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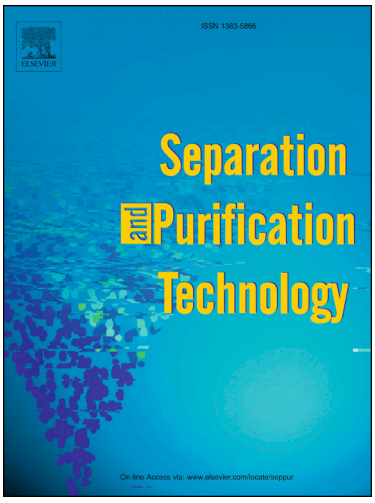

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Downstream separation and purification of succinic acid from fermentation broths using spent sulphite liquor as feedstock

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Abstract

Spent sulphite liquor produced as side stream from sulphite pulping of *Eucalyptus globulus* hardwood could be used for the separation of lignosulphonates by nanofiltration in the retentate stream and succinic acid production via fermentation of the permeate stream by *Actinobacillus succinogenes* or *Basfia succiniciproducens*. The potential integration of this process in conventional pulp mills towards the development of a novel biorefinery is dependent on the efficient downstream separation of succinic acid crystals at high yield and purity. This study focuses on the evaluation of five downstream separation processes, namely calcium precipitation, direct crystallisation using acidification or cation-exchange resins, salting-out and reactive extraction, for the purification of succinic acid from crude fermentation broths. Reactive extraction using trioctylamine in 1-hexanol and direct crystallisation coupled with cation-exchange resins led to succinic acid recovery yields of 73% and 79%, respectively. ^{1}H-NMR analysis showed that these downstream separation processes led to succinic acid crystal purities of *ca* 98.5% for reactive extraction and higher than 99% for the direct crystallisation method coupled with cation-exchange resins with no detectable acetic acid content when re-crystallisation was employed. It has been demonstrated that succinic acid produced via fermentation using side streams from pulp and paper mills could be separated at high purity and yield from crude fermentation broths rendering feasible its utilisation for poly(butylene succinate) production.

Keywords: succinic acid, spent sulphite liquor, downstream separation process, *Basfia succiniciproducens*, *Actinobacillus succinogenes*

1. Introduction

Succinic acid is an important platform chemical with various industrial uses as it is a precursor for the production of numerous products, such as 1,4-butanediol, resins, coatings, pigments and many others [1]. Succinic acid is conventionally produced in the petrochemical industry via hydrogenation of maleic anhydride followed by hydration or direct hydrogenation of maleic acid, in a process that requires heavy metals as catalysts, organic solvents and high temperature and pressure [2]. However, in the last decade, it is also produced by biological means. The market for bio-based succinic acid has potential to reach a capacity of 600,000 t by 2020 [3].

It has been demonstrated that spent sulphite liquor (SSL), a byproduct stream from the pulp and paper industry, can be used as fermentation feedstock for bio-based succinic acid production [4-7]. SSL is produced from the acidic sulphite pulping process and is rich in sugar monomers (mainly xylose) and lignosulphonates (LS). LS are products of lignin degradation and together with phenolic compounds present in SSL are inhibitory compounds in fermentation processes [4]. SSL constitutes an important feedstock for the bio-economy era as its annual production reaches 90 billion liters [8]. The combination of phenolic extraction followed by nanofiltration of SL for the removal of LS led to the production of a sugar-rich permeate that was used as fermentation medium for the production of 39 g/L succinic acid using the bacterial strain *Basfia succiniciproducens* [5]. The efficient production of succinic acid in a crude fermentation feedstock should be combined with the efficient recovery of succinic acid crystals at high purity and yield [9]. High recovery yields are required for the development of cost competitive bio-based succinic acid production processes. High purities are required especially for the production of biopolymers, such as the poly(butylene succinate). The fermentation broth occurred from cultures carried out on SSL-based media is

a multicomponent stream the composition of which could vary in wide proportions and thus succinic acid separation at high purity and yield is a challenging task.

The presence of monocarboxylic acids, such as acetic and formic acid that are produced as by-products during fermentation, inhibit the polymerisation process [10]. They act as chain stoppers during polymerisation preventing the polymer reaching high molecular weight, even if they are present in very low concentrations. For high quality and commercially viable poly(butylene succinate), its average molecular weight should be at least from 100 kDa to 200 kDa [11]. Poly(butylene succinate) with molecular weight of 200 kDa contains about 1160 units of succinic acid in an average chain. To reach this chain length, monomeric succinic acid should have concentration of the impurities capable to be chain stoppers of less than 0.09 mol%. The presence of a small amount of di-acids (e.g. maleic acid) or hydroxy-acids (such as lactic acid, 2- or 3-hydroxybutyric acid, etc.) should not affect the polymer significantly, as their incorporation into the growing poly(butylene succinate) chain does not stop the polymerisation. Therefore, it is important to develop an efficient downstream separation process (DSP) for the removal of impurities originated from both the SSL-based media and the microbial metabolism leading to succinic acid crystals with purity sufficient for further polymerisation, but not excessively prohibitive in costs.

Many DSP have been evaluated in the literature (e.g. calcium precipitation, reactive extraction, electrodialysis, and crystallisation coupled with acidification of succinate salts) towards the separation and purification of succinic acid [11-17]. In the traditional calcium precipitation process, $Ca(OH)_2$ is added in excess in order to precipitate succinic acid in the form of calcium succinate. Succinic acid is then released after the addition of H_2SO_4. This process results in high amounts of $CaSO_4$ and relatively low purities of the final product [12]. Reactive extraction using amines in an organic solvent has been reported in literature-cited publications [13-15]. In DSP based on electrodialysis, succinic acid is separated from the non-

ionised compounds that are present in the fermentation broth through ion-exchange membranes [16]. Crystallisation is based on the precipitation of succinic acid at low temperatures and it is mainly the final step after acidification or treatment with resins and evaporation [9,17]. The highest succinic acid recovery yield (89.5%) and purity (99%) have been achieved by Lin et al. [17] when fermentation broths of the bacterial strain *Actinobacillus succinogenes* cultivated on wheat-derived hydrolysate were used. Another recovery process is salting-out, which is applied in order to separate a hydrophilic compound from an aqueous solution using an organic solvent as an extractant and an inorganic salt as the salting-out reagent at appropiate concentrations [18]. Furthermore, Orjuela et al. [19,20] proposed simultaneous acidification and esterification of succinic acid from pure solutions and fermentation broths, that is recovered as a solution of free succinic acid, monoethyl succinate and diethyl succinate in ethanol, reporting around 95% recovery for both media. The number of unit operations employed in DSP for the purification of succinic acid could be achieved by developing coupled fermentation–separation system [21] or conducting fermentations with genetically engineered yeast strains at low pH [22].

The development of economically viable bioprocesses for succinic acid production can be achieved through refining of renewable resources including the production of value-added co-products. Biorefinery development based on SSL will generate fermentation broths with additional impurities, besides fermentation by-products and unconsumed nutrients. SSL is a low-value industrial side stream that can be fractionated into phenolic compounds and lignosulphonates, prior to fermentation of the remaining hemicellulose derived sugars to succinic acid [5]. This study focuses on the evaluation of five DSP in order to identify the most efficient process for the separation and purification of succinic acid from crude fermentation broths where nanofiltrated SSL was used as feedstock. The calcium precipitation method, salting-out, reactive extraction and direct crystallisation using acidification or cation-

exchange resins have been tested on simulated and actual fermentation broths. Succinic acid production was carried out by the bacterial strains *A. succinogenes* and *B. succiniciproducens*.

2. Materials & Methods

2.1. Chemicals, microorganisms and spent sulphite liquor

The bacterial strains *A. succinogenes* 130Z (DSM 22257) and *B. succiniciproducens* JF 4016 (DSM 22022) were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. Preservation of the microorganisms, strain precultures and fermentation conditions for succinic acid production were previously described [5,23].

The chemicals used in this study were purchased from Sigma-Aldrich. The thick SSL used in fermentation processes after pretreatment and recovered LS from the thick SSL stream were supplied by Sniace S.A. (Torrelavega, Spain). The LS were added in synthetic solutions in order to formulate simulated fermentation media and thus evaluate the separation of succinic acid by different DSP. The SSL was derived via the acidic sulphite pulping process using the hardwood *Eucalyptus globulus* and dolomite. The composition of the thick SSL (e.g. sugars, LS, phenolics, dry matter) used in this study has been presented by Alexandri et al. [5].

2.2. Evaluation of crystallisation duration

A solution containing 190 g/L of commercial succinic acid was prepared and heated to 80 ^{o}C in order to achieve complete dissolution. It was subsequently placed in a cooling chamber at constant temperature of 4 °C under continuous stirring. The decreasing temperature of the solution was monitored using an EasyLog USB (Lascar electronics, UK). Samples were taken every approximately 30 min for the analysis of the succinic acid concentration in the solution.

2.3. Succinic acid recovery process

Four succinic acid rich media, either synthetic solutions or actual fermentation broths (Table 1), were used for the evaluation of succinic acid separation and purification using the five DSP (Figure 1). The first medium used was a synthetic solution (medium 1) containing pure-commercial compounds that was prepared based on the fermentation results achieved by *B. succiniciproducens* when it was cultivated on pretreated SSL via liquid-liquid extraction for removal of phenolic compounds coupled with nanofiltration as it was reported by Alexandri et al. [5]. The second medium used was a simulated fermentation broth (medium 2) with the same composition of succinic, lactic, acetic and formic acids as in the case of medium 1 containing also 10 g/L of LS as in the case of actual fermentation broths produced by *B. succiniciproducens* cultivation on pretreated SSL as reported by Alexandri et al. [5]. The third medium used was an actual fermentation broth obtained from an *A. succinogenes* fermentation (medium 3) carried out using a synthetic medium containing mixed sugars at the same proportion as the one contained in SSL, as reported by Pateraki et al. [6]. The fourth medium was obtained from a *B. succiniciproducens* fermentation using pretreated SSL via nanofiltration, according to the methodology described by Alexandri et al. [5], as fermentation feedstock supplemented with yeast extract and various salts as sources of minerals (medium 4). For every test of DSP (besides reactive extraction), 100 mL of each solution was used in order to evaluate the recovery efficiency of succinic acid.

2.3.1. Calcium precipitation method

The calcium precipitation method used was based on the study of Luque et al. [9]. Specifically, 100 mL of fermentation broth (media 3 and 4) were centrifuged (15 min, 9,000 rpm, 4 ^{o}C) in order to remove the bacterial cells. The supernatant was then filtered through

Whatman No. 1 paper. The filtrate was mixed with 3% (w/v) activated carbon (medium 3) or 12.5% (w/v) (medium 4) for 1 h in order to decolorise the sample. The suspension was then filtered and the pH of the clear broth was adjusted to around 13.5 by adding approximately 20% (w/v) calcium hydroxide solution and the mixture was placed on a shaker at 200 rpm and 39 ^{o}C for 20 h. Precipitated calcium succinate was filtered off and washed with water. Distilled water was used and an excess of H_2SO_4 was added to form calcium sulfate. When the pH reached a value of around 2.5, the calcium sulfate precipitate was removed via filtration. The precipitate was washed twice with 40 mL distilled water in order to remove the remaining succinic acid. Vacuum evaporation was then carried out in order to remove the residual volatile carboxylic acids. The final solution was concentrated to 20% of its original volume and the crystallisation of succinic acid was carried out at 4 ^{o}C for 24 h. The crystals were filtered and washed with a cold saturated synthetic succinic acid solution and then dried for 12 h at 70 ^{o}C. Final yield and purity were calculated according to the following equations:

$$Recovery\ yield\ (\%) = \frac{Dry\ weight\ of\ recovered\ succinic\ acid\ (g)}{Dry\ weight\ of\ succinic\ acid\ in\ the\ initial\ liquid\ medium\ (g)} \quad \text{Eq.1}$$

$$Purity\ (\%) = \frac{Dry\ weight\ of\ the\ recovered\ succinic\ acid\ (g)}{Total\ dry\ weight\ of\ the\ recovered\ sample\ (g)} \quad \text{Eq.2}$$

2.3.2. Direct crystallisation method

The direct crystallisation method was applied in two synthetic media (media 1 and 2) and two actual fermentation broths (media 3 and 4). Fermentation broths (100 mL) were centrifuged (15 min, 9,000 rpm, 4 ^{o}C) to separate the bacterial cells. The supernatant was filtered through Whatman No.1 paper. Activated carbon was mixed with the filtrate for 1 h to decolorise the broth. The concentration of the activated carbon varied depending on the fermentation medium used. The suspension was then filtered and the clear fermentation broth

was treated either via acidification with H_2SO_4 or with cation-exchange resins. The resulting solution was vacuum evaporated at 60 ^oC to remove the volatile carboxylic acids, such as acetic acid and formic acid.

When synthetic media were used, the steps involving centrifugation, treatment with activated carbon and filtration were omitted. Acidification of the solutions was not required since the pH value was around 2. Vacuum evaporation was directly employed, which was carried out in the same way as in the case of actual fermentation broths.

The evaporation was terminated when each solution was concentrated to approximately 20% of its original volume. Subsequently, crystallisation of succinic acid was carried out at 4 ^oC for 24 h in all cases. The final slurry was filtered through Whatman No.1 paper and the crystals were carefully washed with a saturated synthetic solution of succinic acid to remove impurities. The succinic acid crystals were dried at 70 ^oC for 12 h.

2.3.2.1. Direct crystallisation method with acidification

This method was tested on the two actual fermentation broths (media 3 and 4). The pH of the actual fermentation broth was adjusted to 2.0 using 98% sulfuric acid. Vacuum evaporation and crystallisation were then carried out as previously reported.

2.3.2.2. Direct crystallisation using a cation-exchange resin.

The conversion of the sodium and magnesium salts into acids was carried out using a cationic resin of sulfonic (SO_3H) type, Amberlite IR 120H, based on a polystyrene-divinylbenzene copolymer, according to the process developed by Lin et al. [17]. The fermentation broth (100 mL) was passed through 50 g of resin. The pH of the effluent was 2.0. The acidified medium was then vacuum evaporated, crystallised and dried as described in section 2.3.2. This method was employed on media 3 and 4.

When re-crystallisation was carried out in the case of medium 4, the crystals obtained after washing were dissolved in ultrapure water. The volume of the solution was the same as the one obtained by vacuum evaporation. Using heating and stirring they were fully dissolved and then subjected to crystallisation. Washing and drying was carried out as previously described. Re-crystallisation was carried out only in the case that cation-exchange resins were used. The obtained recovery yield and purity of the succinic acid crystals were determined according to equations (1) and (2).

2.3.3. Salting-out

Salting-out extraction was carried out according to the methodology presented by Sun et al. [18] on an actual fermentation broth produced by *A. succinogenes* (medium 3) . The fermentation broth (100 mL) was treated with 76% (v/v) H_2SO_4 until the pH reached a value of 3. Then, 30% (v/v) acetone and 20% (w/v) $(NH_4)_2SO_4$ were added and the mixture was shaken. The mixture was allowed to settle for 8 h in order to allow phase separation. Succinic acid was extracted in the acetone phase. Activated carbon was added to the succinic acid-acetone phase in order to remove the residual organic impurities. The next step involved filtration in order to remove the activated carbon and the filtrate was vacuum evaporated to recover acetone and to concentrate the succinic acid solution. Subsequently, crystallisation was carried out at a pH value of 2.0 at 4 ^{o}C for 24 h. Finally, succinic acid crystals were washed as previously stated and dried at 70 ^{o}C for 10 h. Yield and purity were calculated according to equations (1) and (2).

2.3.4. Reactive extraction

The list of amines and solvents used for reactive extraction are shown in Table 2 together with some of their main properties. The selection of the chemicals was carried out

based on the study of Kurzrock and Weuster-Botz [14]. The amine concentration used in all cases was 0.5 mol/kg solvent. When medium 1 was used, no pH adjustment was carried out. When medium 4 was employed, centrifugation, treatment with activated carbon were initially employed and pH adjustment was carried out using either cation-exchange resins or 10 M NaOH. Then, 500 μL of succinic acid solution (using medium 1 or medium 4) were mixed with 500 μL of a selected reactive extraction system. The two phases were mixed vigorously for 5 min. Subsequently, the mixture was centrifuged (5 min, 4,500 rpm) and the aqueous phase was used for analysis of the remaining organic acids, whereas the organic phase was kept for further processing.

Back-extraction of succinic acid was carried out by either pH-swing or temperature-swing using the organic phases from the extraction systems trioctylamine in 1-hexanol and dioctylamine in 1-octanol used during reactive extraction of succinic acid from medium 4. In the case of pH-swing, 1 mL of the organic phases, produced by both extraction systems during reactive extraction, was vigorously mixed with 6 mL aqueous solution of NaOH (pH 13) for 12 h. Then, the two phases were separated via centrifugation and the aqueous phase was analysed for organic acids. Three different methodologies of temperature-swing were applied using the organic phases from the extraction systems trioctylamine in 1-hexanol and dioctylamine in 1-octanol applied during reactive extraction. Each organic phase (1 mL) was mixed with 6 mL of distilled water. The organic phase from the extraction system trioctylamine in 1-hexanol was back-extracted either in an ultrasonic bath for 9 h or in a water bath for 9 h at 50 ^{o}C [24]. In all cases, phase separation was achieved via centrifugation and the aqueous phase was analysed for organic acids.

The most efficient process combining reactive extraction and back-extraction was then carried out in 50 mL of medium 4 and the aqueous phase was concentrated using vacuum evaporation in order to increase the final succinic acid concentration. Crystallisation, washing,

and drying of the crystals were also carried out as previously described and crystal yield and purity were calculated using equations (1) and (2).

2.4. Analytical Methods

2.4.1. Determination of sugars, organic acids and LS

Determination of sugars and organic acids was performed by an HPLC SHIMADZU UFLC XR system (isocratic pump, autosampler, RI Detector and column oven) equipped with a Biorad Aminex HPX-87H column with size 300 mm x 7.8 mm or a Phenomenex Rezex ROA Organic acids H+ with the same size. The mobile phase in both columns was 10 mM H_2SO_4 aqueous solution with 0.6 mL/min flow rate and the column temperature was 45 ^oC for the Biorad column and 70 ^oC when the Phenomenex column was used.

LS determination was carried out according to the UNE EN 16109:2012 protocol as described by Alexandri et al. [5].

2.4.2. NMR analysis

The succinic acid crystals obtained by the most efficient DSP were analysed for impurities using NMR. NMR spectra were carried out on a Brucker Ultrafield 400 Plus spectrophotometer in CD_3OD, operating at 400 MHz. Maleic acid was used as internal standard. Singlet was set at 6.32 ppm, 2H.

2.4.3. GC-MS Analysis

GC-MS analyses were performed using Perkin Elmer Clarus 680 GC with 600 GC-MS equipped with an autosampler. Injections were performed with a split ratio of 10:1 at 250 ^oC and the injection volume was 1 µL. The separation was carried out on a DB-5MS column (30m, 0.25 mm ID, 0.25 µm film thickness). Helium flow rate was set at 1 mL/min. The

temperature program was the following: start at 35 ^{o}C, hold for 5 min; ramp rate- 10 ^{o}C/min to 300 ^{o}C; hold for 5 min. The MS in TIC were acquired in the range of m/z of 35-1000 at a scan rate of 2.5 scans/s using EI of 70 eV [25]. Succinic acid samples (0.5 mg) were suspended in MeCN (100 µL) and BSTFA (+ 0.1% TMCS, 50 µL) was added. In addition, the solution became clear and was allowed to stand at room temperature for 15 min to ensure a complete derivatisation. The standard was added (500 µL at 1 mg/mL in MeCN) and a total volume was made up to 1 mL using MeCN. The sample was then analysed using GC-MS. *O*-terphenyl was used as internal standard. Retention time was 24.46 min and m/z = 230.

3. Results & Discussion

3.1. Treatment with activated carbon

The use of activated carbon is necessary for every DSP using simulated or actual fermentation broths for the removal of impurities (e.g. proteins, pigments, LS) that contribute to the dark coloration of the broth. Depending on the origin of the solution used to evaluate the different DSP, varying quantities of activated carbon were required. In pure-commercial organic acid solutions (medium 1), the use of activated carbon was not necessary. When medium 3 (actual fermentation broth obtained via *A. succinogenes* cultivation in synthetic medium) was used, 3% (w/v) of activated carbon was sufficient for complete decoloration of the broth. However, in real SSL-based fermentation broths produced by *B. succiniciproducens* (medium 4) higher quantities of activated carbon were required for effective color removal. Five different quantities of activated carbon (0%, 3%, 7.5%, 12.5% and 20%, w/v) were tested in medium 4 in order to evaluate the efficiency of color removal with respect to the added amount of activated carbon. The color removal was evaluated by measuring the optical density at 400 nm of the five solutions (Figure 2). It is obvious that in order to achieve almost complete color removal in medium 4, around 12.5% (w/v) of activated carbon should be

applied. It is worth noting that fermentation broths containing LS concentrations higher than 100 g/L were not discolorised even with high quantities of activated carbon.

Activated carbon adsorbs succinic acid and the succinic acid losses depend on the amount of activated carbon used for color removal. Hydration of activated carbon with water or even better with fermentation broth should be applied in order to avoid succinic acid losses during decolorisation of the broths with activated carbon.

3.2. Effect of crystallisation duration

The crystallisation step is necessary in order to achieve succinic acid crystals of high purity. In the chemical industries, batch crystallisation is a commonly used unit operation. The crystallisation process should be optimised in order to obtain the appropriate crystal size distribution as well as a good repeatability from batch to batch operation [26]. The cooling rate is very important as it affects directly not only supersaturation but also the crystal size distribution. In industrial applications, there is a lack of kinetic data regarding crystallisation profile. Aiming to optimise this process, the crystallisation rate of succinic acid was monitored by calculating the diluted succinic acid in a synthetic solution containing approximately 190 g/L of succinic acid at 80 ^{o}C. Crystallisation was carried out under continuous stirring. Within 1.5 h of crystallisation, the soluble succinic acid reached a concentration of around 36 g/L at 4 ^{o}C which is close to its theoretical (30 g/L) solubility at 4 ^{o}C. This experiment showed that when the target temperature was reached, crystallisation of succinic acid reached a plateau. The crystallisation is affected by agitation as it influences both mass transfer and crystal formation [26] and by the impurities that are present in the solution. Feng and Berglund [26] studied the effect of the addition of acetic acid in the cooling profile showing that the presence of 2 mol% of acetic acid leads to reduced crystal

growth rate. For this reason, vacuum evaporation prior to crystallisation is very important as it removes the volatile acetic and formic acids leading to succinic acid recovery of high purity.

3.3. Calcium precipitation method

The use of the calcium precipitation method is traditionally applied in the industry mainly for the recovery of citric acid and lactic acid [2]. The recovery of succinic acid using calcium hydroxide in laboratory scale were described by Berglund et al. [27] and Datta et al. [28]. Datta et al. [28] reported a final purity of succinic acid of 94.2%. This method is generally considered quite effective for the removal of residual sugars, proteins, and other impurities. However, this process is very slow and requires high energy consumption [9]. In this study, it was mainly evaluated for comparison reasons.

The calcium precipitation method was applied in two different fermentation broths, one derived from a fermentation using *A. succinogenes* cultivated in a synthetic medium (medium 3) and the other using *B. succiniciproducens* cultivated in SSL-based medium (medium 4). Both samples were treated in the same way with the exception of the activated carbon step. In the case of medium 3, 3% (w/v) of activated carbon was used, whilst in the case of medium 4, 12.5% (w/v) of activated carbon was necessary in order to achieve complete color removal. After evaporation and crystallisation, the succinic acid yield was 8.1% in the case of medium 3 and 13.1% in the case of medium 4, while the purities achieved were 87.2% and 81%, respectively. HPLC analysis showed that the main impurities present in the succinic acid crystals derived from medium 3 were formic and acetic acids, as per 1 g of total crystals recovered approximately 0.003 g of each by-product was detected. In the succinic acid crystals derived from medium 4, formic and acetic acids were not detected, but 0.17 g of lactic acid was detected per 1 g of total crystals.

Luque et al. [9] reported a succinic acid recovery yield of 13% and a crystal purity of 30% when the calcium precipitation method was applied for the recovery of succinic acid from a semi-defined medium containing 35.3 g/L succinic acid, 8.1 g/L acetic acid, 6.3 g/L formic acid and 1 g/L of pyruvic acid. Datta et al. [28] reported a succinic acid purity of 89.6% when the calcium precipitation method was applied in a fermentation broth derived from *Anaerobiospirillum succiniciproducens* culture on dextrose-based defined medium.

3.4. Salting-out

Salting-out extraction is a separation method that is applied in order to extract a hydrophilic molecule from an aqueous solution by using an organic solvent as the extractant and a salt as the salting-out reagent. The advantages of this technique include the low-cost, the low interfacial tension, the good resolution, the high yield and capacity and the simplicity of scaling up the system [29]. Furthermore, bacterial cells and proteins are simultaneously removed from the fermentation broth, avoiding centrifugation and/or filtration steps. The overall succinic acid recovery yields were higher than 60% and the purities were higher than 91% when salting out was applied in synthetic fermentation media as a separation method for the recovery of succinic acid [18,30,31].

Salting-out extraction was applied in media 3 and 4 (Table 1), following the method of Sun et al. [18]. The pH of the fermentation broths was initially adjusted to 3 and then acetone (the extractant) and $(NH_4)_2SO_4$ (the salting-out reagent) were added to the fermentation broths. In this process, biomass removal and activated carbon treatment were not employed as primary separation steps. After 8 h, three distinct phases were observed: a) the upper phase contained the organic solvent (acetone) that is rich in succinic acid, b) the middle phase that contained the biomass, and c) the lower aqueous phase containing residual sugars, proteins and other impurities. Phase separation was carried out via decanting. The succinic acid rich

phase was subsequently treated with appropriate concentrations of activated carbon, (i.e. 3% in the case of medium 3 and 12.5% in the case of medium 4). After color removal, acetone was recovered via vacuum evaporation. The succinic acid recovery yield was 50% in both cases. The purity of the succinic acid crystals from media 3 and 4 was 94% and 86%, respectively. The succinic acid crystals contained a significant quantity of xylose, which accounted for approximately 0.05 g xylose per g succinic acid crystals when medium 3 was used and 0.1 g xylose per g succinic acid crystals when medium 4 was used. The higher xylose content in the case of medium 4 could be attributed to the higher initial xylose concentration in this medium. Sun et al. [18] reported that salting-out coupled with crystallisation led to succinic acid recovery yield of 65 % with a purity of 91 % using a glucose-based defined fermentation medium and a bacterial strain isolated from sea mud.

It should be stressed that actual fermentation broths produced in industrial optimized fermentation processes will not contain any xylose leading to higher purities. However, the achieved yield is considered low to ensure cost-competitiveness.

3.5. Direct crystallisation method

The direct crystallisation method was initially reported by Luque et al. [9]. Bacterial fermentations are carried out at a pH range of 6-7, above the pK_a value of succinic acid rendering acidification of the broth necessary in order to efficiently separate the succinic acid crystals via crystallisation. The pH of the solution should be 2.0 as in this value succinic acid exists in its undissociated form and can be crystallised in higher yields than mono- or di-succinate [17]. After acidification, vacuum evaporation is applied to remove the volatile organic acids, acetic and formic acids, and increase approximately five times the succinic acid concentration. The direct crystallisation method using H_2SO_4 or cation exchange resins was applied in media 1-4 (Table 1).

Figure 3 presents the recovery yield and the purity of the succinic acid crystals obtained by the direct crystallisation methods using both H_2SO_4 and cation exchange resins. In the case of pure organic acid solution (medium 1), the steps involving the addition of activated carbon and acidification were omitted, and subsequent treatment with evaporation and crystallisation led to 72.5% succinic acid recovery yield with 87.1% crystal purity (Figure 3A). The simulated fermentation broth (medium 2) was treated with activated carbon but not with resins or H_2SO_4 as the pH of the broth was already around 2. The succinic acid recovery yield (60%) achieved was lower than the pure organic acid solution, but the crystal purity (89.8%) was higher (Figure 3B).

Both direct crystallisation methods were applied in medium 3. Acidification with H_2SO_4 resulted in a succinic acid recovery yield of 53% and succinic acid crystal purity of 88% (Figure 3C). Higher succinic acid yield (74.6%) and purity (99.3%) were achieved with the use of cation-exchange resins (Figure 3D). When medium 4 was used, the treatment with cation exchange resins led to enhanced recovery yield (79%) and purity (96%) of the separated succinic acid crystals (Figure 3F), than acidification with H_2SO_4 that led to lower recovery yield (57%) and purity (90%) (Figure 3E). The lower purity of the crystals in the case of acidification can be attributed to the formation of salts that could not be detected via HPLC. Lin et al. [17] also reported the formation of potassium sulphate salts that can significantly affect the final succinic acid crystal purity.

Li et al. [32] employed crystallisation as the first recovery step, at pH value lower than 2 and 4 °C, in the separation of succinic acid from a fermentation broth derived by glucose-based cultures of *A. succinogenes* leading to succinic acid recovery yield of 70% and purity of 90%. Lin et al. [17] reported that the application of the direct crystallisation method using cation-exchange resins in a real fermentation broth produced by the bacterial strain *A. succinogenes* led to succinic acid recovery yield and purity of 89.5% and 99%, respectively.

The fermentation medium used by Lin et al. [17] was wheat-derived hydrolysate with glucose as carbon source. The lower succinic acid recovery yield obtained in this study could be attributed to the complexity of the SSL used as fermentation feedstock for the production of succinic acid.

In the succinic acid crystals recovered from direct crystallisation after treatment with cation-exchange resins using medium 4, a re-crystallisation step was also carried out in order to enhance the purity of the sample. Succinic acid crystal purity was increased to approximately 99%.

3.6. Reactive extraction

Reactive extraction, using high molecular weight amines is a well-known method for the extraction of organic acids [14]. The term "reactive" is used in order to differentiate these reactions from the conventional extraction mechanisms. In reactive extraction, the carboxylic acid and the extractant form a complex or a new compound due to intermolecular or chemical-based interactions [10,33]. In the case of succinic acid downstream separation, long-chain aliphatic primary, secondary and tertiary amines have been proposed for its reactive extraction from aqueous solutions [34-36]. Primary amines show high solubility in the aqueous phase. Secondary amines present the highest distribution coefficients but tend to form amides during regeneration by distillation. Primary, secondary and tertiary amines can only extract the undissociated form of the acid. On the other hand, quaternary ammonium salts can extract both dissociated and undissociated forms of the acid, but their regeneration by back-extraction is not easily accomplished [14]. Consequently, tertiary amines are the most promising extractants for reactive extraction of carboxylic acids [24].

The pK_a of the organic acid as well as the pH of the aqueous solution play an important role in reactive extraction. When tertiary amines are used, the pH of the aqueous

solution must be under the pK_a of the organic acid, because at pH values above pK_a the dissociated form of the acid is increased, leading to low extraction yields. Furthermore, in case of dicarboxylic acids, the distribution coefficient (k_d) decreases significantly at pH values between pK_{a1} and pK_{a2} [13].

The selection of the reactive extraction systems used in this study was based on a thorough literature review on reactive extraction systems for the recovery of succinic acid [14]. Secondary and tertiary amines and one primary amine in various solvents were selected for succinic acid separation from fermentation broths. Initially, five amines, four dissolved in 1-octanol and one in 2-octanol, were tested in medium 1 (Figure 4). The extraction yields were higher than 90% in almost all the reactive extraction systems employed. The system trioctylamine in 1-octanol extracted almost 95% of succinic acid, whereas the lowest succinic acid recovery yield (90%) was observed when trihexylamine in 2-octanol extraction system was used.

The efficiency of reactive extraction and the selectivity of the amines selected were then tested in a real fermentation broth derived via *B. succiniciproducens* cultivation in SSL-based media (medium 4). Tables 3 and 4 present the extraction yields achieved for each organic acid present in the fermentation broth by the different extraction systems used at pH values 2 and 5. At pH value of 2, the vast majority (13 out of 15) of the reactive extraction systems were able to remove more than 90% of succinic acid from the fermentation broth, with dioctylamine in 1-octanol reaching a succinic acid extraction yield of 100%. Furthermore, at a pH value of 2, all organic acids, besides formic acid that reacted fairly or not at all, were co-extracted in high removal yields (Table 3). Besides, dioctylamine in 1-octanol, also dioctylamine in 1-hexanol, trioctylamine in 1-hexanol and diisoctylamine in 1-octanol resulted in the extraction of almost all organic acids and only residual sugars

remained in the aqueous phase. In this way, the produced organic acids can be separated from the residual sugars.

At a pH value of 5, the extraction yield of succinic acid was much lower (Table 4) than the extraction achieved in a pH value of 2. However, in many cases, the extraction yields of fermentation by-products were much higher than succinic acid, which remained in the aqueous phase. For instance, the extraction systems dihexylamine in 1-hexanol and tripentylamine in 1-butanol led to complete removal of formic acid, more than 56% removal of lactic acid and more than 42% removal of acetic acid, while the removal of succinic acid was lower than 14.2%. It should be stressed that lactic acid removal from the aqueous phase is beneficial because this organic acid is less easily removed via vacuum evaporation and crystallisation. Especially in the case of the extraction system dihexylamine in 1-hexanol, the extraction yield of lactic acid was 67.1%. Reactive extraction at pH value of 5 could be applied in the case of *B. succiniciproducens* due to the production of lactic acid by this strain.

Two back extraction techniques, namely temperature swing and pH-swing, were evaluated in order to recover the succinic acid from the acid-amine complex and recycle the amine back to the extraction system. The latter method is based on the dependence of the undissociated acid to the pH value of the aqueous solution. A higher pH value than the pK_a of succinic acid inhibits the formation of the amine-acid complex making possible to back-extract the acid to the aqueous phase. An increased temperature is another way to break down the complex because the reactions in the interface of the aqueous and the organic phase involve proton transfer or hydrogen-bond formation and are expected to be exothermic.

The extraction systems that were selected to evaluate the two back extraction methods were trioctylamine in 1-hexanol and dioctylamine in 1-octanol that led to high succinic acid extraction yield at pH 2. In the case of pH-swing using the organic phase obtained from both extraction systems during reactive extraction, the succinic acid recovery (100%) achieved

when the extraction system trioctylamine in 1-hexanol was used for reactive extraction was much higher than the succinic acid recovery achieved (65%) when the organic phase from the extraction system dioctylamine in 1-octanol was used. As trioctylamine is a tertiary amine, the steric hindrance facilitates the back-extraction via pH-swing, while the bonding between succinic acid and dioctylamine, which is a secondary amine, is stronger [35]. The disadvantage of the pH-swing method is that the acid is recovered in a salt form and further acidification is required. Back extraction of succinic acid by temperature-swing was less efficient than pH-swing, as less than 30% of succinic acid was recovered. In the case that the organic phase from the extraction system trioctylamine in 1-hexanol was used, the succinic acid recovery yields achieved were again higher than the ones obtained with the organic phase from the dioctylamine in 1-octanol extraction system. Furthermore, all fermentation by-products were back-extracted in the aqueous phase. Nevertheless, the subsequent unit operations (*i.e.* evaporation and crystallisation) after reactive extraction and back extraction eliminate the residual by-products leading to purified succinic acid crystals.

The reactive extraction system trioctylamine in 1-hexanol followed by back-extraction with pH-swing was the most efficient system. After back extraction, succinic acid is recovered in the form of sodium salts and subsequent treatment with cation-exchange resins, vacuum evaporation and crystallisation was applied. The total succinic acid recovery yield was 73% and crystal purity reached 97.2%.

3.7. Comparison of the different DSP

Figure 1 presents the unit operations involved in each DSP evaluated in this study including the succinic acid recovery yield and purity achieved under the optimum conditions of the five DSP applied for the recovery of succinic acid from medium 4. Direct crystallisation using cation-exchange resins resulted in the highest succinic acid

yield (79%), whereas the highest purity was obtained via reactive extraction using back-extraction with pH-swing (97.2%).

3.8. Succinic acid crystal analysis by NMR and GC-MS

GC-MS and NMR analyses were carried out in the succinic acid crystals obtained via direct crystallisation using cation exchange resins and reactive extraction using trioctylamine in 1-hexanol followed by back-extraction of succinic acid with pH-swing. GC-MS analysis of purified succinic acid crystals showed that they were free of formic, maleic, lactic and hydroxybutyric acids (Table 5). Palmitic acid was present in all samples, while linoleic acid was detected in the succinic acid crystals derived from process 2 (reactive extraction) and process 3 (direct crystallisation with cation-exchange resins followed by re-crystallisation). The presence of fatty acids is probably attributed to *Eucalyptus globulus* wood, the raw material used in the sulphite pulping process [37].

Low quantities of acetic acid are masked by derivatisation agents and their by-products in MS total ion current chromatogram. Therefore, ^{1}H NMR analysis was employed for acetic acid quantification in the samples of succinic acid crystals. Furthermore, the final purity of succinic acid crystals was established by ^{1}H NMR analysis. Process 1 (direct crystallisation with cation-exchange resins) provided succinic acid crystals with purity of *ca* 99 % and acetic acid content of 0.24% (0.56 mol%). The purity of succinic acid crystals was increased to higher than 99% when direct crystallisation with cation-exchange resins was combined with re-crystallisation (Process 3) resulting also to no acetic acid detection in this sample. Process 2 (reactive extraction with pH swing back extraction) provided succinic acid crystals with purity of *ca* 98.5% and a acetic acid content of 0.14% (0.29 mol%). These results indicate that it is feasible to produce succinic acid crystals with the required purity for further polymerisation into poly(butylene succinate).

4. Conclusions

The development of an intergrated biorefinery in conventional sulphite pulp mills for the production of lignosulphonates, phenolics and succinic acid require also the development of an efficient DSP for the purification of succinic acid that will be suitable for poly(butylene succinate) production. This study showed that it is feasible to recover succinic acid crystals with purity higher than 99%, low acetic acid content (less than 0.09 mol% that is required for efficient polymerisation) and succinic acid recovery yield of 79%. The techno-economic profitability of this process will be evaluated in a forthcoming study.

Supplementary data

The succinic acid losses observed when different quantities of activated carbon were used and the crystallisation profile of succinic acid as a function of time following a temperature ramp to 4 °C are provided as e-supplementary data and can be found in the e-version of this paper online.

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Figure Captions

Figure 1: Schematic diagram of the five DSP evaluated in this study including the final recovery yields and purities of succinic acid recovered from medium 4 utilising each different DSP (higher than 99% purity was achieved when re-crystallisation was applied in the case of direct crystallization carried out with cation-exchange resins)

Figure 2: Color removal from medium 4 using various concentrations of activated carbon

Figure 3: Succinic acid recovery yield (black bars) and purity (grey bars) obtained using the direct crystallisation method. (A) medium 1; (B) medium 2; (C) medium 3 treated via acidification; (D) medium 3 treated via cation exchange resins; (E) medium 4 treated via acidification and (F) medium 4 treated via cation exchange resins

Figure 4: Extraction yield of succinic acid from medium 1 (pure commercial organic acid solutions) using 5 reactive extraction systems

Figure 5: Succinic acid recovery via back extraction using temperature and pH-swing. The black bars correspond to the reactive extraction system using trioctylamine in 1-hexanol and the grey bars correspond to the reactive extraction system using dioctylamine in 1-octanol. (A) temperature swing using treatment in an ultrasonic bath for 9 h; (B) temperature swing using treatment in a water bath for 5 h at 90 ^{o}C; (C) temperature swing using treatment in a water bath for 9 h at 50 ^{o}C; (D) pH-swing

Figure 1

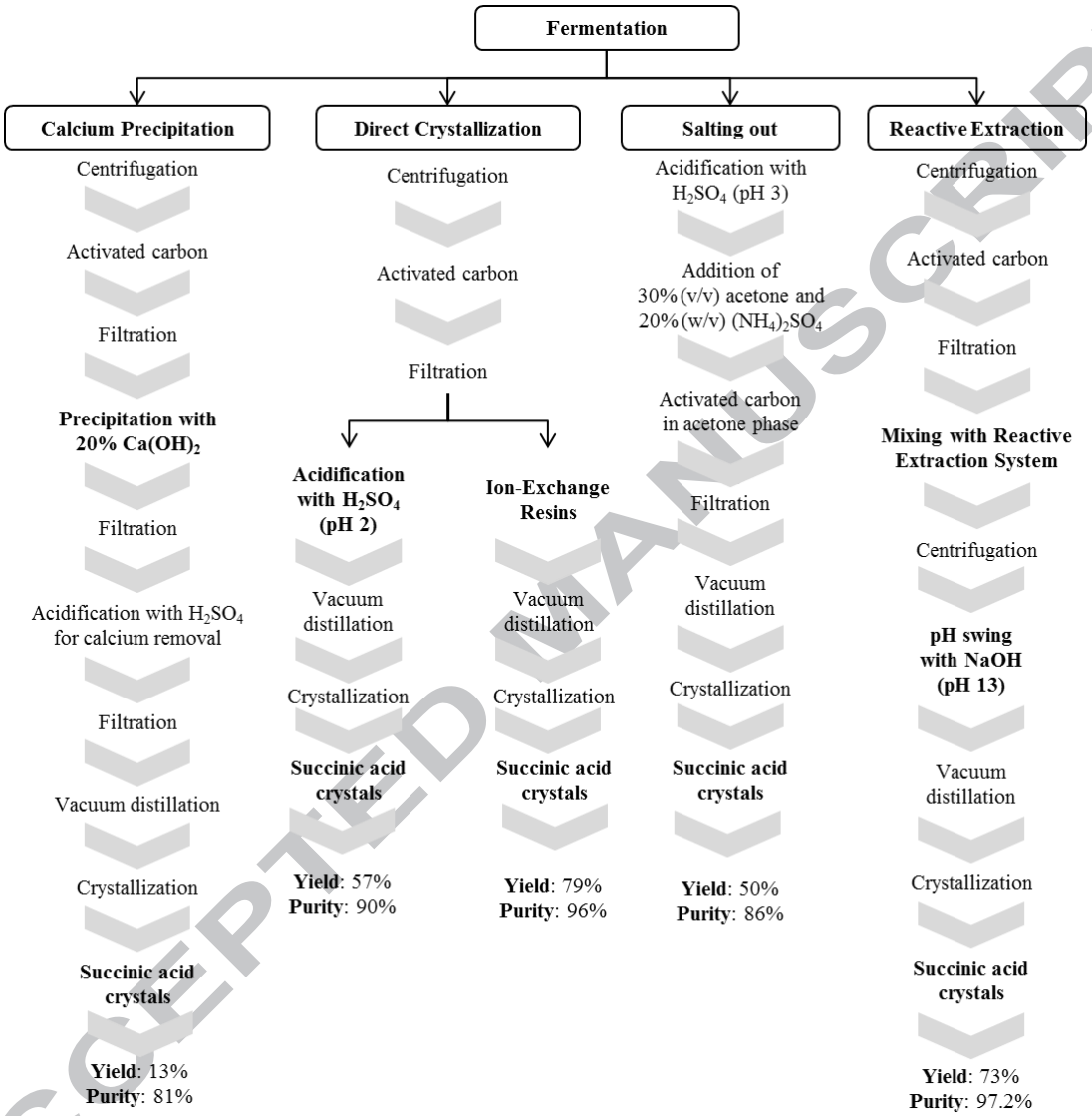

Figure 2

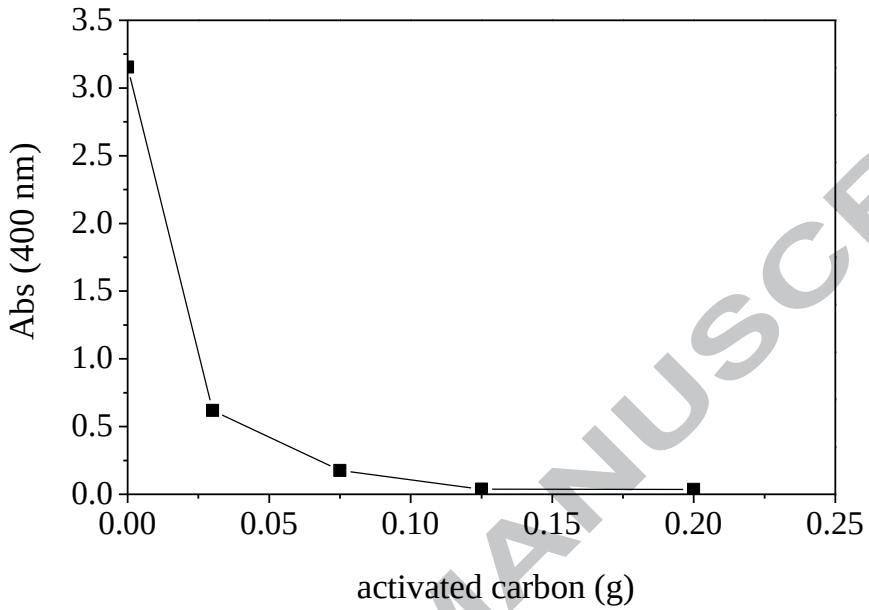

Figure 3

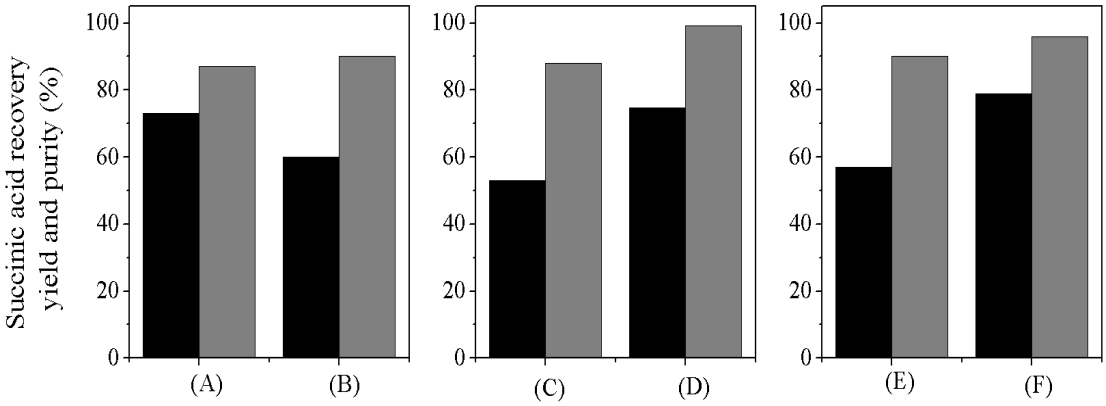

Figure 4

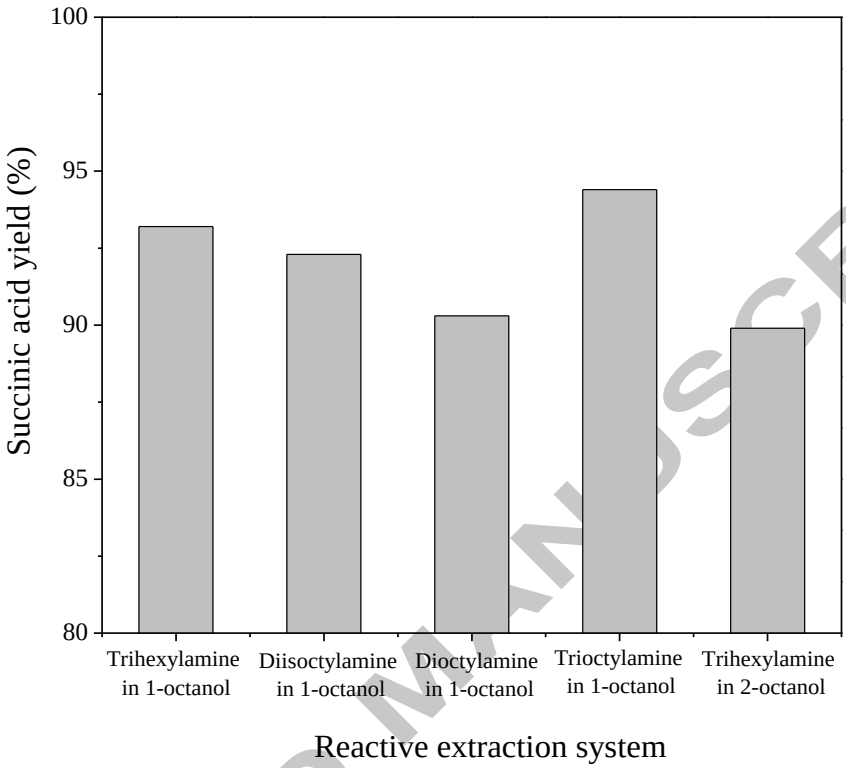

Figure 5

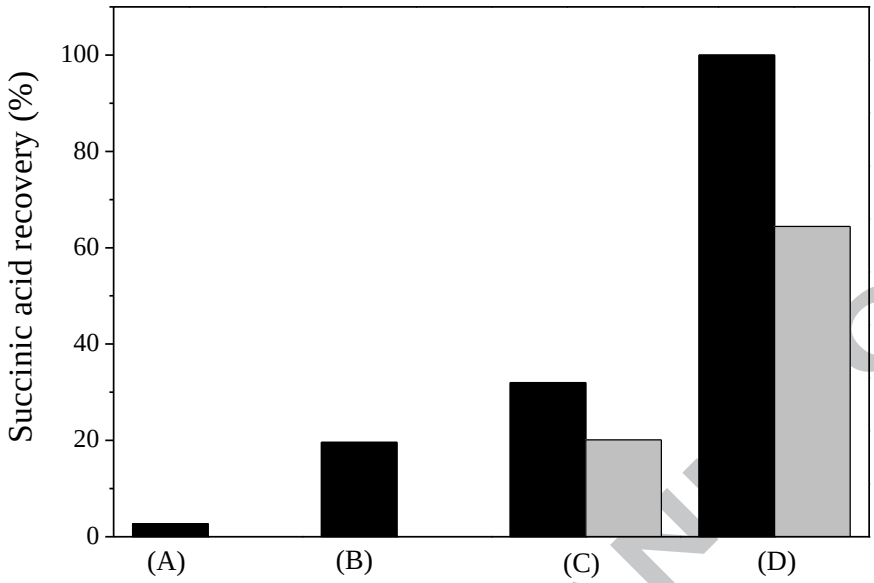

Table 1 Composition of the two synthetic media (1 and 2) and the two actual fermentation broths (media 3 and 4) used in the DSP

Component (g/L)	Medium 1	Medium 2	Medium 3*	Medium 4*
Xylose	10.2	10.2	2.8	9.3
Succinic acid	39.3	39.3	20.3	41.2
Lactic acid	11.1	11.1	0	12.3
Formic acid	2.0	2.0	4.6	2.0
Acetic acid	9.6	9.6	7.6	9.9
LS	0	10.0	0	10.0

* all the acids are present in their corresponding sodium and magnesium salts form

Table 2 Properties of the different amines and solvents used for the reactive extraction of succinic acid

Chemical	Type of amine/solvent	Boiling point (^{o}C)	Solubility in water (25^{o}C)
Octylamine	primary	175-177	0.2 g/L
Dihexylamine	secondary	192-195	0.3 g/L
Dioctylamine	secondary	297-298	< 0.1 mg/L
Diisooctylamine	secondary	123	0.6766 mg/L
Tripentylamine	tertiary	81-83 / 0.2 mmHg	insoluble
Trihexylamine	tertiary	263-265	No data available
Trioctylamine	tertiary	365-367	0.05 mg/L
Methyldioctylamine	tertiary	162-165 / 15 mmHg	No data available
1-butanol	polar/proton donating	117.4	73 g/L
1-Hexanol	polar/proton donating	155-159	5.9 g/L (20^{o}C)
1-Octanol	polar/proton donating	195	0.46 g/L
2-Octanol	polar/proton donating	178.5	1.12 g/L
MIBK (methylisobutylketone)	polar/aprotic	117	19.1 g/L (20^{o}C)

Table 3 Succinic, lactic and acetic acid extraction yields using different reactive extraction systems at pH 2.0 using medium 4

System	Succinic acid yield	Lactic acid yield	Acetic acidy yield
Trioctylamine + 1-octanol	94.0	93.2	71.8
Trihexylamine + 1-octanol	93.4	90.2	70.8
Dioctylamine + 1-octanol	100.0	93.8	100.0
Trihexylamine + 1-hexanol	93.2	82.5	69.2
Trixexylamine + 1-butanol	93.8	91.4	75.4
Trihexylamine + 2-octanol	79.7	67.4	53.0
Diisoctylamine + 1-butanol	97.3	100.0	86.7
Octylamine + 1-hexanol	90.6	100.0	74.0
Dihexylamine + 1-hexanol	93.4	100.0	84.1
Dioctylamine + 1-hexanol	97.4	100.0	94.2
Tripentylamine + 1-butanol	88.5	100.0	78.1
Trioctylamine + 1-hexanol	98.2	100.0	93.7
Methyldioctylamine + MIBK	95.8	100.0	80.2
Diisoctylamine + dixehylamine + 1-octanol + 1-hexanol	97.0	100.0	86.4
Diisoctylamine + 1-octanol	99.0	100.0	90.7

Table 4 Succinic, lactic and acetic acid extraction yields using different reactive extraction systems at pH 5 using medium 4

System	Succinic acid yield	Lactic acid yield	Formic acid yield	Acetic acid yield
Dioctylamine + 1-octanol	34.2	25.6	34.5	12.1
Trihexylamine + 2-octanol	10.7	36.0	38.9	36.4
Trixexylamine + 1-butanol	11.9	46.6	46.9	43.0
Trihexylamine + 1-hexanol	12.4	36.8	41.6	39.8
Trioctylamine + 1-octanol	10.6	34.1	38.1	36.1
Diisoctylamine + 1-octanol	13.2	29.1	32.7	31.7
Diisoctylamine + 1-butanol	17.4	52.5	100.0	54.9
Octylamine + 1-hexanol	14.2	39.6	100.0	37.2
Dihexylamine + 1-hexanol	14.3	67.1	100.0	76.4
Dioctylamine + 1-hexanol	22.4	34.7	100.0	40.1
Tripentylamine + 1-butanol	8.9	56.4	100.0	42.1
Trioctylamine + 1-hexanol	16.6	51.7	100.0	51.0
Methyldioctylamine + MIBK	18.7	44.9	100.0	35.7
diisoctylamine + dixehylamine + 1-octanol + 1-hexanol	3.5	7.0	100.0	47.8
Diisoctylamine + 1-octanol	14.8	19.8	100.0	53.0

Table 5 Characterisation of succinic acid crystals obtained by direct crystallisation with cation-exchange resins (Process 1), by reactive extraction coupled with back extraction using pH swing (Process 2) and direct crystallisation with cation-exchange resins followed by re-crystallisation (Process 3) with GC-MS and ^{1}H-NMR

Process	Compound	MW	% by GC-MS	Retention time (min)	% by ^{1}H-NMR
Process 1	Succinic acid			17.55	*ca.* 99
	Acetic acid		0.3	5.66	0.24
	Oxalic acid	90.03	0.06	12.03	
	Palmitic acid	256.43	0.80	25.80	
	Myristic acid	228.37	0.05	23.87	
	Pentadecanoic acid	242.40	0.05	24.86	
	Heptadecanoic acid	270.45	0.02	26.17	
	Steric acid	284.48	0.07	27.58	
	Linoleic acid	-	BLD	27.23	
Process 2	Succinic acid			17.47	ca. 98.5
	Acetic acid		0.36	5.51	0.14
	Palmitic acid		0.3	25.70	
	Linoleic acid		~1.0	27.24	
Process 3	Succinic acid		>99	17.46	>99
	Acetic acid		BLD	N/A	
	Palmitic acid		0.3	25.71	
	Linoleic acid		0.6	27.24	

Highlights

- Five different downstream methods were evaluated for succinic acid separation from SSL-based media
- Reactive extraction using trioctylamine in 1-hexanol coupled with crystallization and direct crystallization with resins resulted in high purity succinic acid crystals
- Re-crystallisation of the succinic acid crystals led to a purity of more than 99%