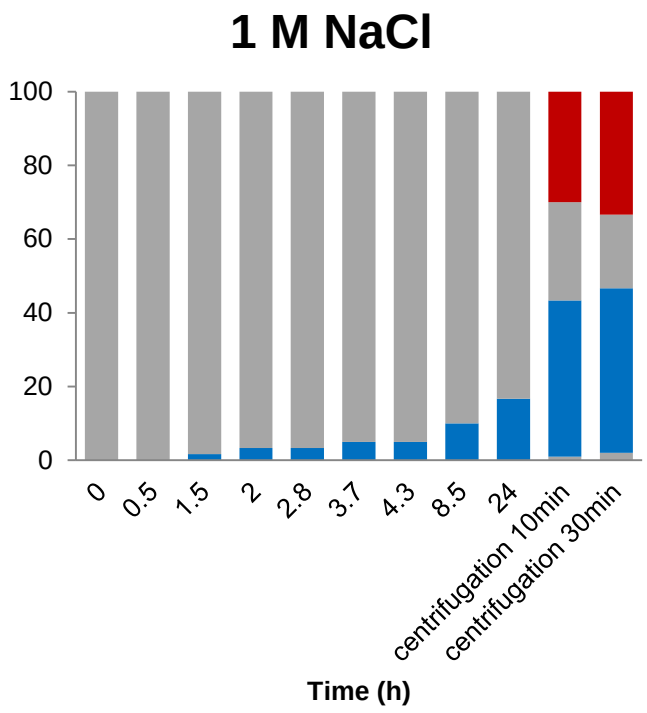
0.5
1.5
2
2.8
3.7
4.3
8.5
24
centrifugation 10min
centrifugation 30min
Time (h)

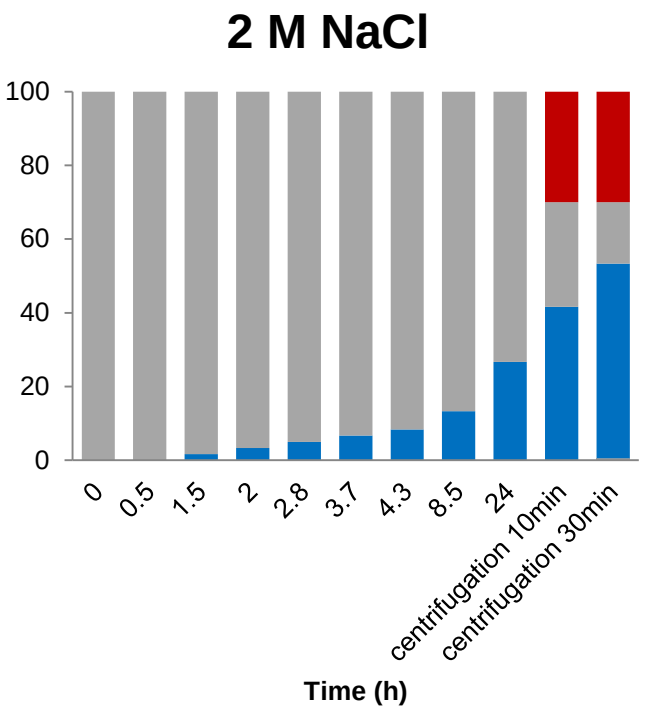

37°C
100
80
60
40
20
0
0
0.5
1.5
2
2.8
3.7
4.3
8.5
24
centrifugation 10min
centrifugation 30min
Time (h)

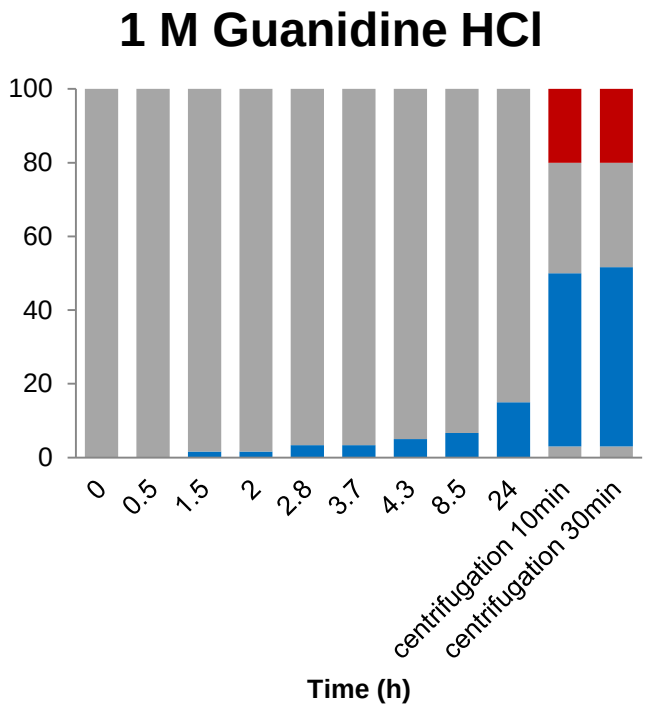

0.2 mM Triton X-100
100
80
60
40
20
0
0
0.5
1.5
2
2.8
3.7
4.3
8.5
24
centrifugation 10min
centrifugation 30min
Time (h)

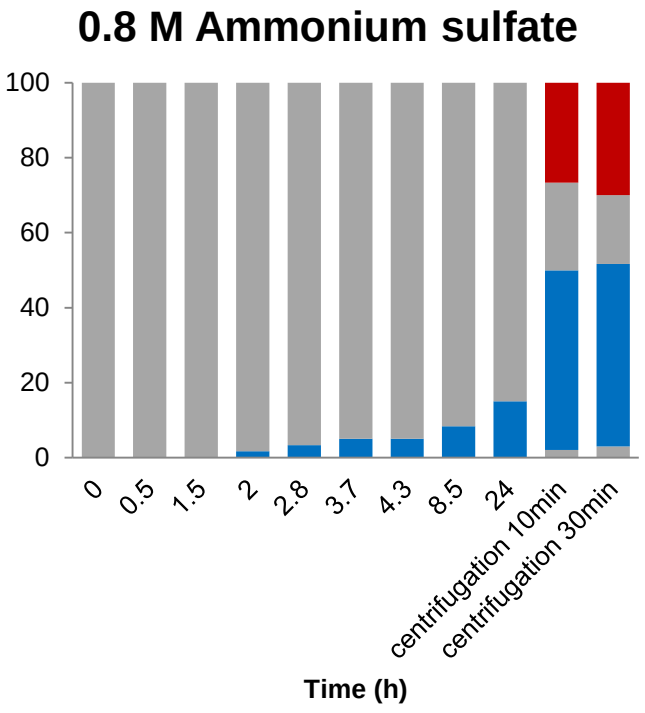

0.9 mM CTABr
100
80
60
40
20
0
0
0.5
1.5
2
2.8
3.7
4.3
8.5
24
centrifugation 10min
centrifugation 30min
Time (h)


8.2 mM SDS
100
80
60
40
20
0
0
0.5
1.5
2
2.8
3.7
4.3
8.5
24
centrifugation 10min
centrifugation 30min
Time (h)


1 M NaCl
100
80
60
40
20
0
0
0.5
1.5
2
2.8
3.7
4.3
8.5
24
centrifugation 10min
centrifugation 30min
Time (h)


2 M NaCl
100
80
60
40
20
0
0
0.5
1.5
2
2.8
3.7
4.3
8.5
24
centrifugation 10min
centrifugation 30min
Time (h)


1 M Guanidine HCl
100
80
60
40
20
0
0
0.5
1.5
2
2.8
3.7
4.3
8.5
24
centrifugation 10min
centrifugation 30min
Time (h)


0.8 M Ammonium sulfate
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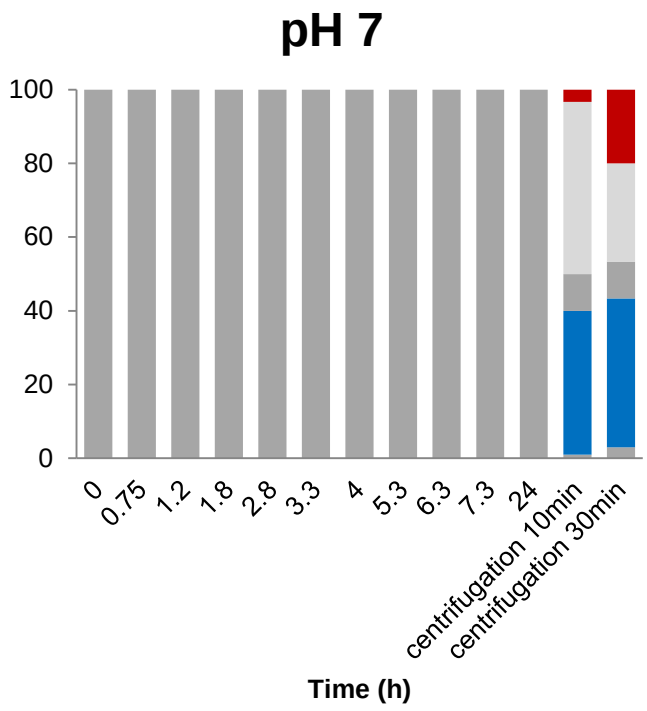
80
60
40
20
0
0
0.5
1.5
2
2.8
3.7
4.3
8.5
24
centrifugation 10min
centrifugation 30min
Time (h)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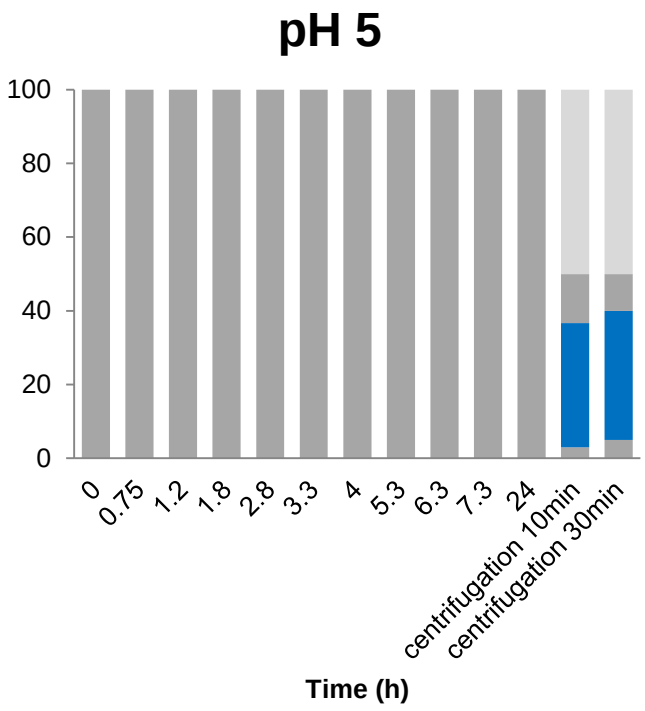
40
20
0
0
0.75
1.2
1.8
2.8
3.3
4
5.3
6.3
7.3
24
centrifugation 10min
centrifugation 30min
Time (h)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60
40
20
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0
0.75
1.2
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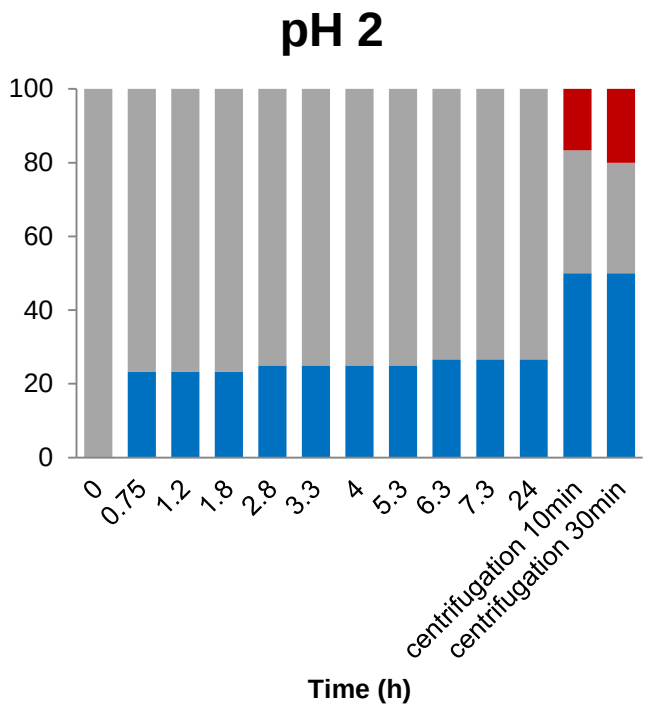
4
5.3
6.3
7.3
24
centrifugation 10min
centrifugation 30min
Time (h)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20
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0
0.75
1.2
1.8
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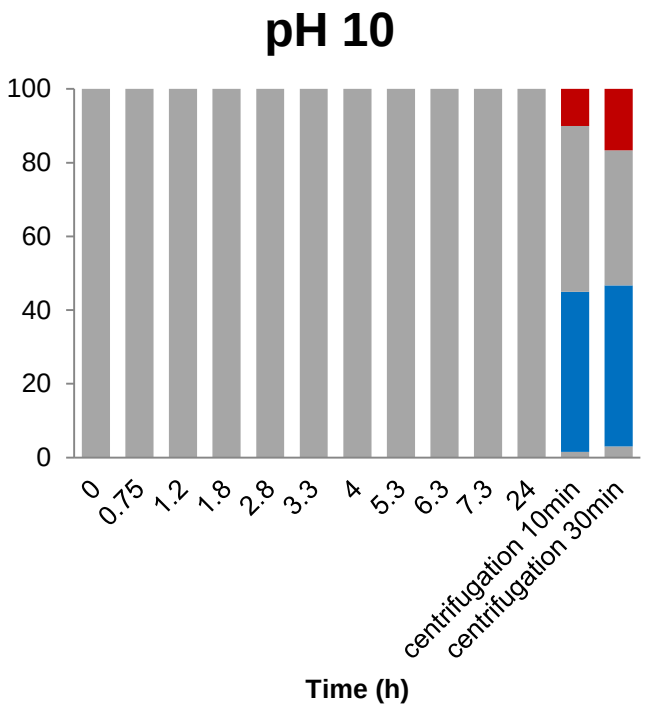
3.3
4
5.3
6.3
7.3
24
centrifugation 10min
centrifugation 30min
Time (h)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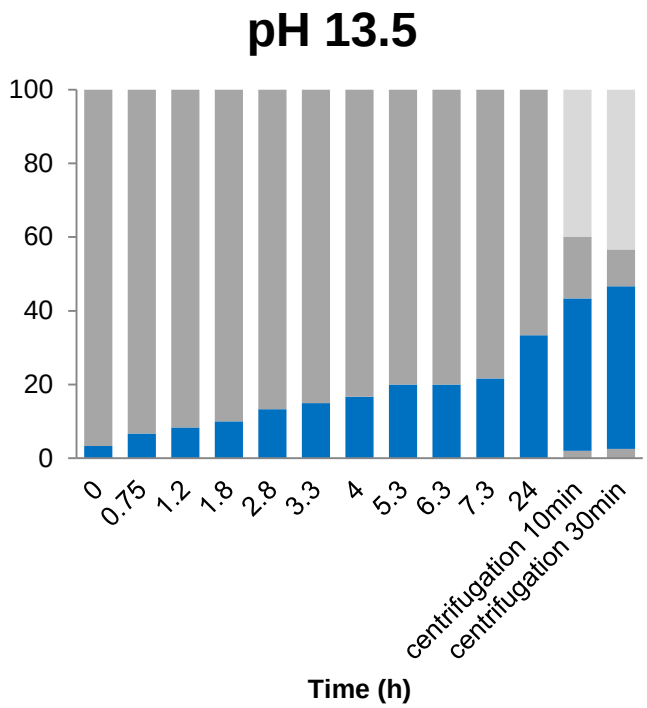
60
40
20
0
0
0.75
1.2
1.8
2.8
3.3
4
5.3
6.3
7.3
24
centrifugation 10min
centrifugation 30min
Time (h)


pH 13.5
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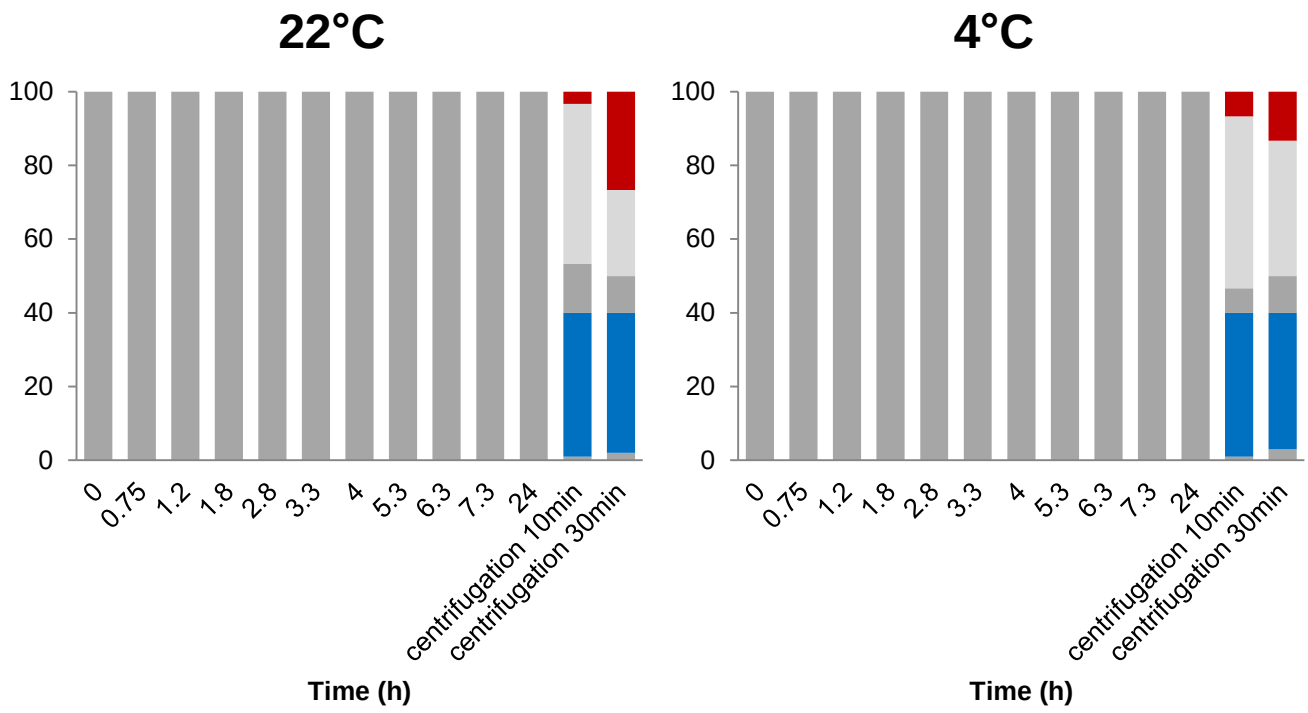
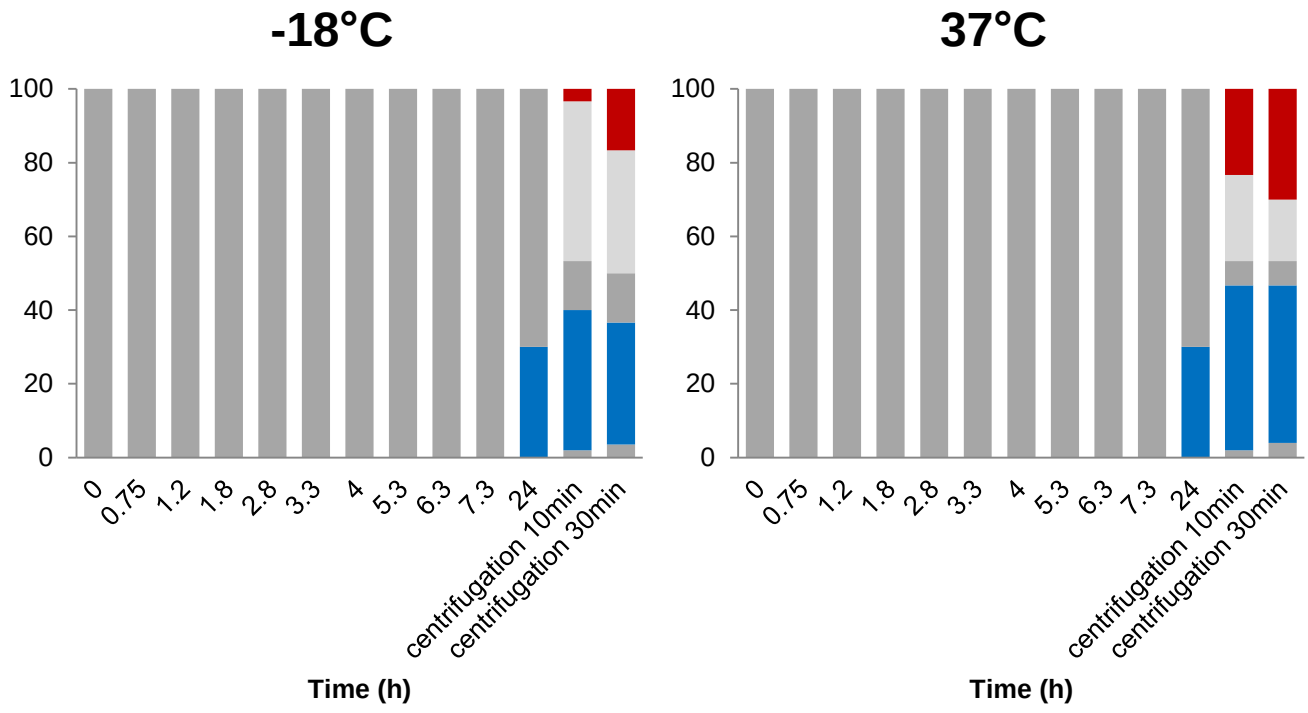
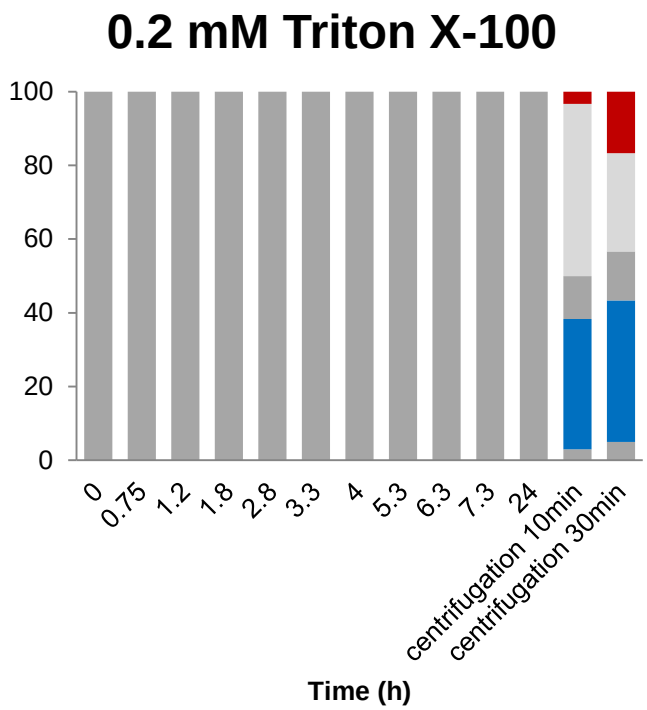
60
40
20
0
0
0.75
1.2
1.8
2.8
3.3
4
5.3
6.3
7.3
24
centrifugation 10min
centrifugation 30min
Time (h)

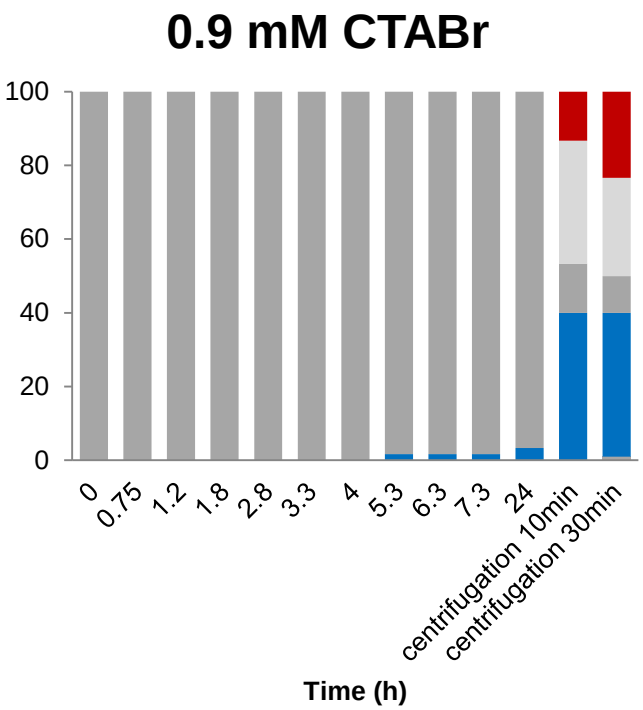

22°C
100
80
60
40
20
0
0
0.75
1.2
1.8
2.8
3.3
4
5.3
6.3
7.3
24
centrifugation 10min
centrifugation 30min
Time (h)

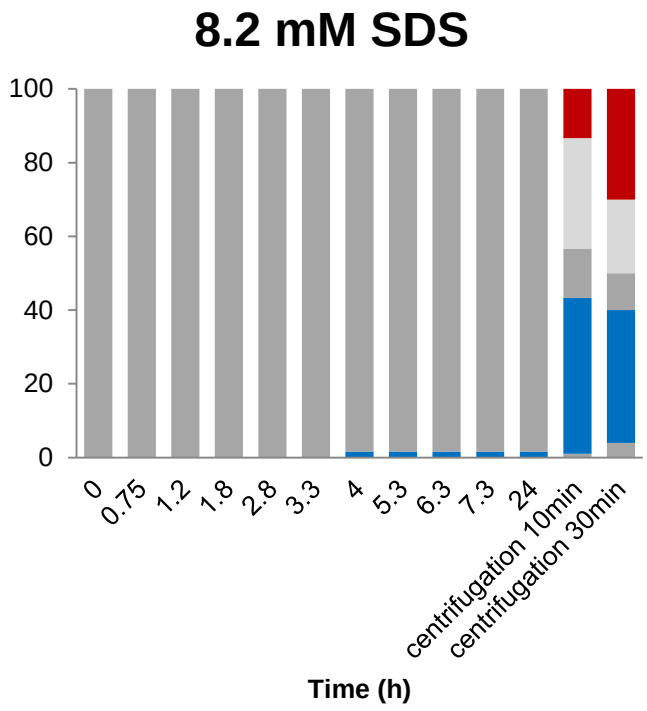
4°C
100
80
60
40
20
0
0
0.75
1.2
1.8
2.8
3.3
4
5.3
6.3
7.3
24
centrifugation 10min
centrifugation 30min
Time (h)

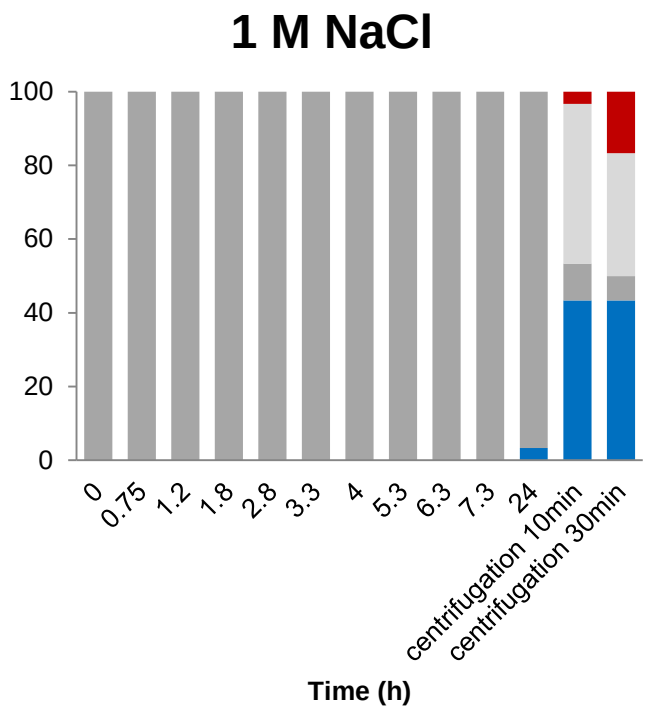

-18°C
100
80
60
40
20
0
0
0.75
1.2
1.8
2.8
3.3
4
5.3
6.3
7.3
24
centrifugation 10min
centrifugation 30min
Time (h)

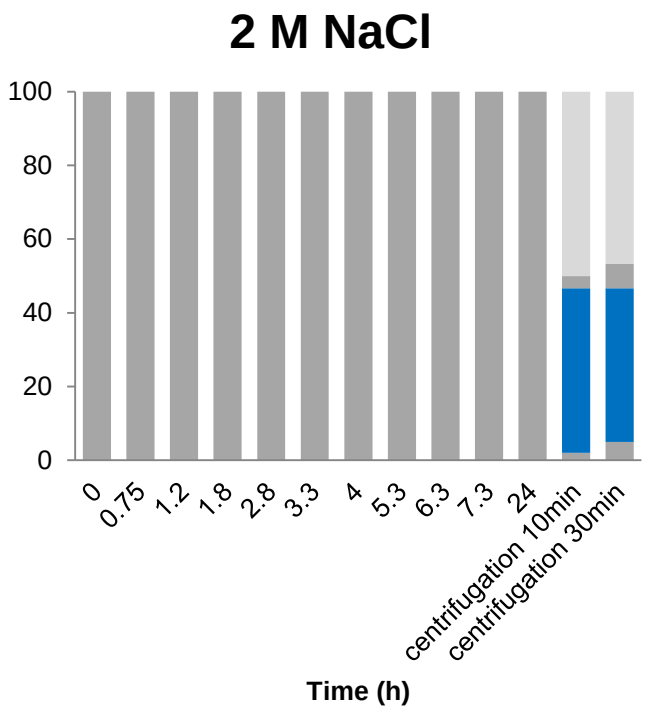
37°C
100
80
60
40
20
0
0
0.75
1.2
1.8
2.8
3.3
4
5.3
6.3
7.3
24
centrifugation 10min
centrifugation 30min
Time (h)

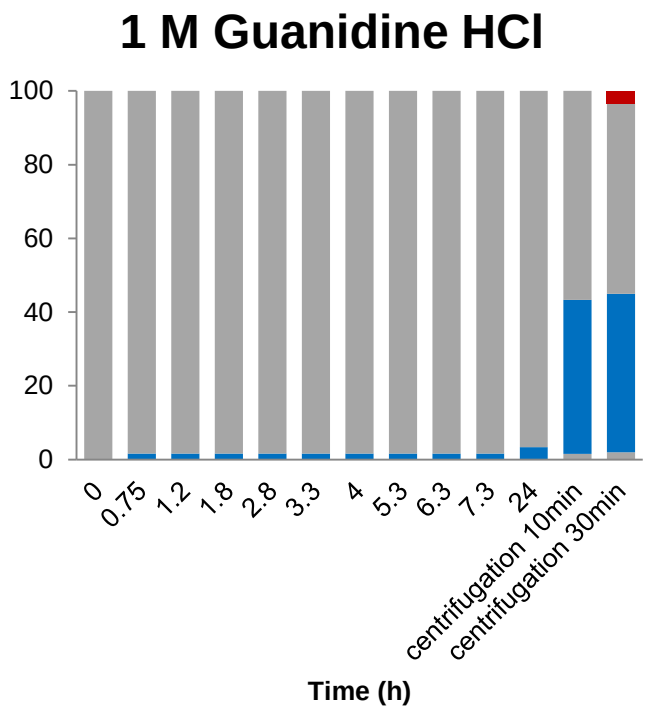

0.2 mM Triton X-100
100
80
60
40
20
0
0
0.75
1.2
1.8
2.8
3.3
4
5.3
6.3
7.3
24
centrifugation 10min
centrifugation 30min
Time (h)

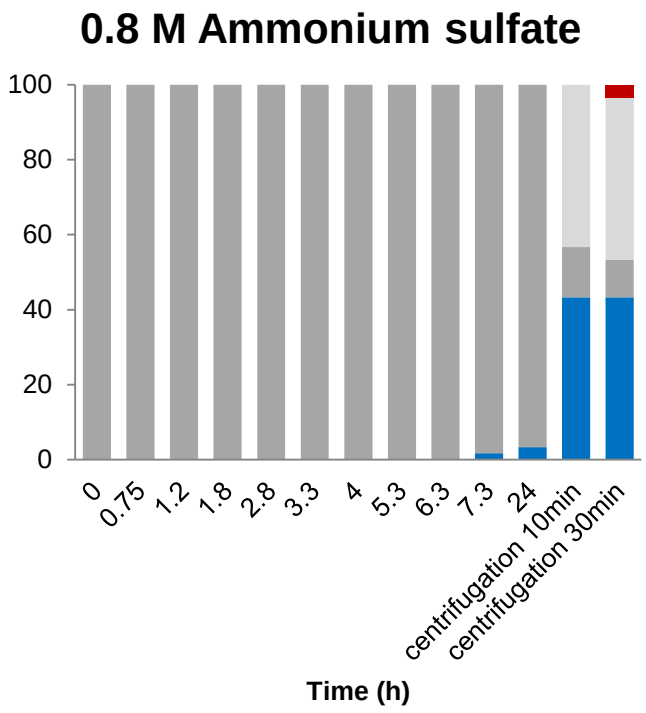

0.9 mM CTABr
100
80
60
40
20
0
0
0.75
1.2
1.8
2.8
3.3
4
5.3
6.3
7.3
24
centrifugation 10min
centrifugation 30min
Time (h)

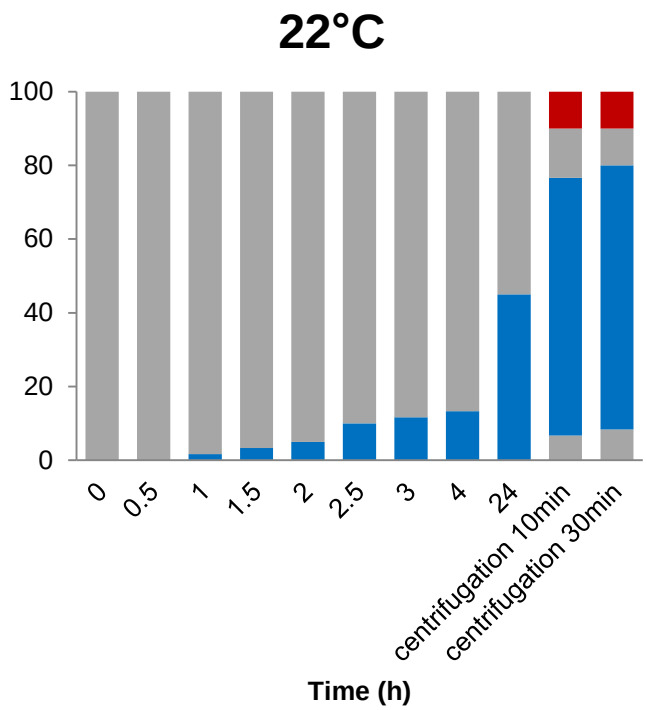

8.2 mM SDS
100
80
60
40
20
0
0
0.75
1.2
1.8
2.8
3.3
4
5.3
6.3
7.3
24
centrifugation 10min
centrifugation 30min
Time (h)

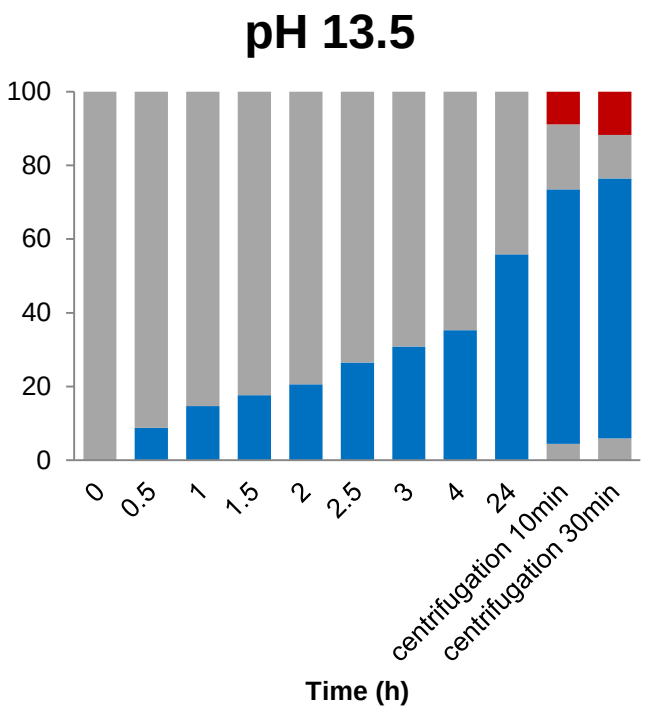

1 M NaCl
100
80
60
40
20
0
0
0.75
1.2
1.8
2.8
3.3
4
5.3
6.3
7.3
24
centrifugation 10min
centrifugation 30min
Time (h)

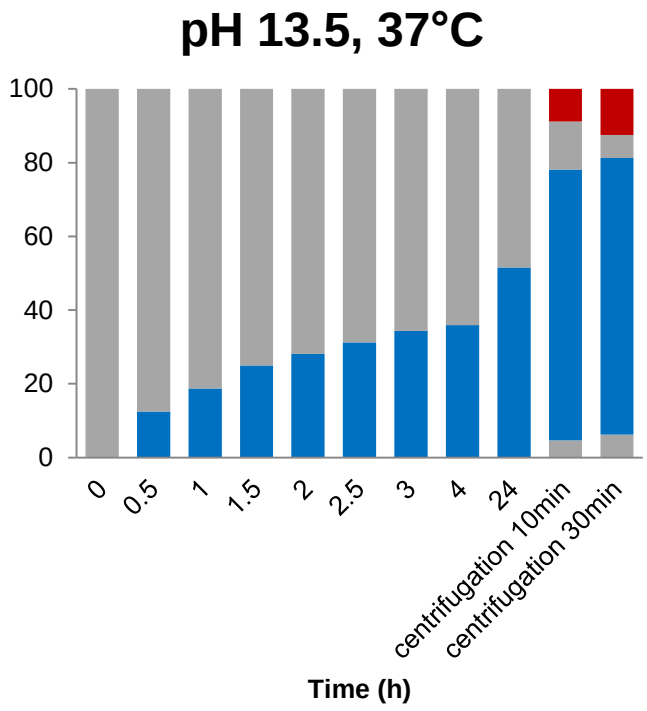

2 M NaCl
100
80
60
40
20
0
0
0.75
1.2
1.8
2.8
3.3
4
5.3
6.3
7.3
24
centrifugation 10min
centrifugation 30min
Time (h)

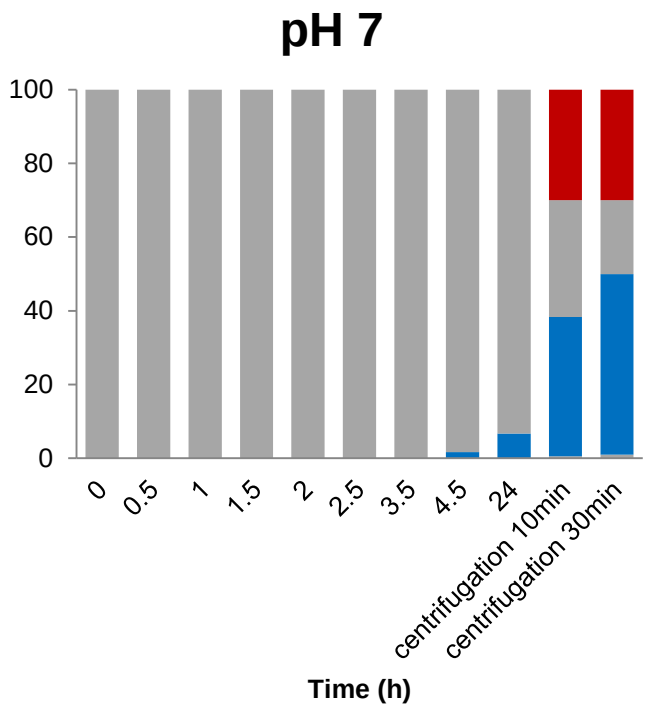

1 M Guanidine HCl
100
80
60
40
20
0
0
0.75
1.2
1.8
2.8
3.3
4
5.3
6.3
7.3
24
centrifugation 10min
centrifugation 30min
Time (h)

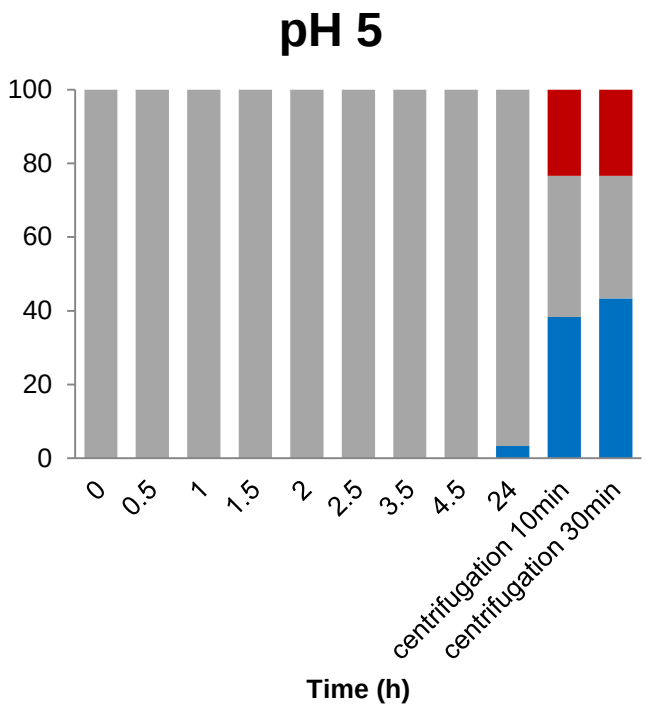

0.8 M Ammonium sulfate
100
80
60
40
20
0
0
0.75
1.2
1.8
2.8
3.3
4
5.3
6.3
7.3
24
centrifugation 10min
centrifugation 30min
Time (h)

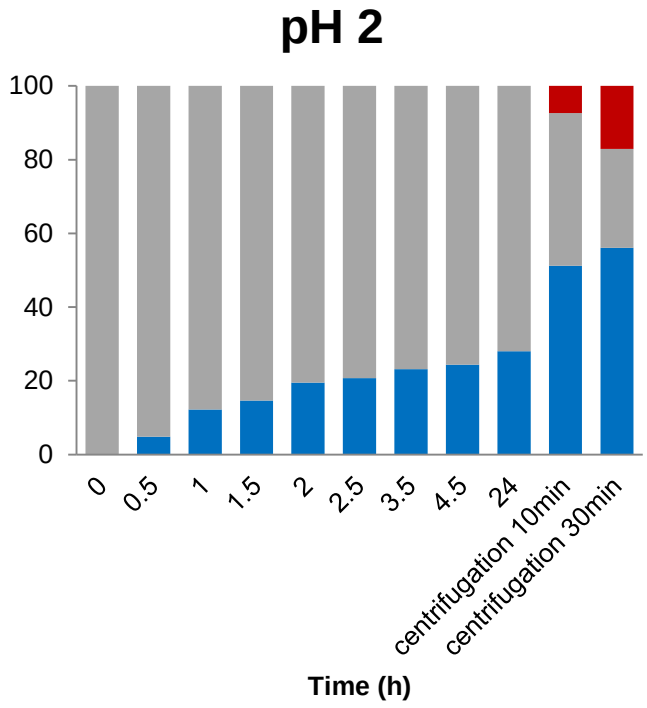

22°C
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3
4
24
centrifugation 10min
centrifugation 30min
Time (h)

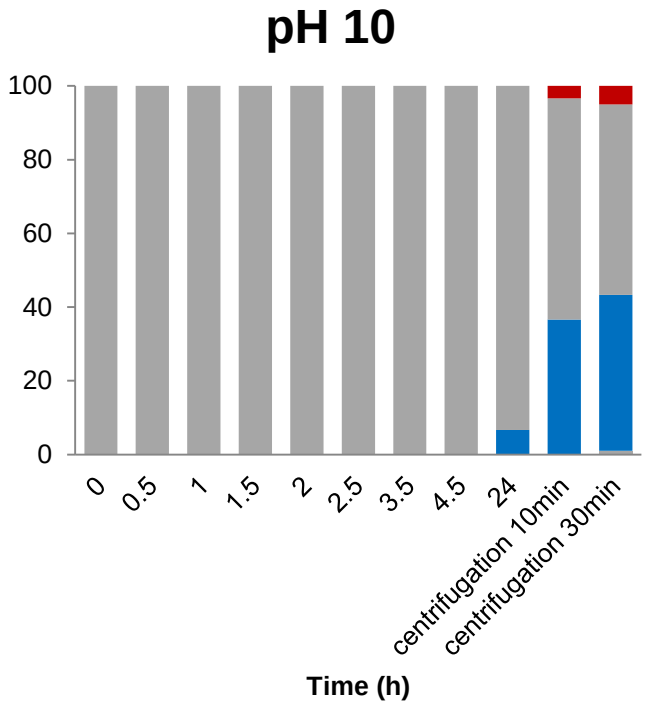

pH 13.5
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3
4
24
centrifugation 10min
centrifugation 30min
Time (h)

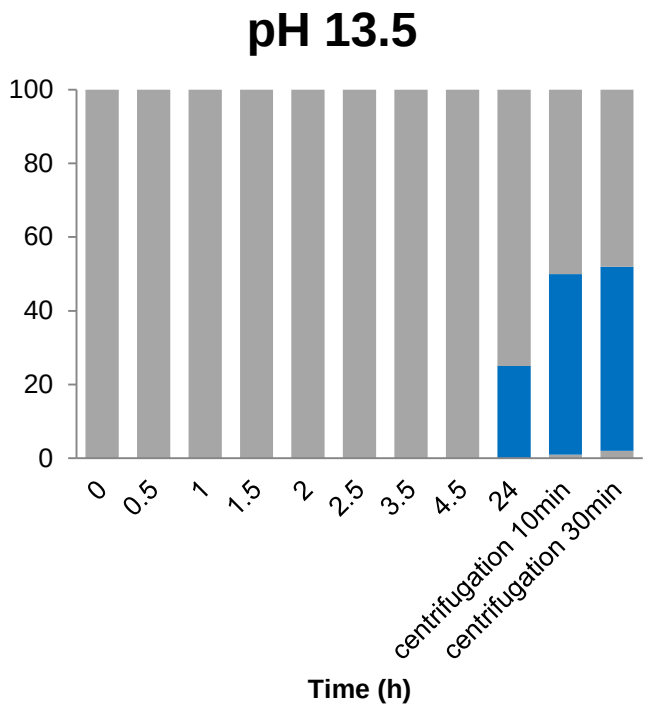

pH 13.5, 37°C
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3
4
24
centrifugation 10min
centrifugation 30min
Time (h)

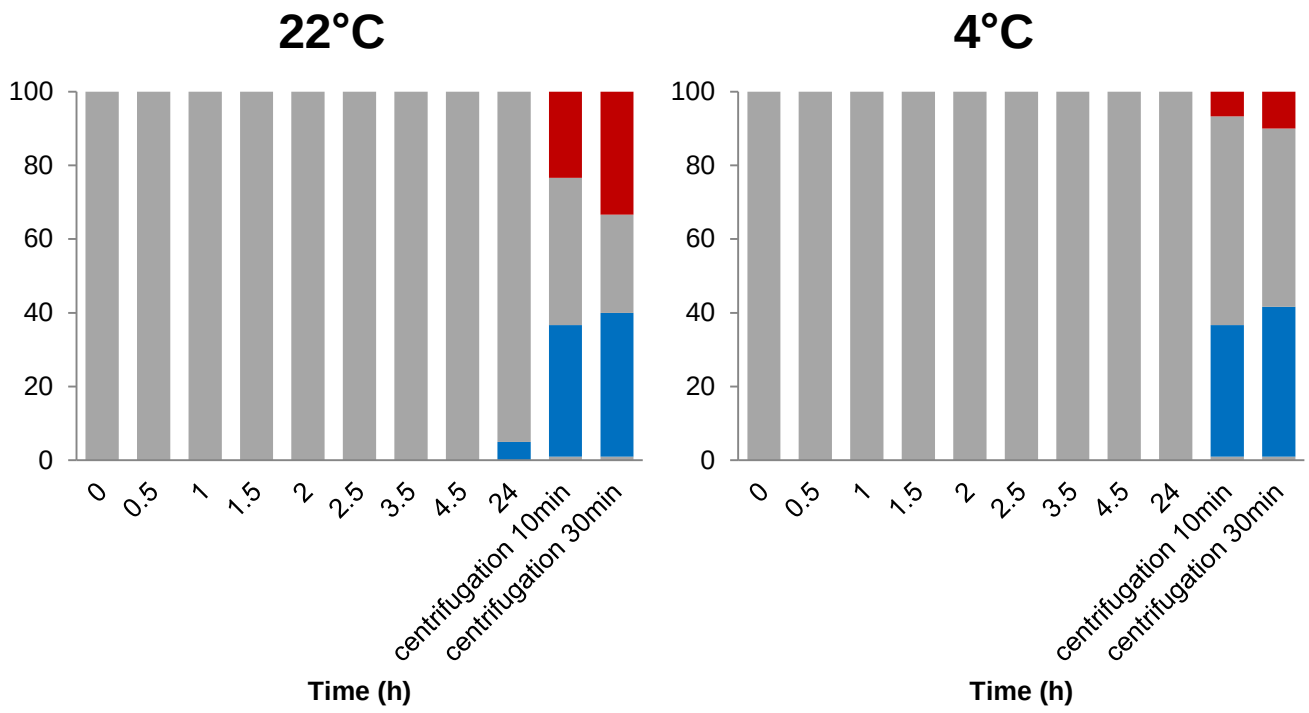

pH 7
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3.5
4.5
24
centrifugation 10min
centrifugation 30min
Time (h)


pH 5
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3.5
4.5
24
centrifugation 10min
centrifugation 30min
Time (h)

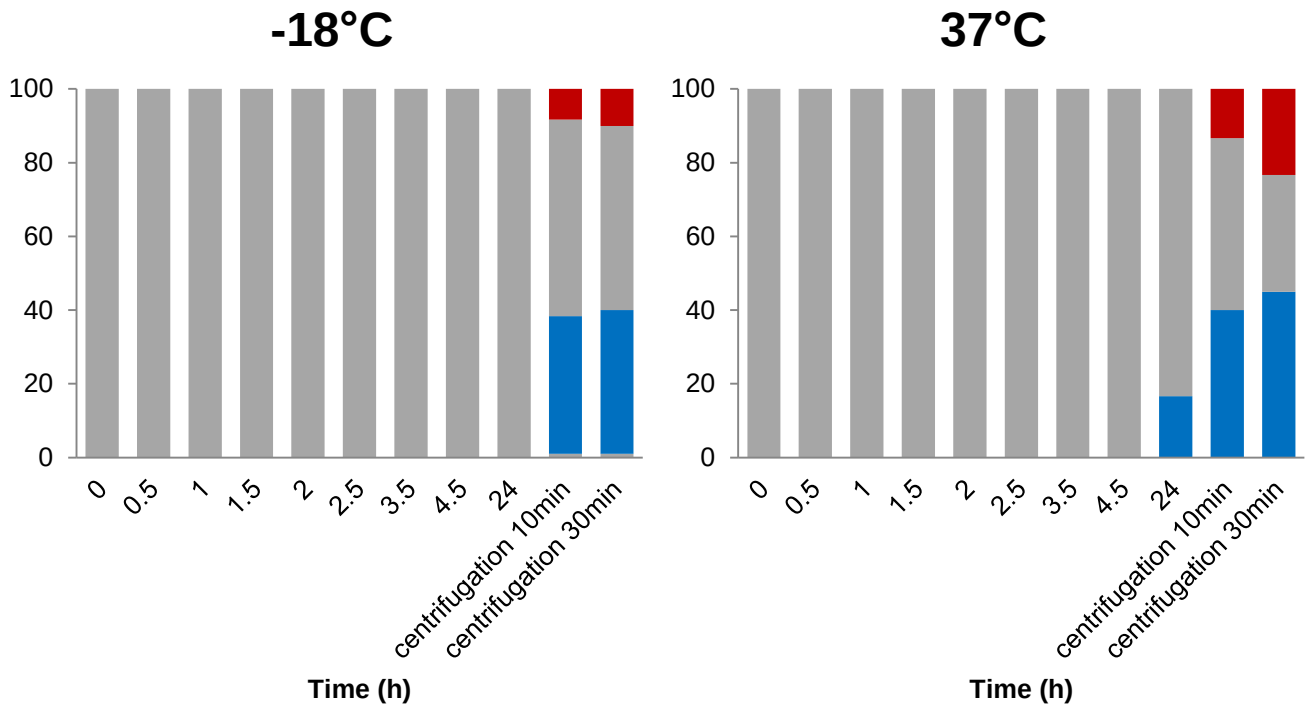

pH 2
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3.5
4.5
24
centrifugation 10min
centrifugation 30min
Time (h)


pH 10
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3.5
4.5
24
centrifugation 10min
centrifugation 30min
Time (h)

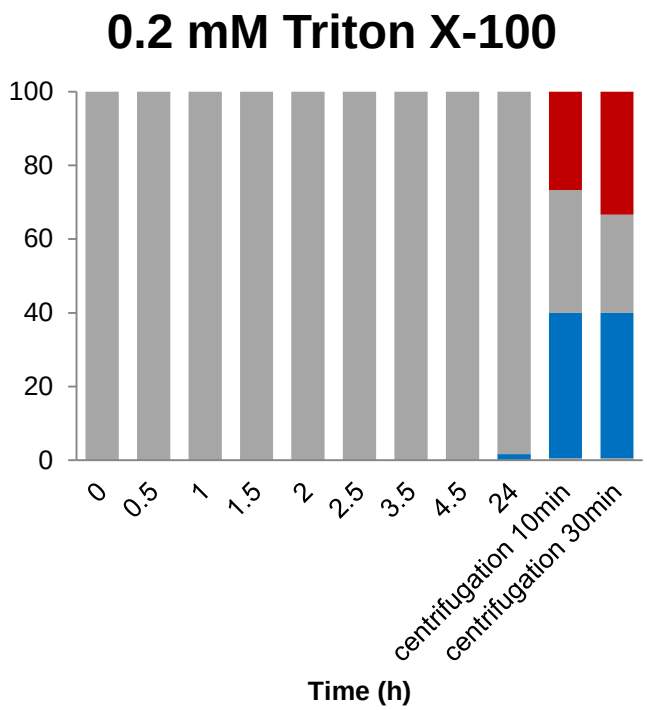

pH 13.5
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3.5
4.5
24
centrifugation 10min
centrifugation 30min
Time (h)

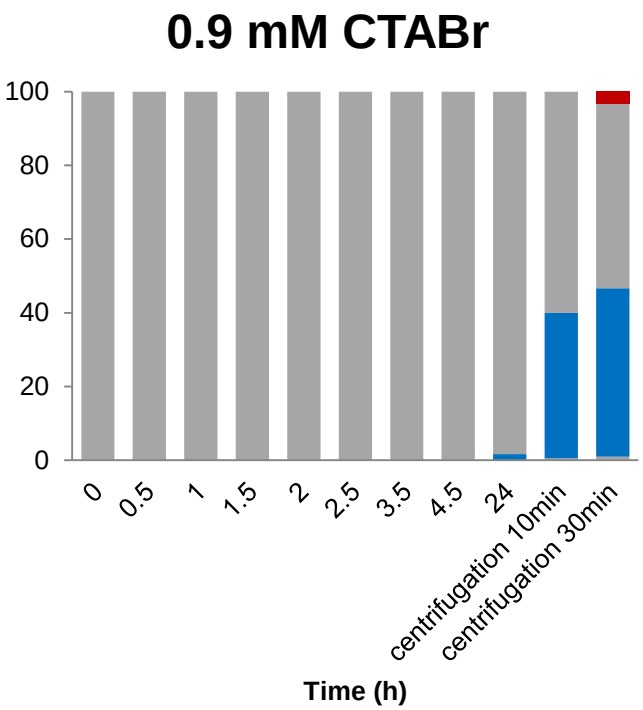

22°C
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3.5
4.5
24
centrifugation 10min
centrifugation 30min
Time (h)

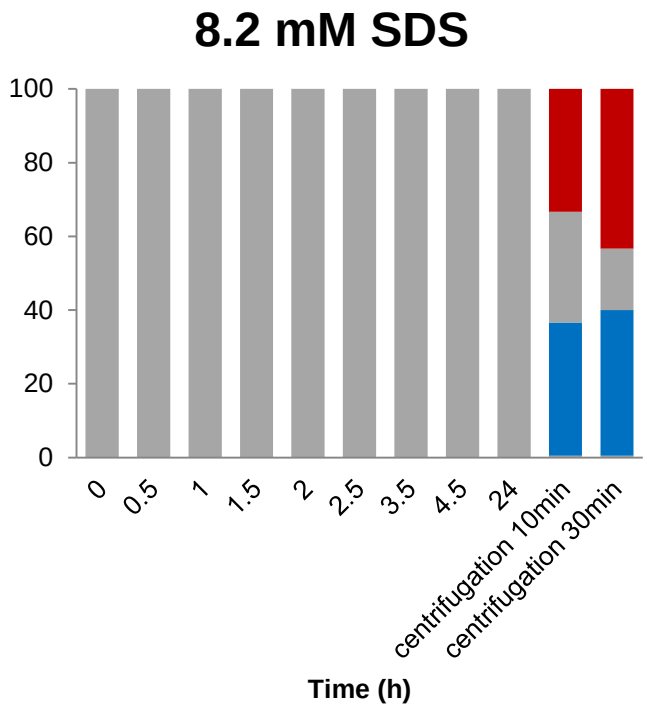
4°C
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3.5
4.5
24
centrifugation 10min
centrifugation 30min
Time (h)

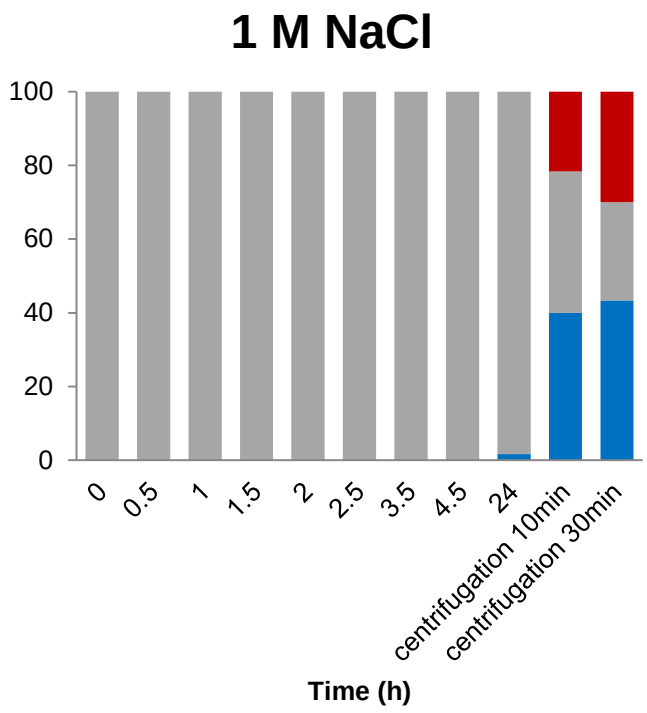

-18°C
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3.5
4.5
24
centrifugation 10min
centrifugation 30min
Time (h)

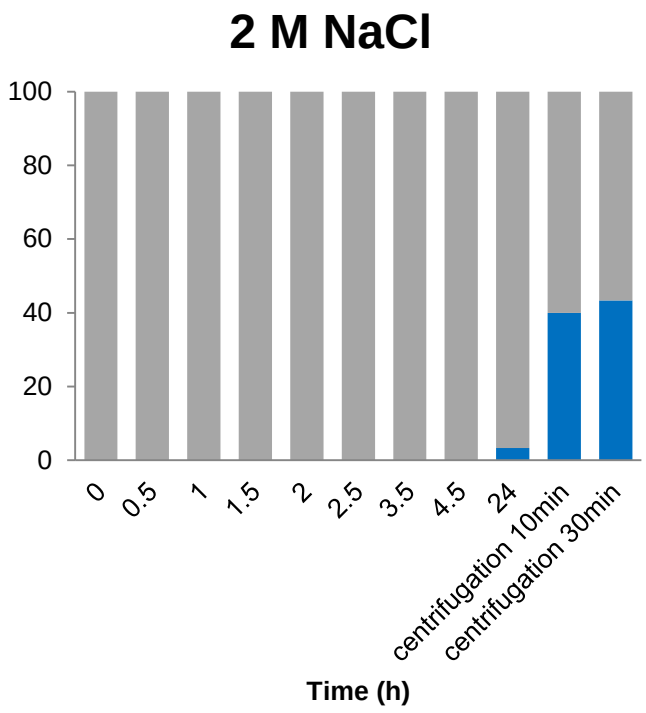
37°C
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3.5
4.5
24
centrifugation 10min
centrifugation 30min
Time (h)

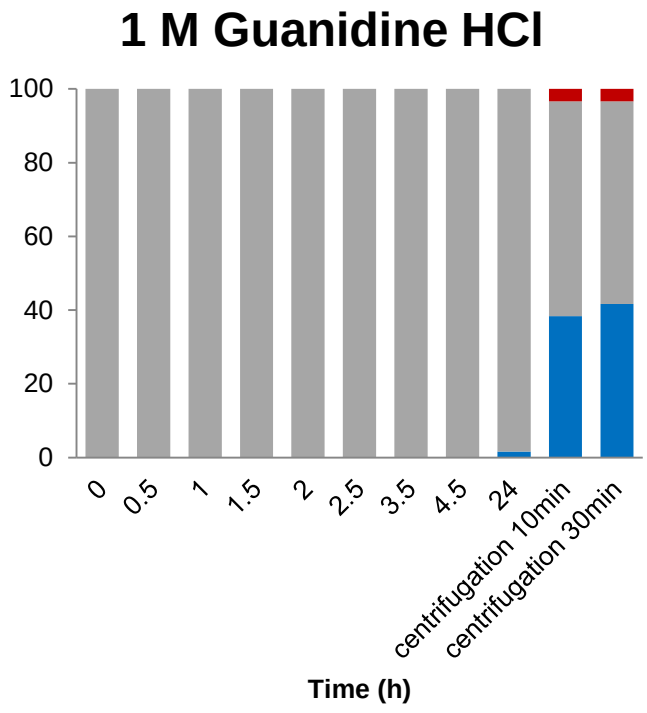

0.2 mM Triton X-100
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3.5
4.5
24
centrifugation 10min
centrifugation 30min
Time (h)

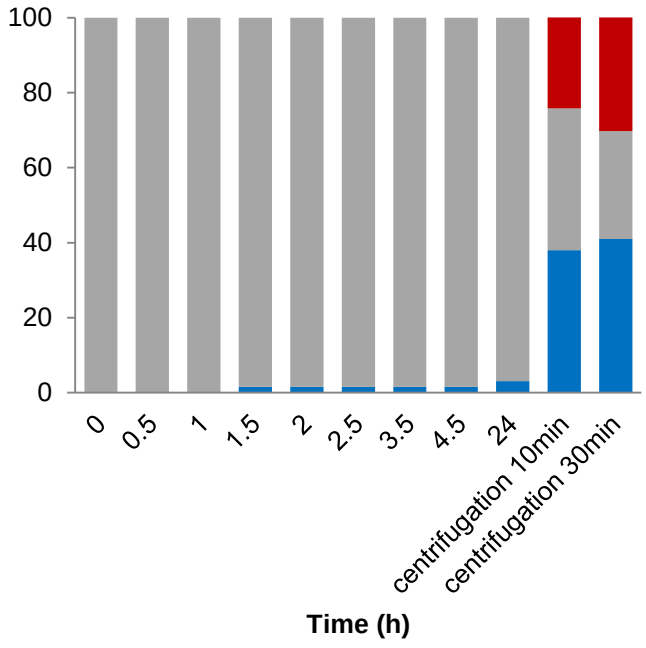

0.9 mM CTABr
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3.5
4.5
24
centrifugation 10min
centrifugation 30min
Time (h)

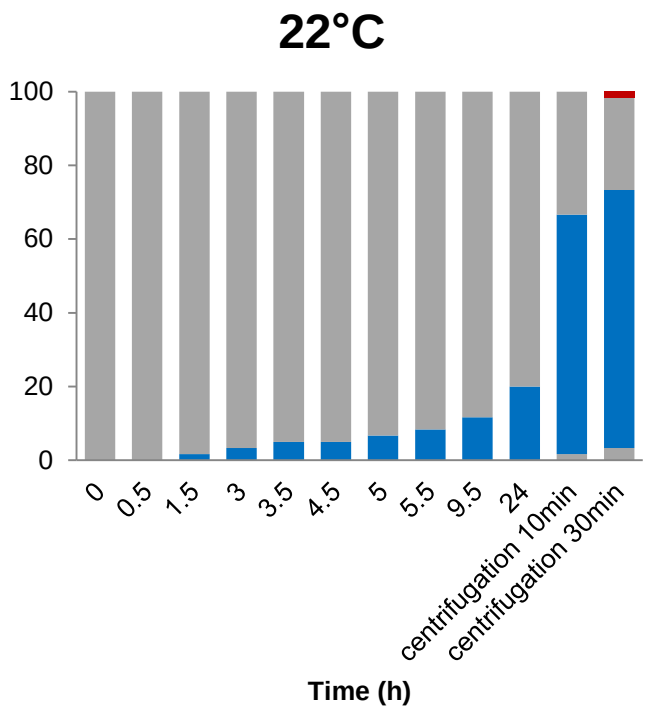

8.2 mM SDS
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3.5
4.5
24
centrifugation 10min
centrifugation 30min
Time (h)

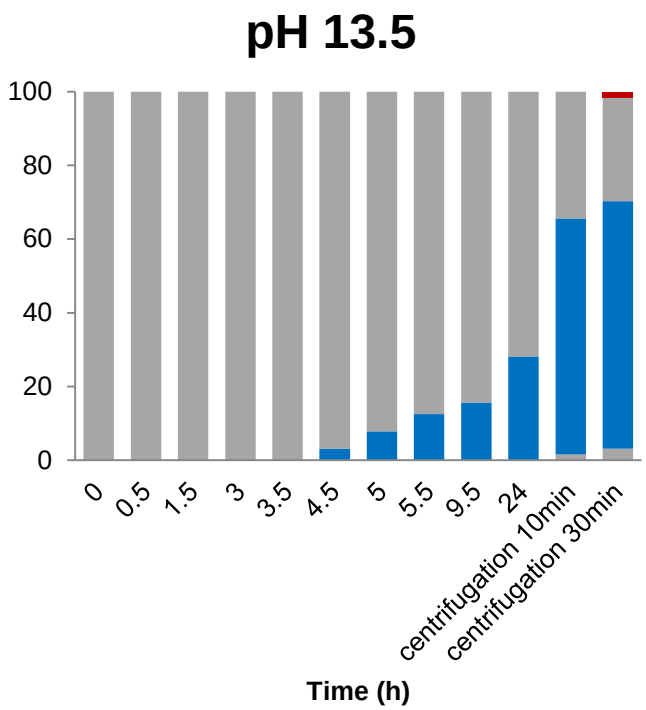

1 M NaCl
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3.5
4.5
24
centrifugation 10min
centrifugation 30min
Time (h)

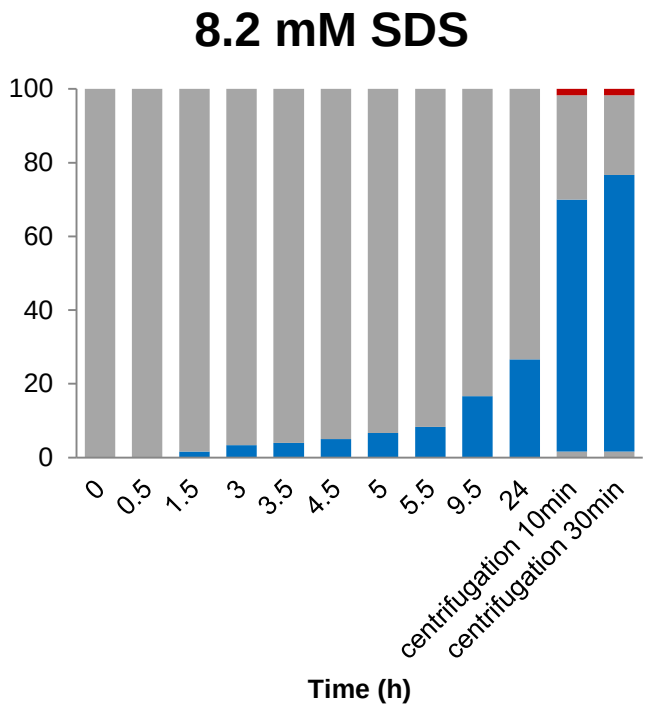

2 M NaCl
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3.5
4.5
24
centrifugation 10min
centrifugation 30min
Time (h)

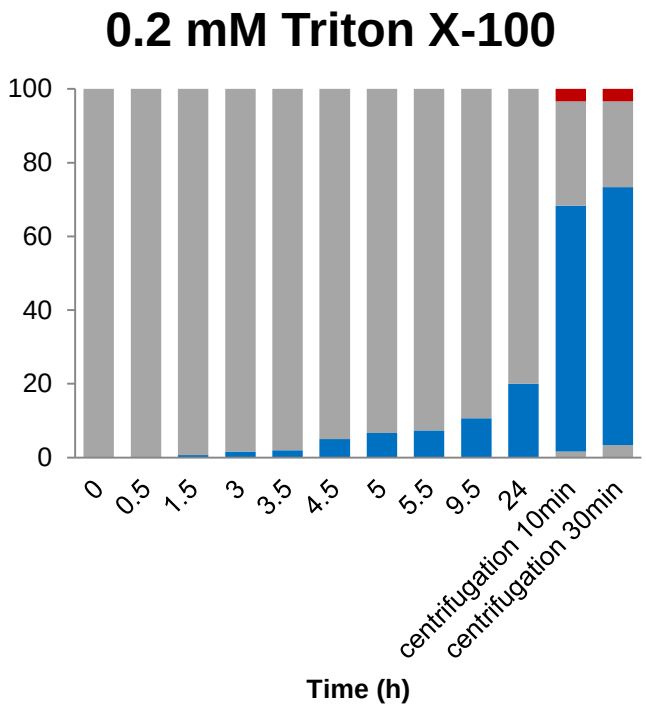

1 M Guanidine HCl
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3.5
4.5
24
centrifugation 10min
centrifugation 30min
Time (h)

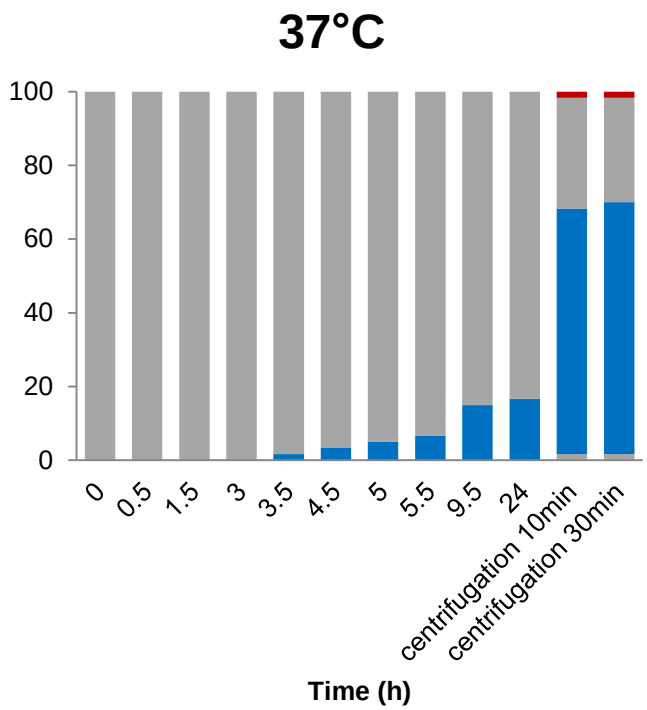

0.8 M Ammonium sulfate
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3.5
4.5
24
centrifugation 10min
centrifugation 30min
Time (h)

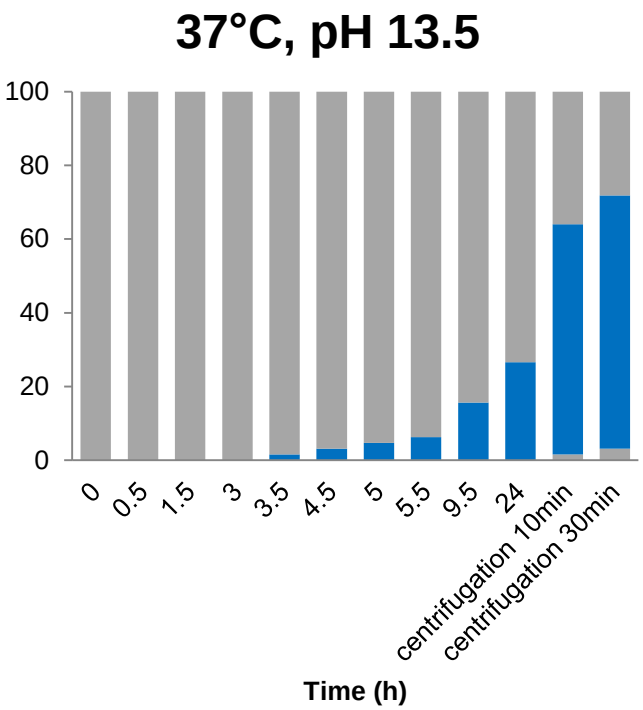

22°C
100
80
60
40
20
0
0
0.5
1.5
3
3.5
4.5
5
5.5
9.5
24
centrifugation 10min
centrifugation 30min
Time (h)

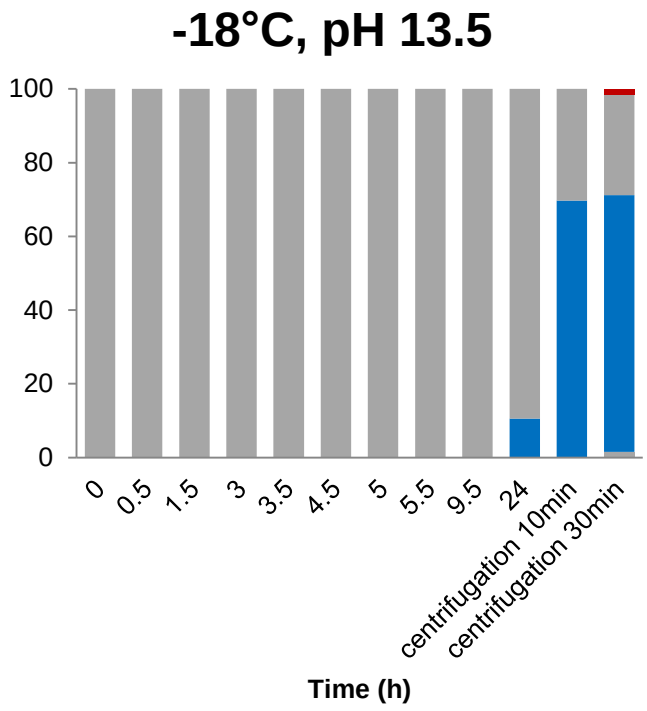

pH 13.5
100
80
60
40
20
0
0
0.5
1.5
3
3.5
4.5
5
5.5
9.5
24
centrifugation 10min
centrifugation 30min
Time (h)


8.2 mM SDS
100
80
60
40
20
0
0
0.5
1.5
3
3.5
4.5
5
5.5
9.5
24
centrifugation 10min
centrifugation 30min
Time (h)


0.2 mM Triton X-100
100
80
60
40
20
0
0
0.5
1.5
3
3.5
4.5
5
5.5
9.5
24
centrifugation 10min
centrifugation 30min
Time (h)


37°C
100
80
60
40
20
0
0
0.5
1.5
3
3.5
4.5
5
5.5
9.5
24
centrifugation 10min
centrifugation 30min
Time (h)


37°C, pH 13.5
100
80
60
40
20
0
0
0.5
1.5
3
3.5
4.5
5
5.5
9.5
24
centrifugation 10min
centrifugation 30min
Time (h)


-18°C, pH 13.5
100
80
60
40
20
0
0
0.5
1.5
3
3.5
4.5
5
5.5
9.5
24
centrifugation 10min
centrifugation 30min
Time (h)