Electronic Supplementary Information

Temperature-responsive extraction of violacein using a tuneable anionic surfactant-based system

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METHODOLOGY

Chemicals and biomass

The aqueous systems studied in this work were composed of pre-established molar ratios of sodium dodecyl sulfate (99% purity from Acros Organics) and tetrabutylammonium chloride (97 % purity from Sigma Aldrich). Analytical grade ethanol (Fisher Scientific) and Triton X-114 (Acros Organics) were used as a representative organic solvent and non-ionic surfactant respectively for comparative purposes during solid-liquid extraction and cloud point separation (for Triton X-114). L(-)-menthol (99.5 % purity) and thymol (99.0 % purity) were obtained from Acros Organics and their confirmed by ^{1}H and ^{13}C NMR analysis. All chemicals were used as received. Ultrapure, double distilled water, passed through a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus, (18.2 MΩ.cm at 298 K) was used for all experiments. Violacein standard extracted from *Janthinobacterium lividum* (98 % purity, Sigma Aldrich) was used to obtain a UV-Vis calibration curve for violacein.

The yeast *Y. lipolytica* strain JMY7019 carrying the expression cassettes *URA3ex*-VioABE and *LEU2ex*-VioCD contained the *E. coli* codon optimized genes of the vioABCDE cluster of *Chromobacterium violaceum*. For biomass production, JMY7019 cells were pre-grown in minimal media YNB supplemented with tryptophan (25 $mg.L^{-1}$) for two days at 301 K with constant shaking. YNB is composed of 0.17 % (w/v) yeast nitrogen base (without amino acids and ammonium sulfate, YNBww, Difco), 0.5 % (w/v) NH_4Cl, and 50 mM KH_2PO_4/Na_2HPO_4 buffer (pH 6.8). The pre-culture was used to inoculate minimal enriched media YNBD3YP which consist of YNB with glucose (3 %), Yeast extract (0.05 %) and peptone (0.05 %) supplemented with tryptophan (25 $mg.L^{-1}$) for five days at 301 K with constant shaking.

Instrumentation

All tests were performed in triplicate with the average value reported. System compositions were determined by the weight quantification of all components added within an uncertainty of $\pm 10^{-3}$ g. Total protein and violacein concentrations were determined by UV-Vis analysis using a Biotek Synergy HT microplate reader. $[N_{4444}]DS$ concentration in the aqueous phase and HES phase after phase separation and violacein back extraction respectively was analysed by quantitative ^1H-NMR with benzene as internal standard (300 MHz Bruker Avance III spectrometer). Viscosities were measured at atmospheric pressure and in the temperature range 283 to 298 K using an automated SVM 3001 Anton Paar rotational Stabinger viscometer–densimeter. The uncertainty of temperature is ±0.02 K and the relative uncertainty of the dynamic viscosity is ±0.35 %. Chloride concentration of the surfactant-rich phase after extraction was determined using a Metrohm 904 Titrando with a chloride electrode. An 831 KF Coulometer (Metrohm) was used to determine the water content of the surfactant-rich phase. Solution conductivity was monitored using a SevenExcellence multiparameter pH and conductimeter (Mettler Toledo). Samples were agitated during solid-liquid extraction using a temperature-controlled Eppendorf thermormixer C. Violacein and protein concentrations were calculated by UV-Vis analysis using a Synergy HT microplate reader from BioTek.

Cloud point and DLS measurements

The cloud points of the [N_{4444}]Cl-SDS systems at various surfactant to salt ratios were measured using the cloud point methodology. The cloud point temperature of each mixture was visually determined by monitoring the solution onset turbidity with temperature. The systems were heated from 274 K to 372 K at a ramping rate of 0.5 $K.min^{-1}$ in a temperature-controlled water bath with a precision of ±0.01 K (ME-18 V Visco-Thermostat, Julabo).

Dynamic Light Scattering (DLS) and zeta potential measurements were measured using a Malvern Zetasizer Nano-ZS in a temperature controlled cell. Each system was analysed a minimum of five separate times over a 2 hr period to ensure that equilibration was achieved, with the average values reported. All data was analysed by the software DTS v 7.03. Measurements were performed using ultrapure water and left to equilibrate for 30 min at the tested temperature prior to measurements. As part of DLS analysis, samples were irradiated with a red light (HeNe laser, wavelength of 565 nm) and the intensity fluctuations of the scattering light were detected at a backscattering angle of 173° to generate an autocorrelation function.

Solid-Liquid extraction of violacein from Y. lipolytica

The cell suspension was homogenised with aqueous solutions of [N_{4444}]Cl-SDS to test its ability to release violacein under different conditions. SDS and [N_{4444}]Cl were independently used as controls. A fixed time of extraction of 30 minutes and solid-liquid ratio (SLR) of 0.025 (mass of wet cells (in g) *per* volume of solvent (in mL) was established for all extractions. Multiple factors were studied to assess their impact on violacein and total protein extraction, including the [N_{4444}]Cl to SDS ratio, extraction temperature and the [N_{4444}]Cl-SDS concentration (at equimolar ratio of [N_{4444}]Cl to SDS). Unless otherwise specified, standard extraction conditions were at 293 K, an equimolar [N_{4444}]Cl to SDS ratio, a concentration of 0.2 M and a total system volume of 1 mL. The biomass was placed in contact with aqueous solutions of surfactant and the samples were subjected to constant stirring (1000 rpm) for 30 minutes in a temperature-controlled thermomixer. The extractions performed in this work were carried in the dark to better preserve the violacein stability (Ahmad et al., 2012). After the extraction, the samples filtered using Millipore seringe filters (pore size 0.45 µm)

to separate the cell debris from the aqueous solutions rich in violacein. The resultant pellet was discarded while the violacein-rich aqueous supernatant was collected, and its absorption spectra determined between 200-700 nm in a UV-Vis microplate reader (Synergy HT microplate reader – BioTek). The violacein content was quantified at the violacein maximum peak of absorbance observed, 571 nm. The quantification of the protein contaminants was also assessed by UV-Vis using the PierceTM BCA Protein Assay Kit. All the extractions were carried in triplicate, being the results presented as the average of the three. For comparative purposes, the same procedure was also carried out using 0.1 M Triton X-114 at 293 K under identical experimental conditions as those described.

Cloud point extraction and separation of Violacein

The extracted violacein present in the aqueous solutions of $[N_{4444}]$Cl-SDS was further concentrated by heating the samples above their cloud point (323 ± 1 K), inducing the formation of two liquid phases. The aqueous solutions were kept in an air oven at the desired temperature for *ca.* 2 h, to reach the equilibrium. The two phases were separated, and the volume of each phase recorded. The violacein and total protein content in the aqueous phase was evaluated through UV-Vis spectrophotometry using the methodology described previously. The small volumes of the surfactant-rich phase prevented their reproducible quantification, with only the aqueous phase measured and the extraction efficiency (EE) estimated by mass balance as presented in equation (1). At least three independent replicas were prepared, being the results presented as the average of the three.

$$\%EE = \frac{([M]_{aq,i} - [M]_{aq,f})V_{aq,i}}{[M]_{aq,i} \quad V_{aq,f}} \times 100 \qquad \text{- (1)}$$

Where $[M]_{aq}$ is the concentration of either violacein of total protein of the aqueous phase ($\mu g.mL^{-1}$), V_{aq} is the volume of the aqueous phase (mL) and the subscripts 'i' and 'f' denote the systems before and after phase separation at 323 K.

Back-extraction of violacein using natural hydrophobic eutectic solvent (HES)

The hydrophobic eutectic solvent (HES) used as back extraction solvent is a binary mixture of menthol with thymol prepared in a 1:1 molar ratio composition. The mixture was heated

under agitation at a temperature 10 K above the melting of pure thymol (333K) until a homogenous liquid was formed and stirred in the liquid state for 30 minutes.

Following the solid-liquid extraction of violacein under optimised conditions (T= 298 K, an equimolar $[N_{4444}]Cl$ to SDS ratio, concentration of 0.1M) and enrichment by cloud point separation at 323 K, the violacein-rich phase was isolated and re-diluted using ultrapure water to 1 mL. The violacein-rich aqueous solution was agitated with a 1:1 menthol:thymol solution at an organic:aqueous phase ratio of 0.5 and room temperature for 5 min. The solution was centrifuged at 10000 rpm and the separated phase isolated. Complete violacein extraction was confirmed by UV-Vis analysis of the aqueous phase. Partition of $[N_{4444}]DS$ to the HES phase was determined by quantitative ^{1}H-NMR. The HES phase was further scrubbed using a 0.5 M KCl solution (organic: aqueous phase ratio of 0.2) to precipitate co-extracted anionic dodecyl sulphate.

REFERENCES

Ahmad, W.A., Yusof, N.Z., Nordin, N., Zakaria, Z.A., Rezali, M.F., Production and characterization of violacein by locally isolated chromobacterium violaceum grown in agricultural wastes. Appl Biochem Biotechnol., 2012, 167, 1220–1234.

Martins, M., Wei Ooi, C, Neves, M.C., Pereira, J.F., Coutinho, J.A.P., Ventura, S.P., Extraction of recombinant proteins from Escherichia coli by cell disruption with aqueous solutions of surface-active compounds. J. Chem. Technol. Biotechnol., 2018; 93, 1864–1870.

Rettori D, Dura N. Production, extraction and purification of violacein: an antibiotic pigment produced by Chromobacterium violaceum. World J. Microbiol. Biotechnol., 1998, 14, 685-688.

FIGURES

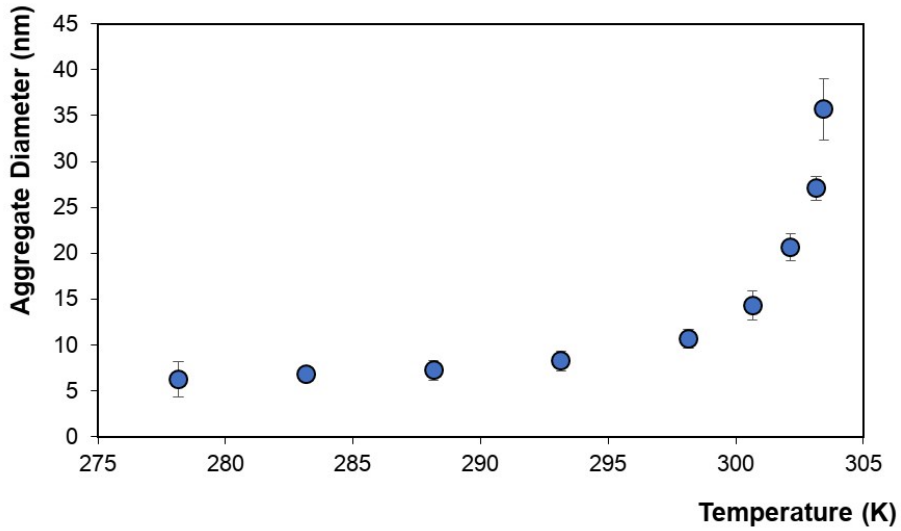

Figure S1. Average aggregate diameter as a function of temperature for the 1:1 $[N_{4444}]Cl$:SDS system ([SDS]=0.2M). **Figure 1** in the manuscript indicates this system undergoes phase separation at 304.9 K.

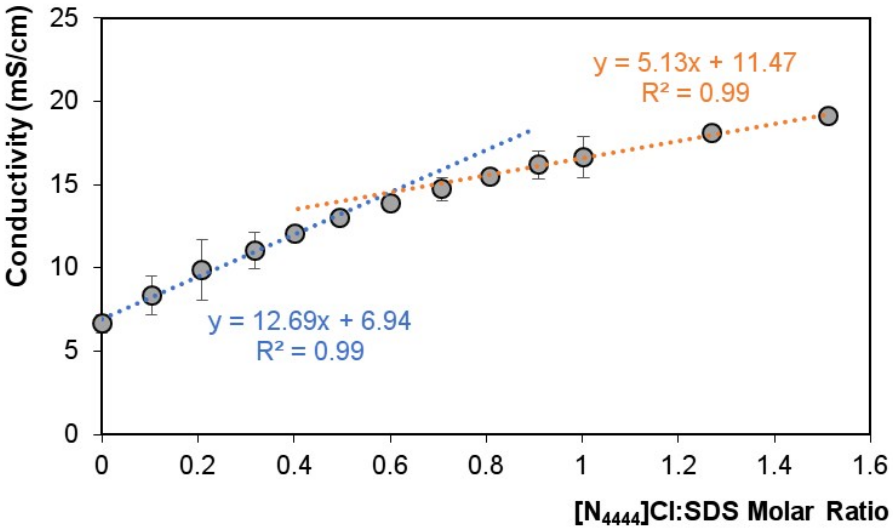

Figure S2. Conductivity of aqueous mixtures for varying $[N_{4444}]Cl$ to SDS molar ratios ([SDS]=0.2M, T= 293 K).

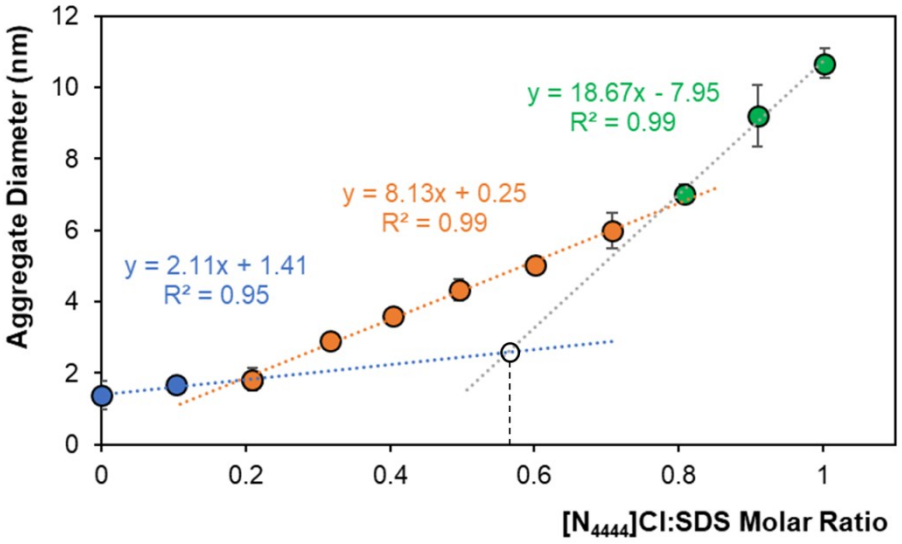

Figure S3. Aggregate diameter in aqueous mixtures for varying $[N_{4444}]Cl$ to SDS molar ratios ([SDS]=0.2M, T=293K).

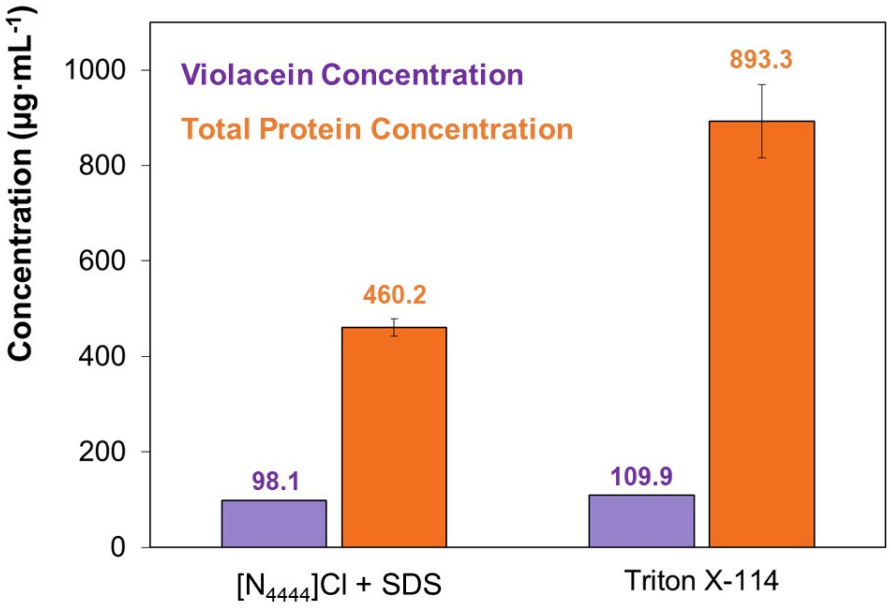

Figure S4. Final violacein and total protein concentration in the final extraction solution of equimolar 0.1 M $[N_{4444}]Cl$-SDS or 0.1 M Triton X-114 at 293 K. The final values are listed above each bar. A different *Y. lipolytica* culture was used to that employed for the extraction tests in Figure 2 of the manuscript, explaining the greater obtained values for violacein and protein extraction in the $[N_{4444}]Cl$-SDS system compared to Figure 2.

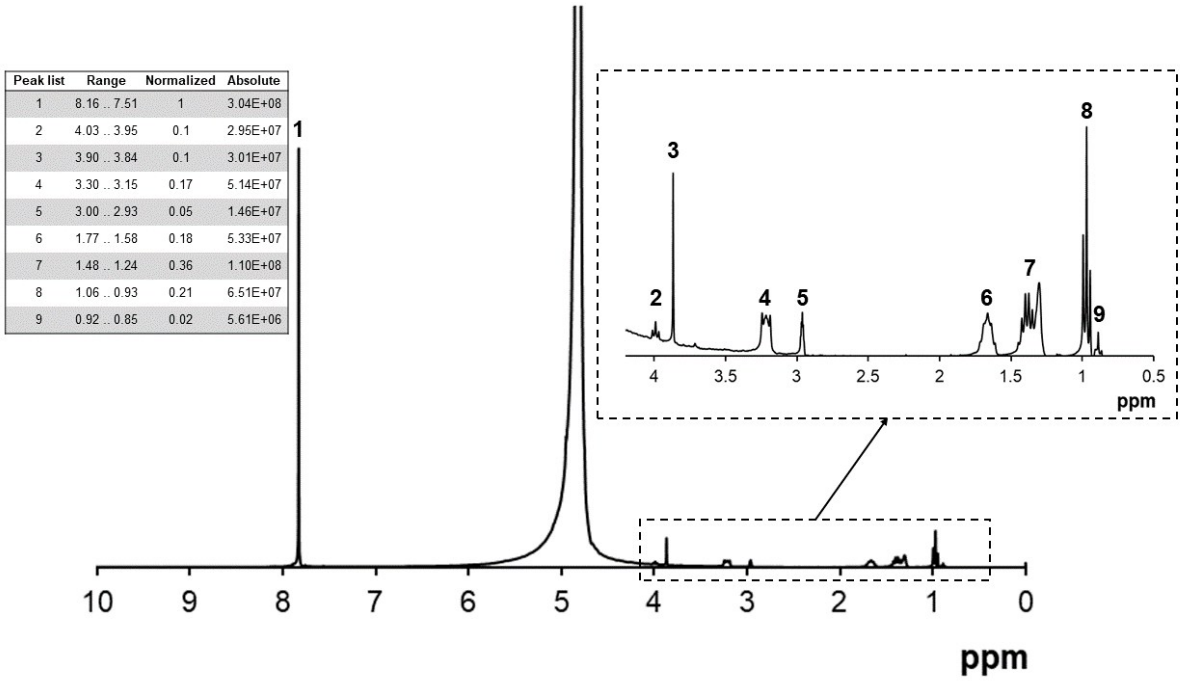

Figure S5. Quantitative ^{1}H-NMR analysis of the aqueous phase after phase separation at 323 K for the [N_{4444}]Cl-SDS system (0.1 M, equimolar ratio) in the absence of violacein with benzene as internal standard. All peaks were standardized with respect to benzene (δ=7.8 ppm – peak 1, [benzene]= 3.96 x 10^{-5} mol, 6 hydrogen) and adjusted by the number of hydrogen corresponding to each peak and the dilution factor (x3). $[N_{4444}]^{+}$ and $[DS]^{-}$ concentrations where estimated from their respective CH_3 peaks at δ=1.02 ppm (12 hydrogen, peak 8) and δ=0.9 ppm (3 hydrogen, peak 9).

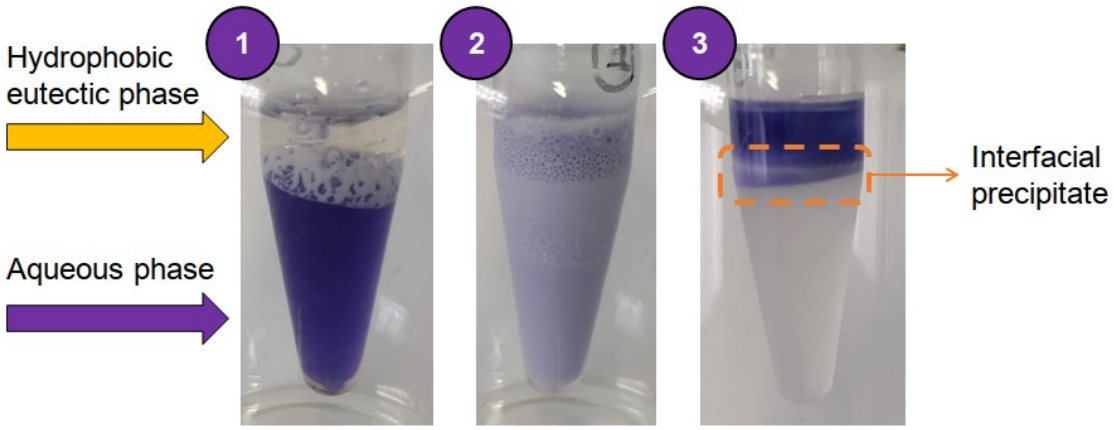

Figure S6. Illustration of violacein back-extraction from the diluted surfactant-rich phase using a 1:1 menthol:thymol eutectic solvent .

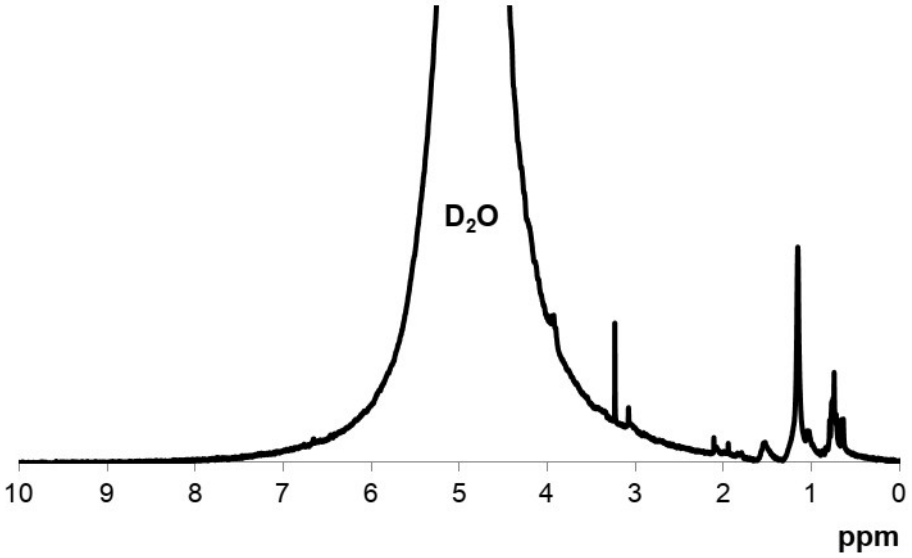

Figure S7. ^{1}H-NMR spectra of the aqueous phase after violacein back extraction into the thymol-menthol HES (organic to aqueous phase ratio =0.5) following cloud point separation in the [N_{4444}]Cl-SDS system (0.1 M, equimolar ratio). Peak identification of the thymol-menthol HES is presented in Figure 4 of the manuscript.