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Novel resin-based vacuum distillation-crystallisation method for recovery of succinic acid crystals from fermentation broths

Sze Ki Carol Lin,*[a,b,c] Chenyu Du,[b] Alexandra Cristina Blaga,[d] Maria Camarut,[d] Colin Webb,[b] Christian V. Stevens[e] and Wim Soetaert[a]

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In this paper, a novel resin-based crystallisation method was developed for the recovery of succinic acid (SA) crystals from fermentation broths (FB) using *Actinobacillus succinogenes*. Direct crystallisation of synthetic fermentation broths was firstly investigated and the results indicated that the synthetic fermentation broth consisting of a mixture of organic acids (in the free acid form rather than in the salt form) could significantly benefit in succinic acid recovery. Therefore, a commercially available cation-exchange resin Amberlite IR 120H was employed to convert the fermentation end-products such as succinate, formate, acetate and pyruvate from the salt form into the free acid form. Then, succinic acid was selectively separated from the acid mixture by vacuum evaporation and crystallisation. Highly purified SA crystals were successfully recovered from both synthetic and real fermentation broths using this method. A higher SA crystal purity (99%) and yield (89.5%) were obtained in the direct crystallisation method using an ion-exchange resin as compared to the direct acid addition method (46% and 35%, respectively). The work presented here sets the stage for the development of an efficient resin-based vacuum-distillation and crystallisation system for the recovery of succinic acid crystals from fermentation broths.

Introduction

Succinic acid (1,4-butanedioic acid, SA) is identified as a potential platform chemical for the production of various high value-added derivatives from renewable resources.[1,2,3] It can be used as a precursor of many chemicals,[4] which are used in food and pharmaceutical products, solvents, biodegradable polymers, surfactants and detergents. The current market price of succinic acid is at US$1.22 per kg, and the potential market size of succinic acid and its derivatives worldwide is estimated to be between 30,000 and 50,000 tons a year. It is predicted that the global annual market size will be over US$1 billion a year by 2015.[3]

Succinic acid is traditionally manufactured from petrochemicals, *e.g.* by catalytic hydrogenation of maleic acid or male anhydride.[4] However, the inevitable future depletion of fossil feedstocks and the escalating environmental concerns has spurred the need for an alternative sustainable production route. Extensive research efforts have been devoted to bio-based succinic acid production from renewable resources in the past decade. Very recently, a succinic acid fermentation demonstration unit by BioAmber with a production capability of 2,000 tonnes/year is currently under construction.[5] Glucose derived from wheat and sugar beet is used as feedstock and the carbon dioxide by-product could be utilised in an adjacent biorefinery for succinic acid production using an *E. coli* mutant strain.[6,7] The fermentation unit is expected to start up by the end of 2009. Similarly, DSM (The Netherlands) and Roquette (France) announced a plan to build a demonstration plant in Lestrem, France in 2009.[8]

Escherichia coli mutants[9,10,11] and *Actinobacillus succinogenes*[12,13,14] are two of the most promising bacteria reported in the literature for fermentative succinic acid production. *E. coli* has been used industrially, can utilise a broad range of sugars and has only simple nutrient requirements. Intensive research has been conducted on metabolic engineering of *E. coli* due to the well-understood physiology and established tools for genetic manipulation. *A. succinogenes* was first isolated from the bovine rumen in 1999.[15] It is a gram-negative facultative anaerobic bacterium that has several strain identifiers such as 130Z, ATCC 55618 and CIP 106512. It is a moderate osmophile and it has a high tolerance to succinate salts. Our previous study showed that *A. succinogenes* utilised hydrolysate produced from the enzymatic hydrolysis of wheat flour milling by-products to produce 62.1 g L^{-1} succinic acid using a batch fermentation strategy.[16] *A. succinogenes* was chosen for this study presented in this paper due to its ability to produce high concentrations of

[a]*Laboratory of Industrial Microbiology and Biocatalysis, Department of Biochemical and Microbial Technology, Faculty of Bioscience Engineering, Ghent University, Belgium*
[b]*Satake Centre for Grain Process Engineering, School of Chemical Engineering and Analytical Science, The University of Manchester, United Kingdom*
[c]*Bioengineering Program, Department of Chemical and Biomolecular Engineering, The Hong Kong University of Science and Technology, Hong Kong. E-mail: keclin@ust.hk*
[d]*Department of Biochemical Engineering, Faculty of Chemical Engineering and Environmental Protection, Technical University Gh Asachi Iasi, Romania*
[e]*Research Group SynBioC, Department of Organic Chemistry, Faculty of Bioscience Engineering, Ghent University, Belgium*

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succinic acid naturally. In addition, succinic acid production by this micro-organism requires CO_2 fixation, creating a potentially novel CO_2 sequestration process.

The critical factors that affect the production cost of succinic acid are productivity and yield, raw material cost, and the downstream recovery method.[17,18] Downstream processing is the key step for industrial scale bio-based succinic acid production, as downstream purification cost for fermentation-based processes normally runs up to about 80% of the total production costs.[19] For bio-based succinic acid production to be economically competitive with the petrochemical-based feedstocks, breakthroughs in downstream recovery techniques are essential. The purification process consists of the removal of proteins, sugars and by-products such as acetic, formic and pyruvic acid. Also, the conversion of succinic salts into the acid form is required for commercial application. Several recovery techniques such as crystallisation, extraction, adsorption and electrolysis with biopolar membranes (EDBM) have been reported.[20,21,22,23] Among these recovery methods, electrodialysis is a relatively environmentally benign process. However, the disadvantage of this process is its high cost associated with membrane and electricity consumption. According to Sauer *et al.*,[24] it remains questionable whether this recovery method is economically feasible for industrial large-scale production processes.

Our previous study demonstrated that succinic acid crystals can be separated using a direct crystallisation method with acidification.[25] However, succinic acid yield (28%) and purity (45%) were low when a real fermentation broth was used. In this study, we report the development of two processes (crystallisation with direct acid addition, Process I and crystallisation using an ion-exchange resin, Process II) for the effective separation and purification of a SA-enriched fermentation broth.

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Materials and methods

Chemicals, micro-organism and growth conditions

Chemicals used throughout this study were obtained from Sigma-Aldrich and Fisher Scientific, except where otherwise specified. *Actinbacillus succinogenes* (CIP 106512) was obtained from the Collection de I'Institut Pasteur (CIP) in France. An inoculum was prepared by incubating *A. succinogenes* cells from a cryopreservation vial in 100 mL Duran bottles containing 50 mL of trypticase soya broth (TSB; Fluka, BioChemika, Buchs, Switzerland) at 30 °C on a rotary shaker at 100 rpm for 48 h.

Defined fermentation broths

Two synthetic fermentation broths (Solutions A and B) were prepared with compositions listed in Table 1. Solution A was a mixture of organic acids consisting of acetic, formic, pyruvic and succinic acid and their concentrations were based on the typical *A. succinogenes* fermentations.[13,26,27] Solution B was an aqueous solution consisting of various sodium salts (acetate, formate, pyruvate and succinate) with equivalent acid concentrations as in Solution A. In all cases, 100 mL of fermentation broth was used for the recovery of the succinic acid crystals.

Table 1 Compositions of defined fermentation broths A, B and actual fermentation broth produced by *A. succinogenes*

Solution	A
Component	Concentration/g L^{-1}
Pyruvic acid	5
Acetic acid	10
Formic acid	10
Succinic acid	50

Solution	B
Component	Concentration/g L^{-1}
Sodium pyruvate	6.25
Sodium acetate	13.6
Sodium formate	14.9
Sodium succinate	68.7

Table 2 Compositions of actual fermentation broths produced by *A. succinogenes*

		Concentration/g L^{-1}			
Recovery process	Fermentation broth	Acetic acid	Formic acid	Pyruvic acid	Succinic acid
I	I	10.8	9.4	4.6	42.0
II	II	11.0	9.2	2.6	46.0

Fermentation broths produced by *A. succinogenes*

The inoculation procedure and batch fermentation conditions using *A. succinogenes* were described in previous publications.[13,26] Two batches of bacterial fermentations were conducted at 37 °C with a working volume of 0.6 L semi-defined and wheat-derived media separately in a 1.8 L bench-top bioreactor (Electrolab 351, Tewkesbury, UK). The semi-defined medium (L^{-1}) comprised of: 50 g glucose; 10 g yeast extract; 1.16 g $NaH_2PO_4{\cdot}H_2O$; 0.31 g Na_2HPO_4; 1.0 g NaCl; 0.2 g $MgCl_2{\cdot}6H_2O$; 0.2 g $CaCl_2{\cdot}2H_2O$; 10 μg B_{12} vitamin; 200 μg biotin; 200 μg folic acid; 500 μg thiamine; 500 μg riboflavin; 500 μg niacin; 500 μg pantothenate; 500 μg p-aminobenzoate; 500 μg lipoic acid; 1 μg B_6 vitamin, 10 g $MgCO_3$ and 1 mL silicone antifoam. The pH was automatically controlled at 6.6–6.8 with the addition of 10 M NaOH solution. The broth was sparged with 0.5 vvm CO_2 and agitated at 800 rpm. Two resultant fermentation broths produced by *A. succinogenes* were denoted as Fermentation Broth I and II, containing 42.0 and 46.0 g L^{-1} SA, respectively (Table 2).

Downstream process for SA recovery

The fermentation broth (100 mL) was centrifuged for 30 min at 7,500 rpm and 4 °C to separate the cell biomass. The supernatant was further filtrated through Whatman No.1 paper in order to separate the trace solid residues. Activated carbon (12.5% w/v) was mixed with the filtrate for 1 h to remove the organic impurities that contributed to the dark brown colour of the broth. The suspension was then filtered and a clear fermentation broth obtained was further treated using either Process I or Process II to remove the by-products and salts.

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Direct crystallisation method with acidification (Process I). Both synthetic fermentation broths and actual fermentation broths were used in Process I. The initial pHs of synthetic fermentation broths, Solution A and Solution B were 2.1 and 7.8, respectively. The pH of Solution B was adjusted to 2, 3, 4, 5 and 6 using 98% sulfuric acid. In each case, 100 mL solution was vacuum distilled at 48 °C for 1 h to eliminate residual volatile carboxylic acids such as acetic, formic and pyruvic acid. It was concentrated to around 20% of its original volume and the crystallisation of SA was carried out at 4 °C for 24 h. The final slurry was filtrated through Whatman No. 1 paper and the SA crystals were dried at 70 °C for 12 h. The purity and purification yield of the recovery process are defined as follows (eqn (1) and 2):

$$\text{Purity}(\%) = \frac{\text{SA concentration in crystals recovered}(g/L)}{\text{Total acid concentrations in crystals recovered}(g/L)} \times 100 \quad (1)$$

$$\text{Yield}(\%) = \frac{\text{Dry weight of SA in crystals recovered}(g)}{\text{Initial dry weight of SA in the fermentation broth}(g)} \times 100 \quad (2)$$

Direct crystallisation method using an ion-exchange resin (Process II). The ion-exchange resin employed in this study was Amberlite IR 120H, a cationic resin of sulfonic (SO_3H) type based on a polystyrene-divinylbenzene copolymer. In order to convert the sodium salts into acids, the clear aqueous fermentation broth was passed through the resin (50 g) packed in a teflon column (90 cm × 3.2 cm diameter) at a rate of 10 mL min^{-1}. The pH of the effluent was 2.0 after the ion-exchange process. It was then followed by vacuum distillation and crystallisation as described in Process I for the recovery of SA crystals. The ion-exchange resin was regenerated with 4% (v/v) hydrochloric acid for 30 min. It was then followed by elution with deionised water until the effluent was shown to be pH neutral.

Analytical techniques

Concentrations of SA, acetic, formic and pyruvic acid were determined by high performance liquid chromatography (HPLC) as previously described.[13] All samples were analysed at least in triplicate to ensure consistent results. Calculations were generally based on the average values of the individual readings taken.

High resolution ^{1}H-NMR (300 MHz) of succinic acid crystals recovered from fermentation broth produced by *A. succinogenes* was recorded with a Jeol JNM-EX 300 NMR spectrometer. The crystals were dissolved and diluted in deuterated water and were recorded in a 5 mm probe. Peak assignments were obtained using distortionless enhancement by polarisation transfer (DEPT).[28]

Results and discussion

For the recovery of SA from the fermentation broth, two methodologies were proposed: a direct acid addition method using sulfuric acid (Process I, Fig. 1A) and a direct crystallisation method using an ion-exchange resin (Process II, Fig. 1B). As previously reported,[25] the direct crystallisation method with acidification employs a simple and environmentally benign vacuum distillation and crystallisation methodology (employed as the final steps of recovery in Processes I and II), allowed the isolation of pure SA crystals. However, the yield of SA recovery was low (35% when real fermentation broth was used).

Table 3 Summary of SA crystals recovered in Processes I & II. An initial volume of 100 mL was used for all solutions and fermentation broths

Process	Solution	Initial succinic acid concentration/g L^{-1}	Purity (%)	Yield (%)
I	A	48.1	91	62
I	B (pH 2)	49.5	65	58
I	B (pH 3)	49.5	40	42
I	B (pH 4)	49.5	34	29
I	B (pH 5)	49.5	0	0
I	B (pH 6)	49.5	0	0
I	Fermentation Broth I	42.0	46	35
II	B	49.5	91	48
II	Fermentation Broth II	46.0	99	89.5

In order to improve the SA recovery yield, we first investigate the effect of different forms of the synthetic fermentation broths on the SA recovery yield through vacuum evaporation and crystallisation. In this study, Solution B, an aqueous solution consisting of various sodium salts (acetate, formate, pyruvate and succinate) was adjusted to pH 2.0, using sulfuric acid which is the pH of this acid mixture in the free acid form. Sulfuric acid was used instead of hydrochloric acid,[25] as sulfuric acid is a diprotic acid that has two acidic protons, whereas hydrochloric acid is a monoprotic acid with only a single hydrogen atom that can dissociate. Therefore, it is expected that Solution B can acidify more effectively using sulfuric acid. The solution was subjected to vacuum evaporation and crystallisation subsequently. As a comparison, Solution A was also subjected to vacuum evaporation and crystallisation. As shown in Table 3 and Fig. 2, the SA recovery yield of Solution A was 41% higher than that of Solution B, indicating that the fermentation broth in the free acid form facilitates the SA recovery significantly using the direct vacuum evaporation-crystallisation method.

Based on the above conclusion, we proposed a direct crystallisation method with an ion-exchange step (Fig. 1B, Process II), employing a cation-exchange resin to convert the fermentation broth from the salt form to the free acid form. It was then followed by vacuum distillation to increase the SA concentration 5-fold, as well as to remove the residual volatile organic acids. As the final step of purification process, the crystallisation was carried out at 4 °C and colourless SA crystals were obtained after drying. The feasibility and effectiveness of the two proposed methodologies will be evaluated in the recovery of SA from both synthetic and actual fermentation broths.

Succinic acid recovery from synthetic fermentation broths

Table 3 presents a summary of the purity and yield of SA crystals recovered from synthetic fermentation broths (Solutions A and B) in Processes I and II. Overall, the purity of the recovered SA crystals was relatively high (91–96%). However, the crystals yields of Solution B obtained by means of Process I decreased from 51% to 35% as the pH of the salt mixtures increased from

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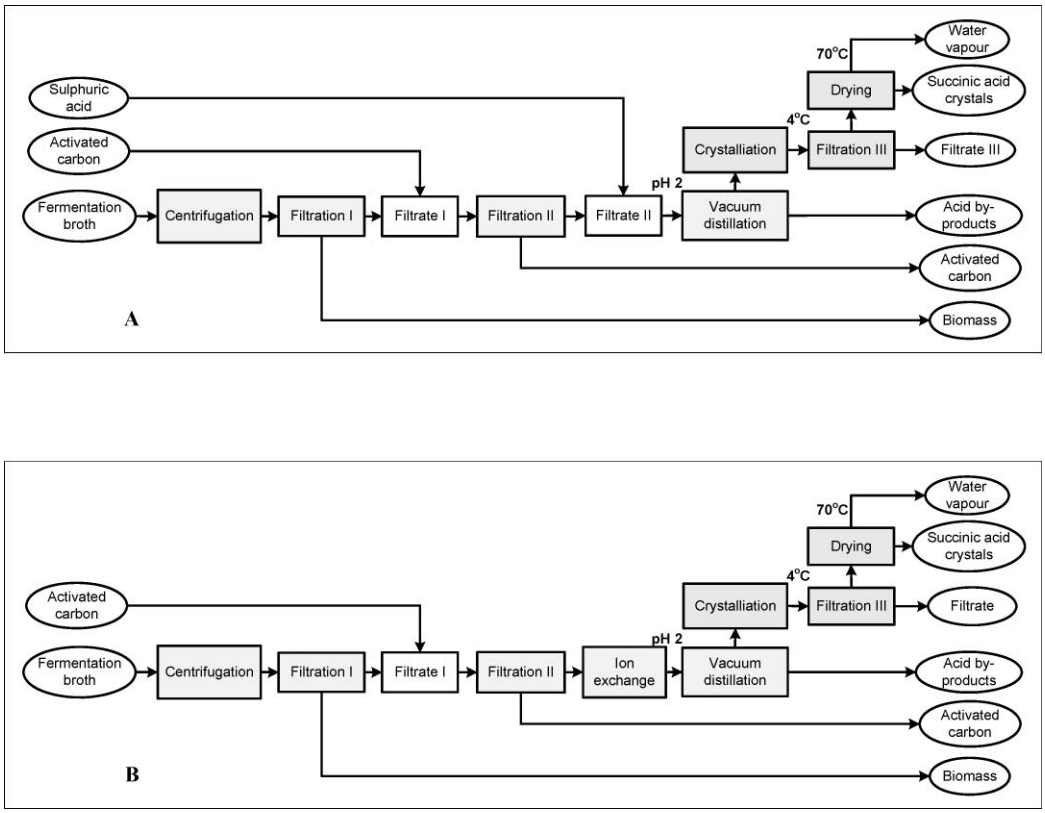

Fig. 1 Schematic diagrams of two recovery processes of SA crystals (A) direct crystallisation method with acidification (Process I) (B) direct crystallisation method with an ion-exchange resin (Process II).

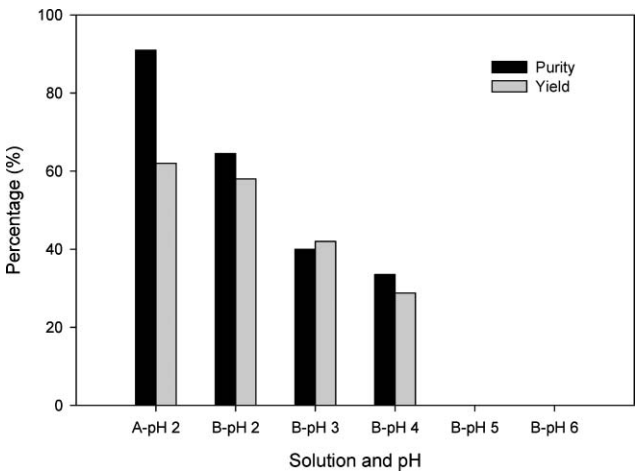

Fig. 2 Purity and yield of SA crystals recovered in Process I using Solutions A & B with a range of pH values (pH 2 to 6).

pH 2 to 4. This was due to the formation of sodium sulfate precipitate by the addition of H_2SO_4 to lower the pH of the salt mixture. No SA crystals formed when the pH of the salt mixture was set to 5 and 6 prior to the vacuum distillation step (Fig. 2). Significant amounts of sodium sulfate were formed in SA crystals from Solution B, explaining the decreasing yields obtained in Process I with the lower pH of the salt mixture. These results also imply the direct acid addition method was not the most appropriate one to recover SA crystals with high purity and yield.

A significant improvement in the recovery of SA crystals was obtained using the direct crystallisation method using an ion-exchange resin (Process II) as compared to the direct acid addition (Process I). As shown in Table 3, SA recovery yield using Solution B without pH adjustment in Process II was 48%, resulting in high crystal purity (91%). The results are very similar to the one using Solution A in Process I (91% purity and 62% yield). This indicates that the cation-exchange resin has both high affinity and capacity for converting organic acids from salt form to its acid form.

Succinic acid recovery from fermentation broths produced by *A. succinogenes*

We then moved on to study the SA crystal recovery from actual fermentation broths. Table 3 shows the purity and yield of SA crystals obtained from actual fermentation broths produced by *A. succinogenes*.

The initial SA concentration in Fermentation Broth I was 42.0 g L^{-1} but only 35 g SA crystals per L were isolated using the direct acidification method (Process I). In addition, the formation of potassium sulfate precipitate contributes to poor SA crystals purity (46%, Table 3).

Compared to Process I, both purity and recovery yield were improved in Process II due to the efficient conversion of the salt mixture into the acid form. The process employs Amberlite IR 120H cation-exchange resin that exhibited high affinity for both sodium and potassium ions.[29] The purity of the SA crystals from the *A. succinogenes* fermentation broth was

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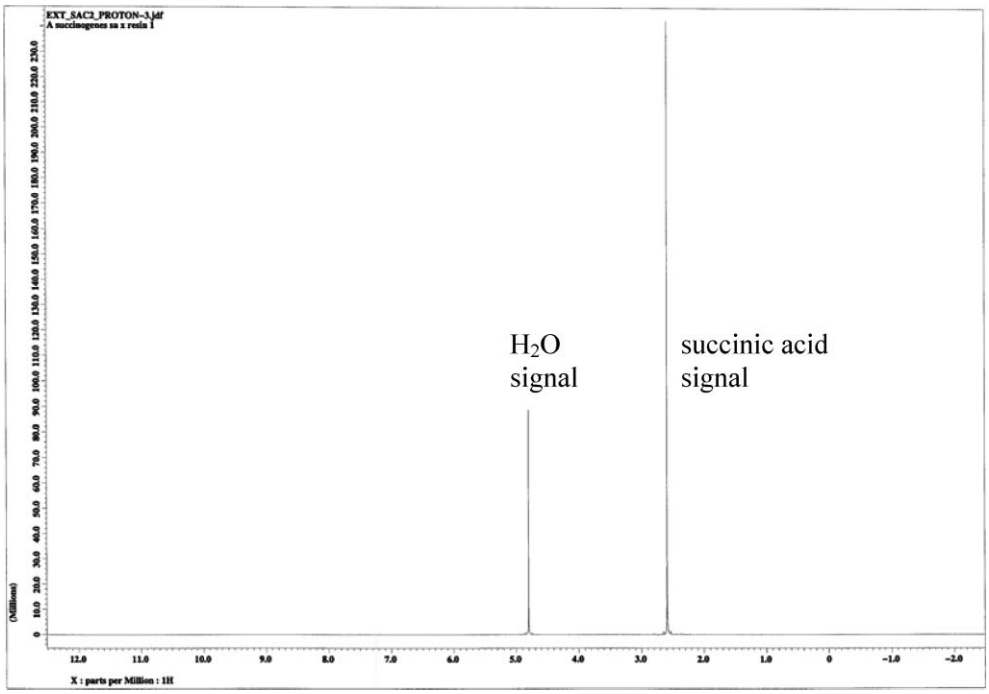

Fig. 3 Spectrum of ^{1}H NMR analysis (300 MHz; D_2O as solvent and internal standard) of succinic acid crystals recovered in Process II using fermentation broth produced by *A. succinogenes*.

considerably increased (up to 99%, Table 3). Also, significant improvement of the recovery yield (89.5%) was obtained in Process II. This is the highest recovery yield of succinic acid crystals from actual fermentation broths reported, to date, and is significantly higher than the 67.05% reported by Song *et al.*[30] using reactive extraction followed by a vacuum distillation-crystallisation method.

Fig. 3 shows the ^{1}H NMR spectrum of succinic acid crystals recovered in Process II using the fermentation broth produced by *A. succinogenes*. Signals from deuterium oxide and succinic acid were observed. The spectrum illustrates that the organic by-products have been successfully removed and highly purified succinic acid crystals were obtained.

In this study, activated carbon was used to remove the organic impurities that contributed to the dark brown colour of the fermentation broth.[25] Also, we have carried out studies in determining the effect of the added amount of activated carbon on the optical density of the resultant fermentation broth. The optical density decreased when the added amount of activated carbon increased. No change in optical density was observed when the concentration of activated carbon exceeded 12.5% w/v (results not shown). Another study showed that activated carbon is an efficacious adsorbent in reducing inherent proteins of wine solution.[31] This is beneficial for the ion-exchange process as the effect of organic fouling could be prevented.[32] As a consequence, no change in resin capacity was observed in this study. However, regeneration of resin using hydrochloric acid was necessary after every cycle as the resins were in a fully dissociated state.

Conclusions

A simple, effective and environmentally friendly cation-exchange resin-based process (*via* vacuum distillation and crystallisation) has been developed for the recovery of SA crystals with high purity from fermentation broths. Compared to the direct acidification method, the purity and recovery yield of SA crystals improved by 2.2 and 2.6-fold, respectively, using actual fermentation broths produced by *A. succinogenes*. Activated carbon was used as an effective adsorbent to remove the protein impurities from fermentation broth. The cation-exchange resin has shown to have high affinity and capacity for the removal of cations (*e.g.* sodium and potassium) from organic acid salts. The work presented here sets the stage for the development of an efficient resin-based system for the recovery of succinic acid crystals from fermentation broths.

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