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Downstream Processing of Biosurfactants

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Sophorolipids (SL) are a promising biosurfactant due to their biodegradability and broad range of application in cleaning, cosmetics or agriculture. Downstream processing of biotechnological products is often challenging because of the complex mixture of many components in low concentrations, unknown thermodynamic data and high water content. With regard to sophorolipids, mainly poorly water-soluble lactonic SL can be produced in high concentrations of up to 400 g/L so that a product-rich phase is formed during cultivation. Consequently product isolation and purification can be achieved very easily by phase separation and subsequent washing steps. To optimize those process steps, the solubility of SL in water and protein removal was measured and implemented in an equilibrium stage model. The solubility of SL decreases with higher temperature, whereas the separation of proteins could not be investigated at high temperatures due to denaturation. Instead the distribution of proteins was determined at lower temperatures and used for the whole temperature range in the model. Based on this data, the optimal washing process to remove 90 % of protein consists of three stages and a product-phase to water ratio of 4:1 (w:w) per stage.

1. Introduction

The production of sophorolipids (SL) by non-pathogenic *Candida species* was already discovered in 1961 [Gorin, 1961] and their biosynthesis was investigated more closely in the past years by many groups. Basically, SL consist of the hydrophilic moiety sophorose - a glucose disaccharide - and a fatty acid as the hydrophobic moiety. Usually a mixture of SL is produced, where two main classes can be discriminated, internally esterified (closed) lactonic and acidic (open) SL. Additionally the sophorose can be acetylated and the fatty acid chain length and saturation may vary depending on the hydrophobic substrate. Due to the different structure, acidic and lactonic SL differ in their properties. Acidic SL are more water-soluble and have a better foam performance compared to lactonic SL, which are poorly water soluble and can crystallise [Van Bogaert, 2007]. Both show a strong effect on surface tension of water and decrease it to 30 - 40 mN [Ashby, 2008].
The focus of previous work was primarily on the impact of cultivation conditions and different substrates on the productivity and product spectrum. High concentrations of up to 400 g/L can be produced if renewable resources like glucose as the hydrophilic and vegetable oil as the hydrophobic building block are fed under growth limiting conditions in fed-batch mode. Due to the high concentration and low solubility, a second SL-rich phase is formed, which consists of approximately (app.) 50 % water and a mixture of app. 40 % lactonic and 10 % acidic SL [Pekin, 2005]. This product-phase is heavier than the cultivation suspension and settles quickly after turning off the stirrer as an oily viscous fluid phase. Consequently product isolation from the cell containing cultivation broth can be achieved easily by phase separation.

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Depending on the point of cultivation termination the product phase contains not converted oil and fatty acids, which have a negative effect on the foaming properties. Additionally co-settled cells and secreted proteins can also be present, which have to be separated since they may lead to allergic reactions if the product is used as a detergent. The separation of cells and hydrophilic components can be achieved by washing the product-phase with water. Important for the design of the washing process are the removal of impurities and the loss of product to the washing water to maximise purity and yield. With respect to the phase behaviour of SL, thermodynamical modelling is not applicable, since too many components with unknown properties are present. Additionally SL form micelles and superstructures in solution, which affects the phase behaviour in a very complex way. Since no solubility data of SL in water is published up to now, it is investigated in equilibrium experiments. To take the complex mixture of different SL and their amphiphilic and emulsifying properties into account, an untreated product-phase was used. Only in the context of crystallisation some solubility data of SL in ethanol and aqueous buffer solutions is published [Hu, 2001]. Increasing solubility from 0.12 – 0.62 g SL / g ethanol was measured in ethanol within the range of 10 – 45 °C. The temperature dependence of SL solubility in aqueous buffer was not measured; merely the effect of pH in the range of 4.0 to 8.0 at 25 °C was investigated and resulted in solubility of 0.017 – 0.025 g SL / g buffer.
Protein removal is used as the benchmark to evaluate the washing process, since cells as particulate matter are not properly measureable in equilibrium experiments due to their co-settlement. The acquired results are then used in a cross-flow equilibrium model to simulate the washing process under different conditions. Using this model, the process was analysed with respect to temperature, number of stages and amount of water.

2. Materials and Methods

2.1 Equilibrium experiments for SL solubility

The SL-rich product-phase was produced by cultivation of *Candida bombicola* ATCC 22214 in a two-step fermentation process according to [Casas, 1999]. First biomass is produced in a nitrogen-rich medium and harvested by centrifugation after 24 h. The cells are then resuspended in a nitrogen-lean medium, containing 100 g/L of each glucose and rapeseed-oil. After a cultivation of five to seven days the substrates are depleted and the product-phase is separated from the cultivation broth by sedimentation.
Preliminary tests were conducted to verify that there is no difference in SL concentration between deionised water and cultivation broth. The influence of pH on SL solubility was investigated by varying the pH of the washing water (3; 3.5; and 6.5). In addition it was tested if equilibrium is achieved and phase settling completed after 24 h. To investigate the effect of the amount of used washing water, different ratios of product-phase and washing water were weighed in extraction vessels, which allow both mixing and phase separation. Table 1 summarises the investigated product-phase to washing water ratios, which were measured in parallel at all temperatures.

Table 1: Conditions of equilibrium experiments.

Mass ratio PP: H_2O [g/g]	5:1	3:1	2:1	1:1	1:2	1:3
Temperature [°C]	20	30	40	50	60	70

Vessels were placed in a tempered water bath and mixed with a stirrer speed of 300 rpm. After one hour the vessel was turned and equilibrating and phase settling could proceed within 24 h under tempered conditions. Phase separation was achieved by a burette and samples were taken for HPLC analysis, measuring the water content of the product-phase by titration and the protein content with the Popov assay.

2.2 Analytics

To quantify the composition of the product-phase and the washing water, samples were analysed in duplicate by HPLC (Agilent 1200 Series). Samples of the product-phase were diluted in a mixture of

acetonitrile and water (v/v= 4:1), since it was too concentrated and viscous for direct injection. Samples of the water phase could be analysed without dilution. All samples were filtered through a 0.45 µm filter. Due to the similarity of the components a gradient of acetonitrile and water was necessary to separate them on a Zorbax Eclipse XDB – C18 column (diameter: 4.6 mm, lenght: 50 mm, particle: 1.8 µm) at 40 °C. A constant flow of two mL/minute is used for elution. Starting with 95 % water the fraction of acetonitrile is increased to 100 % within four minutes. After two minutes of eluting with 100 % acetonitrile the initail composition is reached within one minute. For detection an UV detector was used at a wavelengh of 210 nm. External standards were used for quantification of lactonic and acidic SL seperately.
Automatic titration (Mettler Toledo DL 31) according to [Fischer, 1935] was applied to measure fivefold the water content of the product-phase. The measurement of the proteins in surfactant containing solutions is challenging due to micelles, which falsify the spectroscopic measurement. Therefore the Popov assay was used, since here proteins are precipitated and separated from the matrix by centrifugation [Popov, 1975]. After two washing steps the protein pellet is resuspended in 0.1 M NaOH and analysed at 620 nm (Shimadzu UV mini 1240). Quantification was done with an external standard of bovine serum albumin. Nevertheless a measurement of the cloudy product-phase was not possible due to solubility problems and only the aqueous phase could be analysed in triplicate with good reproducibility.

2.3 Model for simulation studies

A cross-flow equilibrium-stage model was used for modelling the washing process. Necessary input parameters are the amount and composition of the entering product-phase, the temperature and the amount of pure washing water. The SL concentrations of the exiting streams are calculated by using the experimentally acquired correlation, overall mass balance and SL balance. Other component concentrations are calculated by using distribution coefficients and corresponding component balances.

3. Results and discussion

3.1 Equilibrium experiments for SL solubility

Preliminary tests showed that there is no difference in the composition and concentration of SL in aqueous phases of cultivation broth and pure water. No change in composition of the aqueous phase was detected after six hours. Anyway phase separation was conducted after 24 h for all following experiments. The pH showed no influence on SL solubility so that all other experiments were conducted with unmodified deionised water (pH of 6.5).
An influence of different phase ratios could not be demonstrated; therefore the average values of the conducted experiments at different ratios are depicted in Figure 1. In the investigated temperature range two main aspects of the phase behaviour of a SL product-phase could be observed. First the solubility of SL in water decreases with increasing temperature (Figure 1 left). Contrary to the expected composition there is no higher concentration of acidic SL in the aqueous phase compared to lactonic SL. The ratio of lactonic to acidic SL in the product-phase is app. 4.5 to 1 and only app. 2.5 to 1 in the aqueous phase. Considering the total amount of SL, the concentration in the aqueous phase increases from 0.8 w% at 70 °C to 1.6 w% at 30 °C, which is in the range of the results published by Hu (0.017 – 0.025 g SL / g buffer at 25 °C). The corresponding SL concentration in the product-phase decreases from 52.7 w% at 70 °C to 49.3 w% at 30 °C. Measuring the water content of the product-phase confirms this behaviour, showing an increase from 41.6 w% at 70 °C to 53.6 w% at 30 °C. This behaviour implies a lower critical solution temperature, which cannot be determined due to superposed crystallisation taking place below 30 °C.
Crystallisation has a strong effect on SL concentration, which is illustrated in Figure 1 considering the solubility of SL at 20 °C. A significant increase of total SL of app. 5 w% is observed in the aqueous phase. The difference is even larger in the product-phase, a decline of app. 12 w% compared to the total solubility at 30 °C is measured. The ratio of lactonic to acidic SL is increased to 6.4 to 1 in the

product-phase, indicating an enrichment of lactonic SL due to selective crystallisation; although the ratio of lactonic to acidic SL remains app. the same in the aqueous phase.

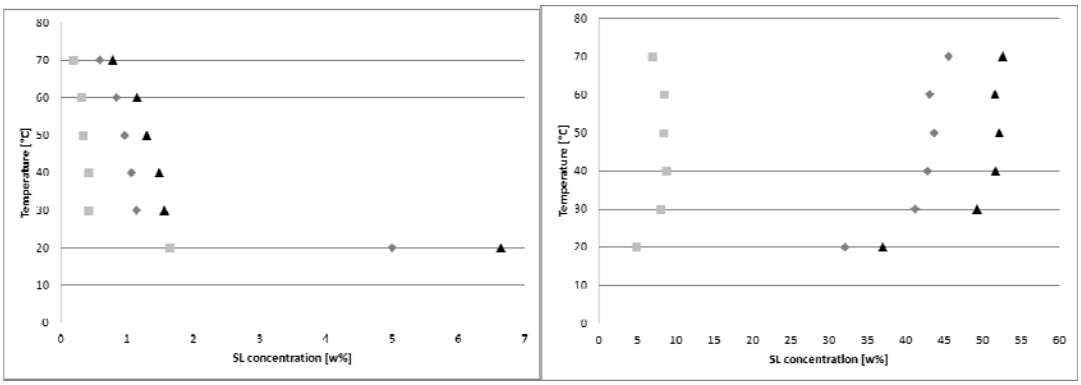

Figure 1: Solubility of SL in aqueous (left) and product-rich phase (right). ▪ acidic SL; ◆ lactonic SL ▲ total SL

The removal of proteins could be observed by comparing the protein concentration in the cultivation broth and the aqueous phases of different washing steps (Table 2). The initial protein concentration of app 0.4 g/L is reduced to app. 0.1 g/L, if a phase ratio of 1:1 is used, indicating the reduction of protein in the product-phase, even though a direct measurement is not possible due to the afore mentioned solubility problems. A pH-dependence of the protein removal could not be shown (Table 2) so that experiments investigating the phase ratio were conducted with unmodified deionised water (pH of 6.5). If less water is used, the protein concentration increases (ratio of 3:1) since the less water is available but absolute removal is better, if more water is used (ratio of 1:1). Temperature dependence could not be measured since protein denaturation occurred at temperatures higher than 40 °C and resulting amino acids are not detectable with the Popov assay. Therefore the results of the washing steps at 30 and 40 °C were averaged and used for the calculation of protein distribution-coefficients. An average value of 3.1 was determined for all investigated phase ratios (data not shown).

Table 2: Protein removal under different washing conditions.

		washing water (45 °C, 1:1)			washing water (40 °C, pH 6.5)		
	Cultivation broth	pH 3	pH 3.5	pH 6.5	3:1	2:1	1:1
Protein [g/L]	0.398	0.104	0.097	0.107	0.217	0.178	0.115
Removal [%]		50.9	51.8	57.1	51.1	58.8	75.6

3.2 Simulation studies

In our simulation studies with the equilibrium stage model the feed of the washing process is a cell-free product-phase, since cells cannot be considered in the model, with the composition depicted in Table 3. Oil and fatty acids are only considered here for successive purification steps, their removal in the washing step is marginal due the low solubility in water. The temperature range is limited to 30 °C to 70 °C, since the significant increase in solubility due to crystallisation would lead to major losses of SL and is therefore not covered by the correlation.

Table 3: Composition of raw product-phase.

Component	SL	Water	Oil	Fatty acids	Protein
Concentration [w%]	44	51.5	0.985	3.5	0.015

Before analysing the washing process with respect to stage number and amount of washing water, model validation is necessary. Therefore experimental results are compared with corresponding modelling results. Figure 2 shows exemplarily the comparison of experimental and simulated water concentrations in a washed product-phase. In the considered temperature range all simulated results show good agreements (< +/-10 %) with the experimental results.

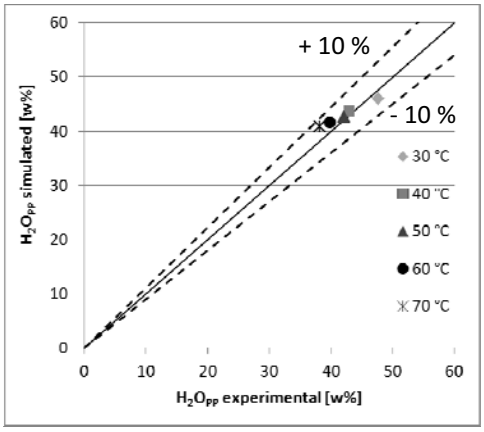

Figure 2: Parity plot of experimental and simulated water concentration in the product-phase.

The objective for the washing process is 90 % removal of the containing proteins, which is with 0.015 w% already low, while minimising the loss of SL. Figure 3 (left) depicts the influence of product-phase to washing water ratio and stage number on protein removal at 70 °C. Due to the decrease in solubility at high temperature, the maximum temperature should be used to maximise the yield of SL in the washing process. It is impossible to remove 90 % of protein with only a single stage, whereas it is possible with two stages to reduce the product-phase to washing water ratio of 2 to 1 per stage and fulfill the requirement. A further reduction of used washing water is possible, if more stages are employed. This is advantageous, since the loss of SL depends only on the total amount of used washing water and is minimised concomitantly. Using a ratio of 2 to 1 per stage results in a total ratio of 1 to 1 and a loss of SL of 2.8 %. If a three stage process with a ratio of 4 to 1 in each stage, resulting in 1.33 to 1 in total, is applied instead, the SL loss can be reduced to 2.2 % (Figure 3 right). Thus this is the optimal setup for the washing process, considering protein removal, minimal loss of SL and processible phase ratios.

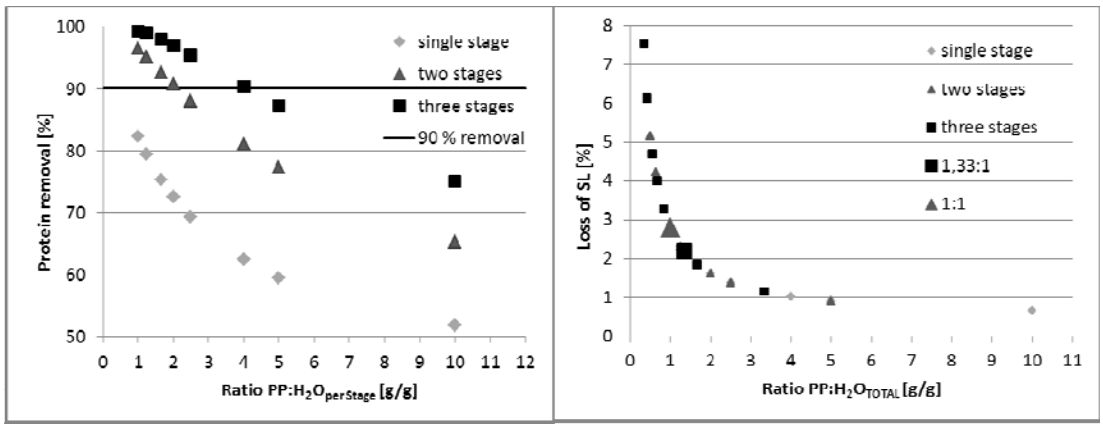

Figure 3: Protein removal depending on product-phase to washing water ratio per stage at 70 °C (left). Loss of SL depending on product-phase to total washing water ratio at 70 °C (right).

4. Conclusion

The solubility experiments with product-phase showed no higher solubility of acidic SL in water compared to lactonic SL. Although a decrease of the ratio of lactonic to acidic SL in the aqueous phase of 2.5 to 1 compared to 4.5 to 1 in the product-phase is observed, lactonic SL are more soluble – probably due to their higher concentration in the product phase. Those observed ratios were constant for all conducted experiments. This might be explained by the complex mixture of different SL in the product-phase and resulting long and short range order within it. Acidic SL can be emulsified by or rather coordinated with lactonic SL in the product-phase instead of solubilized in the washing water. The effect of the coordination in the product phase is also backed by the increased solubility of both SL when selective crystallisation of lactonic SL takes place. It might be, that the increase of the water solubility of the lactonic SL form is due to the absence of the acid form. The interactions in this multi-component system might also be the explanation for the contradictory results of Hu and Ju [Hu, 2001]. A higher solubility was observed at elevated temperatures in contrast to decreasing solubility in this work. They used a different crude product with 95 % lactonic SL, which is due to different cultivation conditions and substrate hexadecane. Furthermore this crude product was purified by solvent extraction with ethyl acetate and hexane, resulting finally in a water-free product.
With respect to the washing process the observed behaviour, decreasing solubility at high temperatures, is favourable since product isolation and purification can be achieved with high yield of SL. Simulation studies showed that a three stage washing process can remove 90 % of initial protein (and other hydrophilic components and cells) with minimal loss of SL of 2.2 %. Crystallisation should be avoided due to higher losses of SL in the spent liquor.

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