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Development of Reactive Extraction systems for Itaconic acid: A step towards *In-situ* Product Recovery for Itaconic acid fermentation

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Abstract

Process optimization by integration of bioconversion with product separation and recovery i.e. *in-situ* product recovery (ISPR) is an important means to develop a sustainable and petrochemical-competitive biotechnological method for itaconic acid production. Since amine-based (reactive) extraction is known to be able to separate carboxylic acids efficiently, this technique was investigated in the present work. With the first step towards development of an integrated itaconic acid fermentation-extraction system, an extensive range of extractants consisting of eight different amines in various combinations with seventeen various types of diluents (alcohols, esters, alkanes) was experimentally evaluated for application in reactive extraction of itaconic acid. Compared to physical extractants using only diluents, reactive extractants based on amine- diluent combinations demonstrated higher extractive yields. Extractants with the highest extractive capacity were selected for further research aimed to optimize the concentration of amine for maximum extraction yield. Additionally equilibrium curves were obtained for the selected systems to evaluate extraction performance as a function of itaconic acid concentration. Experiments to elucidate the effect of medium components on the extraction performance were also performed. Nine reactive extraction systems were found to be most suitable i.e. trioctylamine, dioctylamine and N-methyldioctylamine as amine extractants solved in 1-octanol, pentylacetate and methyloctanoate. The critical results of this study form the basis for future investigations for large scale experiments with reactive extraction systems and development of appropriate ISPR configuration for itaconic acid.

Introduction

Itaconic acid (IA) is a versatile organic compound with many interesting applications, particularly as a monomer (or comonomer) for plastics, resins and synthetic fibres.[1] This is mainly attributed to the presence of conjugated double bonds and two carboxylic groups making it highly reactive for participation in various types of chemical reactions. [2,3,4] IA also finds a multitude of other uses e.g. in paints, films, detergents, cleaners, thickeners or bioactive components with anti-inflammatory and analgesic properties. IA-based products offer several advantages which include high strength and flexibility of IA-polymer based resins, improved adhesive properties of IA containing emulsion paints, high heat-seal strength of IA comprising cellophane films, colour fastness of polyacylonitrile-IA copolymers etc.[5] These properties make the production of IA industrially interesting.

In the recent years there has been an increasing interest in the fermentative production of IA mainly due to the fast depleting petrochemical resources and the increasing global awareness for the use of clean and inexhaustible resources and production protocols. The biotechnological route provides a green, renewable and environmentally favourable route to this industrially important metabolite. However, the present process faces two major bottlenecks. Firstly, product inhibition is already observed in the broth at IA concentrations as low as 20 g L^{-1} which leads to a reduction in production rate by the fermenting fungus and low productivity. [6,7,8] Another challenge concerns the development of efficient methods for separation and recovery of IA from the diluted fermentation broth. In general, the latter accounts for almost 50% of the total production cost of a metabolite and hence presents a major cost driver for bio-based processes. Thus in view of the above considerations, process optimization by integration of bioconversion (fermentation) and separation (downstream) units, i.e. *in-situ* product recovery (ISPR) forms an interesting approach to achieve a cost-efficient and petrochemical-competitive biotechnological IA production method. ISPR has the potential to facilitate in alleviating product inhibition thereby resulting in an enhanced IA productivity while allowing efficient recovery of IA, thereby improving overall process economics. [9,10]

With such a concept in mind, the present study focusses on the development of an efficient reactive extraction system for the separation and recovery of IA from the fermentation broth, mainly serving as the first step towards the design of ISPR-coupled IA fermentation(s). The present study is significant as it aims to contribute towards rarely-existent literature at present on the separation of IA from the fermentation broth in general and ISPR-coupled IA fermentation in particular. The traditional method of precipitation followed by acidification for recovery of acids (at the end of fermentation) is highly undesirable as it generates a lot of waste (gypsum) thus negatively impacting the environment.[11,12] Extraction using conventional diluents, immiscible with the clarified broth, such as alkanes, long chain alcohols or esters is not a practically viable option due to the low partitioning coefficient of the acids which limits their transfer into the diluents. On the contrary, reactive extraction involves a reversible reaction between the acid in the aqueous phase and the extractant in the organic phase (diluent phase) which results in the formation of extractant-acid complexes having a high affinity for the organic phase. The acid can be back extracted from the complex thus regenerating the extractant to be recycled again into the extraction unit.[13,14] To the best of our knowledge, there are only a few reports in the literature on the concept of reactive extraction of IA in general. Wasewar et al. investigated the reactive extraction of IA based on the quarternary amine aliquat 336 or tributylphosphate combined with inert diluents [15,16], while Aşçi and İnci studied trioctylamine-tridodecylamine in combination with several different types of diluents.[17] However, there have been practically no reports on the suitability of reactive extraction for ISPR implementation. The same is therefore the focus of the present study. Long-chain aliphatic amines are reported to be effective extractants for organic acids mainly due to their low solubility in water avoiding the loss of extractant, and their stable thermal nature which allows easy regeneration by simple distillation technique.[18] The amine extractants are typically dissolved in organic solvents (diluents) which provide a range of viscosity and density of the organic solvent (diluent) phase. The equilibrium extraction characteristics in reactive extraction are governed by several factors including the nature of acid, concentration of acid and amine, and type of diluent. [19,20,21] Thus the present study was initiated with a wide spectrum of experiments consisting of various combinations of primary, secondary and tertiary aliphatic amines with seventeen types of diluents comprising alcohols, esters and alkanes for investigating their use in the reactive extraction of IA from aqueous solutions. Optimization of amine concentration in amine-diluent combination(s) for maximum IA extraction yield was also elaborated. Experiments demonstrating the possible influence of varying acid concentrations, and the presence of additional components of fermentation medium such as carbon source (glucose), various salts on the extraction performance were also performed. Finally the reactive extraction systems identified from these studies shall be used in our future investigations for applications in integrated IA fermentation-extraction.

Materials and methods

Materials

Octylamine, trioctylamine, tridodecylamine, N,N-dimethyloctylamine and methyloctanoate were procured from Merck, Germany while dioctylamine and tributylamine were purchased from Acros Organics. Trihexylamine, N-methyldioctylamine, pentylacetate, methyllaurate and all alcohols were supplied by Sigma Aldrich, Belgium. All chemicals were used without further purification. IA, glucose and salts ($MgSO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$) were purchased from Merck, Germany and used as aqueous solution in demineralized water. The pH of the aqueous solutions of IA was measured and found to be very low (~2.3) which is indeed preferred as only undissociated acid molecules are extractable. [22] Therefore no pH adjustment was required in the present study. All the experiments were performed in 1.5 mL microcentrifuge tubes. Since IA inhibition starts at ~20 g L^{-1} and the average IA concentration at the end of the fermentation usually lies in the range of 60-80 g L^{-1}; the lower limit of IA concentration in aqueous solutions for experiments in this study was set at 10 g L^{-1} while the higher limit was 65 g L^{-1}. This was believed to provide a reasonably good range of IA concentration for reactive extraction studies. Nevertheless the initial screening experiments were performed with aqueous solutions containing high concentrations of 65 g L^{-1} IA with the idea that IA concentration of 10 g L^{-1} might be too low to demonstrate the extractive capacity of extractants. All the experiments were performed at least in duplicate.

Experimental design and methodology

Physical extraction using diluents (alcohols, esters and alkanes)

Preliminary experiments were conducted to ascertain the extraction capacity of the pure diluents for IA. 0.5 mL of each diluent was taken in a microcentrifuge tube to which 0.5 mL aqueous solution containing 65 g L^{-1} IA (0.5 M) was added. Vortex mixing was performed for 10 seconds following which the tubes were gently shaken at ambient temperature (22°C) for 16 h on a rotating bench. This allowed an efficient mixing of the two phases and a transfer of the acid from the aqueous phase to the diluent phase. The tubes were then centrifuged for 10 min at 3500 xg and phase separation was confirmed by visual inspection. Samples were analysed for IA concentration as described below.

Reactive extraction of IA using combinations of amines with various diluents

Eight amines were solved in seventeen different diluents in microcentrifuge tubes to form the reactive extractant systems for IA. The final concentration of amines in each system was 1 M. 0.5 mL of this amine- diluent solution was mixed with 0.5 mL aqueous solution of 65 g L^{-1} IA (0.5 M). A total number of 136 reactive extraction systems were investigated in this first phase of screening. The experimental methodology was same as described above for physical extraction. Samples were analysed for IA extraction and a comparison was performed among these 136 systems in order to screen the most probable amine- diluent combinations for efficient extraction of IA from aqueous solutions. The performance of the reactive extraction was judged on four parameters which included:

a) partition coefficient [23]: $K = \frac{C_{a,org}}{C_{a,aq}}$

i.e., the ratio of IA concentration in organic phase ($C_{a,org}$) to its concentration in the aqueous phase ($C_{a,aq}$)

b) extraction yield [23]: $E\% = \frac{K\,(100)}{1+K}$

i.e., the efficiency of extraction.

c) overall loading factor of the amine [23]: $Z = \frac{C_{a,org}}{C_{am,org}}$

i.e., the ratio of the total acid concentration (all forms) in the organic phase ($C_{a,org}$) to the total concentration of the amine in the organic phase ($C_{am,org}$)

d) stoichiometric loading factor of the amine [20] $Z_S = \frac{C_{a,org} - v\, C_{a,dil}}{C_{am,org}}$

i.e., the ratio of the total concentration of complexed acid in the organic phase to the total concentration of the amine (all forms) in the organic phase ($C_{am,org}$). To obtain the concentration of the complexed acid only, the total concentration of acid extracted in the organic phase ($C_{a,org}$) is corrected by a term $v\ C_{a,dil}$ accounting for the acid extracted by the diluent present in the solvent mixture with a volume fraction v.

Influence of varying amine and acid concentrations on reactive extraction

As mentioned above, not only the nature of amine and diluent, but also the concentration of amine and acid can influence the equilibrium extraction characteristics. Therefore experiments were performed to ascertain the possible effect of varying concentrations of different amines on the reactive extraction of IA. These experiments are important for optimizing the amine concentration for maximum IA extraction while also aiming at minimization of the amount of amine required for extraction. Selection of amines and diluents for this experiment was made on the basis of results of previous experiments as described above. The amine concentration was varied from 0.25 to 1 M. In addition, the extraction performance was also evaluated as a function of IA concentration. A concentration range between 10 g L^{-1} (below IA inhibition) and 80 g L^{-1} IA (end of fermentation) was selected.

Influence of carbon source and salts on reactive extraction systems

The fermentation medium invariably contains a carbon source and salts which play a significant role in microbial growth and production of desired metabolite. These medium components can influence the extraction performance of the reactive extraction systems. Therefore, with an aim to establish their possible effect on reactive extraction, experiments were performed by adding defined (reference) concentrations of carbon source (glucose) and salts ($MgSO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$) which are generally found in IA fermentation medium to aqueous solutions containing IA concentrations of 10 and 65 g L^{-1}.[9] Additional experiments with 2-fold reference concentrations of both glucose and salts were also performed to further reinforce the results of influence of these medium components on the reactive extraction of IA. Further, in order to elucidate the selectivity of amine- diluent reactive extraction systems for IA, analysis of glucose concentrations was also performed for these experiments.

Analytical methods

After phase separation had occurred, samples were taken from the aqueous phase and analysed for IA concentration by High Performance Liquid Chromatography (HPLC). Separation was achieved on a Prevail^{TM} Organic Acid 5 μ column (150 mm x 4.6 mm) using 0.05% phosphoric acid as mobile phase at a flow rate of 1.0 mL/ min and temperature of 40 ^{o}C. The IA concentration in the organic phase was calculated by applying a mass balance. Glucose analysis was performed by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) using Dionex Carbopac^{TM} PA1 column (4 x 250 mm) and guard column Carbopac^{TM} PA1 (4 x 50 mm). The mobile phase was isocratic gradient consisting of 50% 250 mM sodium hydroxide and 50% water with a flow rate of 1.0 mL/ min and temperature of 25 ^{o}C.

Results and Discussion

Physical extraction using pure diluents without amine

Experiments with pure diluents were performed to establish their extractive capacity for aqueous solutions containing 65 g L^{-1} IA. These experiments demonstrated that the partition coefficient of IA for the diluent/aqueous system (K_{dil}) was significantly less than 1 for most of the diluents under study (see Table 1), Of all diluents tested, the alkanes gave consistently the lowest partition coefficient K_{dil} (~ 0.10) and extraction yield $E\%_{,dil}$ (~9%). The results were independent of the chain length of the alkane tested. Extraction with esters provided equally low K_{dil}-values (0.05-0.24) and extraction efficiencies (4-11%), with the more polar pentyl acetate having the best extraction performance. The most favourable results were obtained with the alcohols. They demonstrated the highest extraction capacity with K_{dil}-values from 0.27-1.50, and extraction efficiencies from 21-60%. The extraction performance was found to decrease with the chain length of the alcoholic diluent. While the K_{dil} and $E\%_{,dil}$-values were 1.0 and 54% for 1-hexanol, they decreased to 0.63 and 43% for 1-octanol and to as low as 0.21 and 18% for 1-dodecanol. The secondary alcohols performed 6-10% better than their primary isomers.

The overall results can be explained by the chemical nature of the different diluents. The alcohols are proton-donating and more polar and thus better suited to solvate the acid as compared to the esters and alkanes. When the chain length of the alcohol decreases, polarity increases thus improving solvation and extraction capacity. A similar trend has been reported by Uslu and Kibaşlar [25] for the physical extraction of malic acid by a homologous series of 1-hexanol to 1-decanol.[24] Similarly, Aşçi and İnci have found a higher extraction yield for the physical extraction of IA by 1-octanol as compared to 1-decanol.[17] The more performant physical extraction observed with secondary alcohols can't simply be attributed to the polarity of the diluents, since the polarity of the 2-alcohols are (slightly) higher than their corresponding primary isomers. A similar tendency has also been seen for the solubility of IA in alcohols; the solubility of IA being higher in 2-propanol as compared to 1-propanol, despite its lower polarity. [24]

Nevertheless, the diluents alone are by far underperforming to efficiently extract IA from aqueous solutions as reflected by the low partition coefficients and extraction yields above. Thus reactive systems, based on chemical complexation, are indispensable for having an improved performance. In the following sections, chemical extraction of IA with long-chain amines will be discussed.

Reactive extraction systems for IA extraction

The combined effect of the nature of the amine and the diluent influences the extraction capacity of the amine - diluent system in reactive extraction. For amines, and especially tertiary amines, the greater the degree of alkylation the greater its basicity and consequently higher extraction yields are obtained. However, long chain amines may demonstrate lower extraction yields due to steric hindrance. In general, secondary long-chain amines and shorter tertiary amines have higher extraction capacities for organic acids. [22] The chemical characteristics of the diluent are another important factor. Their ability to solvate and stabilize the polar ion-pair acid-amine complex by dipole-dipole interactions or by hydrogen bonding influences the overall extraction performance of the amine-diluent mixture. In this respect, alcohols emerge as the most superior choice as diluents for amines followed by esters and then alkanes. However, when operating the extraction in an ISPR system, the residues of the diluents and amines may be toxic for the fermentation broth. The toxicity will also depend on the nature and solubility of the selected extractants, but will not necessarily correlate with the extractive capacity.

In order to be able to select the best system for extracting IA from an aqueous broth both from an extractive and in a later stage also from a toxicity point of view, a broad screening of amines and diluents was performed. Eight different amines were investigated in various combinations with seventeen different diluents yielding 136 types of reactive extraction systems. The results are summarized in Table 2 (K-values) and Fig. 1 (E%) for the various amine-diluent systems. Evaluation of these systems revealed that the highest extraction capacity was obtained when using alcohols as diluents. For these diluents, all amines under study, except three i.e. octylamine, tributylamine and N,N-dimethyloctylamine, scored high on all three parameters i.e. K, Z and E% . The highest K-values were obtained for dioctylamine (146-337) and trioctylamine (107-294), followed by tridodecylamine (86-227), N-methyldioctylamine (41-123) and trihexylamine (26-84). All these five amines showed a high extraction efficiency ranging between 97 and 99% and an overall loading factor Z of 0.50. The lowest extraction performance was noted for tributylamine, with a K-value ranging between 0.4 and 2.1, and an extraction efficiency between 28 and 68%. This could possibly be attributed to a partial dissolution of tributylamine in the aqueous phase, making it less available for the extraction of IA into the organic phase and resulting consistently in the lowest extraction yields.

The experiments revealed that in most cases the extractability decreased with the size of the alcoholic diluent as could be expected from their reduced polarity [26]. Moreover, it was found to be higher when using primary alcohols as compared to their secondary isomers (see Table 2). The latter is opposite to the trends observed for the physical extraction with pure diluents (see Table 1), indicating that secondary alcohols were less able to solvate and stabilize the IA-amine complexes.

As expected, the ester-based diluents were found to be typically less performant than the alcohols. For most of the amines the partition coefficient decreased from pentylacetate > methyloctanoate > methyllaurate related to a reduction in polarity. For the tertiary amine-based reactants, the extraction performance increased in a similar way as for the alcoholic diluents. The partition coefficient increased with chain length up to trioctylamine, which could be attributed to the increased basicity.

When using tridodecylamine, the extraction performance decreased again. The best results were obtained when using pentylacetate or methyloctanoate as the diluent, and trihexylamine or trioctylamine as the reactant. For these systems, the K-and E%-values ranged between 20-60 and 96-98% respectively, and the overall loading ratio was nearly 0.50. In case of methyloctanoate and methyllaurate, aggregation was observed for some specific amines, i.e., dioctylamine and n-methyldioctylamine. Also for succinic acid, aggregation was observed for these amines when combined with more apolar solvents such as methyl isobutyl ketone. [22]

As can be expected from their chemical nature, the alkanes were the least performing diluents. Primary amines such as octylamine exhibiting the lowest alkalinity in combination with the poor solvating alkanes resulted in considerably low K values (0.2-0.3), low extraction yields of IA (18-20%) and low loading ratios (0.1). Again, trioctylamine appeared to be the better performing amine, with a K-value of 8 and an extraction efficiency of 89%. The performance was found to be independent of the chain length of the alkane applied. Similarly as observed in other studies, in several cases a stable intermediate layer (aggregates) was formed. The aggregate formation occurred with specific amines; i.e., dioctylamine, trihexylamine and N-methyldioctylamine, and was independent of the chain length of the alkane applied. Compared to succinic acid [22], the reactive extraction of IA was found to be less prone to aggregate formation. For instance, no aggregate formation was noted with trioctylamine or tridodecylamine, even when combined with the more heavy alkane diluents like dodecane or hexadecane.

The above findings are consistent with those reported for reactive extraction of succinic acid.[13] The extractive power of a system was found to increase with the chain length of amine and when being diluted in a more polar and protic diluent such as an alcohol. However, it was observed to be more prone to aggregate formation in an inert diluent such as an alkane. The reason for this difference was attributed to the polarity of diluent as the polar complexes tend to form an aggregate (as observed above) to shield themselves from the non-polar environment.

Combining the results of all 136 experiments, 5 amines i.e. dioctylamine, trioctylamine, trihexylamine, tridodecylamine, N-methyldioctylamine and 5 diluents were selected for further studies. As diluents, three linear alcohols, i.e. 1-hexanol, 1-octanol and 1-decanol, as well as the two best performing esters; i.e. pentylacetate and methyloctanoate were chosen. Preference was given to the linear alcohols, because they performed generally better in the reactive extraction experiments. These amine-acid systems shortlisted from the above experiments were used in further investigations with varying amine/acid concentrations and influence of additional medium components on reactive extraction of IA.

Effect of varying amine concentration on reactive extraction

The influence of varying amine concentration on the reactive extraction parameters was investigated for the system of amines and diluents selected in the experiments above. At first, the IA-concentration was taken at 65 g L^{-1} (0.5 M), representative for the concentration at the end of the fermentation. For all systems, an increase in the extraction efficiency E% and corresponding partition coefficient K was observed with increasing amine concentrations (Fig. 3). At low amine concentration (0.25M), already extraction efficiencies of 55-75% were obtained. The individual differences were mostly influenced by the degree of physical extraction of the diluent, and to a lesser extent also by the type of amine for the less solvating diluents. At equimolar amounts of amine (0.5M), clear differences were noted depending on the type of amine and diluent. For the tertiary amines of the study, the best performances were noted for the diluents hexanol, octanol and pentylacetate giving already E%-values of 92-97%. The diluents decanol and especially methyloctanoate performed slightly lower, i.e., with an E% ranging between 85 and 94%. For the secondary amine dioctylamine, the diluents hexanol and decanol were the lesser performing (87-91%) ones, the other diluents having an E% of 94-98%. At double excess amine (1M), the observed trends were very similar, but with the absolute differences in extraction efficiencies overall being smaller (92% and 99+%). Both the overall loading factor as well as the stoichiometric loading factor decreased with increasing amine concentration. Similar results have been reported for the reactive extraction of citric acid and itaconic acid using tertiary amines, indicative of the presence of amine-acid complexes containing more than one amine per complex .[14, 17]

The above information is important as it forms the basis for process flow design and/or process economics e.g. the optimal amount of amine, number of stages etc. required for achieving maximum extraction of IA. It can be further explained as a high amine concentration exhibiting higher partition coefficient would result in efficient separation of IA in one stage only. On the other hand a rather low amine concentration might give similar separation in 2-3 stages while being more cost-efficient. The decision can be made after calculation of an overall process economics. In the present study, the amine concentration could suitably be reduced to 0.5 M which indeed was found to be optimal for achieving maximum extraction of IA from aqueous solutions containing high (65 g L^{-1}) IA concentrations. This amine concentration was then used in all subsequent experiments in the present study, unless otherwise specified.

Since a trade-off exists between performance of a system versus its cost, 1-hexanol, 1-decanol, trihexylamine and tridodecylamine feature as the more expensive alcohols and amines respectively for use in ISPR-based IA process. Also on the basis of results of this experiment, it is evident that 1-decanol was the worst performer among all alcohols. Therefore, considering the overall process including extraction efficiency and economics, three reactive extraction systems comprising 3 amines i.e. trioctylamine, dioctylamine and N-methyldioctylamine and 3 diluents i.e. 1-octanol, pentylacetate and methyloctanoate were found to be the most suitable for extraction of IA and investigated further for an ISPR approach.

Effect of operation at lower amine concentrations

One of the major driving forces to integrate an *in-situ* product removal at the fermenter is to avoid or reduce product inhibition. The starting point of product inhibition can be much lower than what is obtained at the end of the fermentation, and is for instance found to be as low as 20 g L^{-1} for the fermentation of IA. The design of the ISPR-extraction system and especially the concentration at which it should optimally be operated, relies on a counterbalance between two phenomena. On the one hand, the concentration in the fermentation broth should be low enough to avoid significant product inhibition, while on the other hand concentration should be maximized to ensure enough driving force for the extraction. It is therefore essential to have knowledge on the extraction performance as a function of the IA concentration. This was investigated by determining the equilibrium curves for the selected systems as a function of the IA and amine concentration applied (see Fig. 4). The results clearly showed that when extracting a lower concentrated IA-solution while maintaining equimolar amounts of amine, the yield slightly decreased. For instance for the amine trioctylamine, the yield was found to decrease from 95% (0.5 M) to 91% (0.2 M) for octanol, from 92% (0.5 M) to 84% (0.2M) for pentylacetate and from 90% (0.5 M) to 75% (0.2 M) for methyloctanoate. By increasing the molar excess of trioctylamine by 30%, the extraction efficiency could be restored both when applying octanol as well as pentylacetate as diluent. Methyloctanoate was found to be less performant at low IA-concentration, even when increasing the molar excess of trioctylamine.

In all cases, the stoichiometric loading factor Z was the highest at low IA and amine concentrations (see Fig. 5). The stoichiometric loading, which was obtained after correction for the physical extraction, was the highest for the more apolar and lesser solvating diluents, in the order of methyloctanoate > pentylacetate > octanol. The overloading, observed for methyloctanoate and pentylacetate and indicative of the formation of (2,1) and/or (3,1) – acid-amine complexes, is consistent with the expected behaviour for the more inert diluents. The lower (stoichiometric) loading observed with 1-octanol, on the other hand, agrees with the hypothesis that alcohols inhibit overloading and stabilize (1,1) complexes. [23, 27, 28]

Influence of additional medium components on reactive extraction

Since the present study aims at developing reactive extraction systems for applications in ISPR of IA from fermentation broths, it is imperative to elaborate the performance of the investigated systems in the presence of components usually found in the fermentation medium. Such an investigation is usually lacking in the reports on reactive extraction of carboxylic acids. [17] Thus an attempt was made in the present study to demonstrate the possible influence of glucose (used as carbon source) and salts ($MgSO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$) on reactive extraction of IA. Experiments were conducted using two IA concentrations i.e. 10 g L^{-1} and 65 g L^{-1} while the amine concentration was set constant at 0.5 M. Glucose concentration in the aqueous solutions containing IA was 10 g L^{-1} while the same contained 2.08 g L^{-1} $MgSO_4 \cdot 7H_2O$ and 2.36 g L^{-1} $(NH_4)_2SO_4$. These concentrations were selected on the basis of literature reports on IA fermentation. [9] The investigations demonstrated a decrease in extraction yields in the presence of medium components other than IA which was more pronounced for low concentrated IA solutions (10 g L^{-1}) than 65 g L^{-1} solutions. Overall an 8-10% yield reduction was observed for 65 g L^{-1} IA solutions while a (higher) 15-25% decrease in E% values was obtained with solutions containing 10 g L^{-1} IA. In order to further confirm the results obtained above, another set of experiments was performed in the present study which contained 2-fold concentrations of glucose and salts in the aqueous solution than taken in the previous experiment. The experiments distinctly demonstrated a further decrease by 10% in extraction yields with still higher concentrations of salts. In general, strongest effect of medium components on extraction yield was observed for 1-octanol in combination with all 3 amines (Fig. 6a). The decrease in E% was found to be ~40-45% in different cases. A similar reduction in yield was demonstrated by dioctylamine with esters as diluents (Fig. 6 a, b, c). Interestingly the extraction yields were much less affected relative to the base case (control) consisting of only IA in aqueous solutions when trioctylamine was used in combination with esters.

Decrease in extraction yields with increasing ion concentrations has also been reported for succinic acid extraction. [22,29] A probable reason for such an observation was stated to be the presence of salt anions which compete with the carboxylic group of acid for protons thereby resulting in reduced extraction yields due to possible co-extraction of corresponding acids of salts with the target acid. Analysis of glucose in the present study demonstrated that it was almost exclusively confined to the aqueous phase and therefore there was hardly any possibility of its interference with extractant phase and influencing extraction characteristics. In such a case, the observed decrease in IA extraction yields could be attributed to the presence of salt ions in the aqueous phase. Further analysis of anion concentration might be useful to provide a logical explanation and facilitate to ascertain the effect of salt ions on extraction performance. The same is being investigated at present by authors of the present study.

Conclusion and future outlook

136 types of amine- diluent combinations as reactive extraction systems for IA were investigated in the present study with an aim to lead towards development of ISPR-based IA fermentation. Initial experiments demonstrated a higher extractive capacity of reactive extraction than pure diluents alone as extractants for IA. Effect of varying amine concentration demonstrated an increase in K and E% values with increasing amine concentrations when aqueous solutions containing 65 g L^{-1} IA were used. The extraction yields were found to be negatively affected by the presence of additional components (e.g. salts) in the fermentation medium. 3 amines (tricotylamine, dioctylamine and N-methyldioctylamine) and 3 diluents i.e. 1-octanol, pentylacetate and methyloctanoate were found to be the most suitable for efficient extraction of IA from aqueous solutions. Further experiments in this

direction would include large-scale experiments with these 9 selected extraction systems. Since there is particularly no available literature on back extraction of IA from the amine-acid complex, suitable back extraction protocols for IA would also be established in future studies. For ISPR application, several configurations are possible depending upon the location of separation unit with respect to the fermentation system. However, one of the major factors in deciding an appropriate ISPR configuration for a bioprocess is the possible toxic effect of the extractants on growth and hence metabolite production by the fermenting microorganism. In this direction, toxicity tests for ascertaining the effects of reactive extractants on growth of microorganism would be performed. Eventually the selected ISPR configuration would be used in a coupled set-up with a running IA fermentation. A conceptual design for such a process is presented in Fig. 7.

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NOMENCLATURE:

$C_{a,org}$	Concentration of acid in organic phase (mole L^{-1})
$C_{a,aq}$	Concentration of acid in aqueous phase (mole L^{-1})
$C^{o}_{a,aq}$	Concentration of acid in diluent phase at start (mole L^{-1})
$C_{a,dil}$	Concentration of amine in organic phase (mole L^{-1})
$C_{am,org}$	Partition coefficient
K	Partition coefficient for the aqueous/diluent system
K_{dil}	Overall loading factor
Z	Stoichiometric loading factor
Z_s	Extraction yield
E%	Extraction yield
$E\%_{,dil}$	Extraction yield for the aqueous/diluent system
v	Volume ratio of the diluent in the amine-diluent system

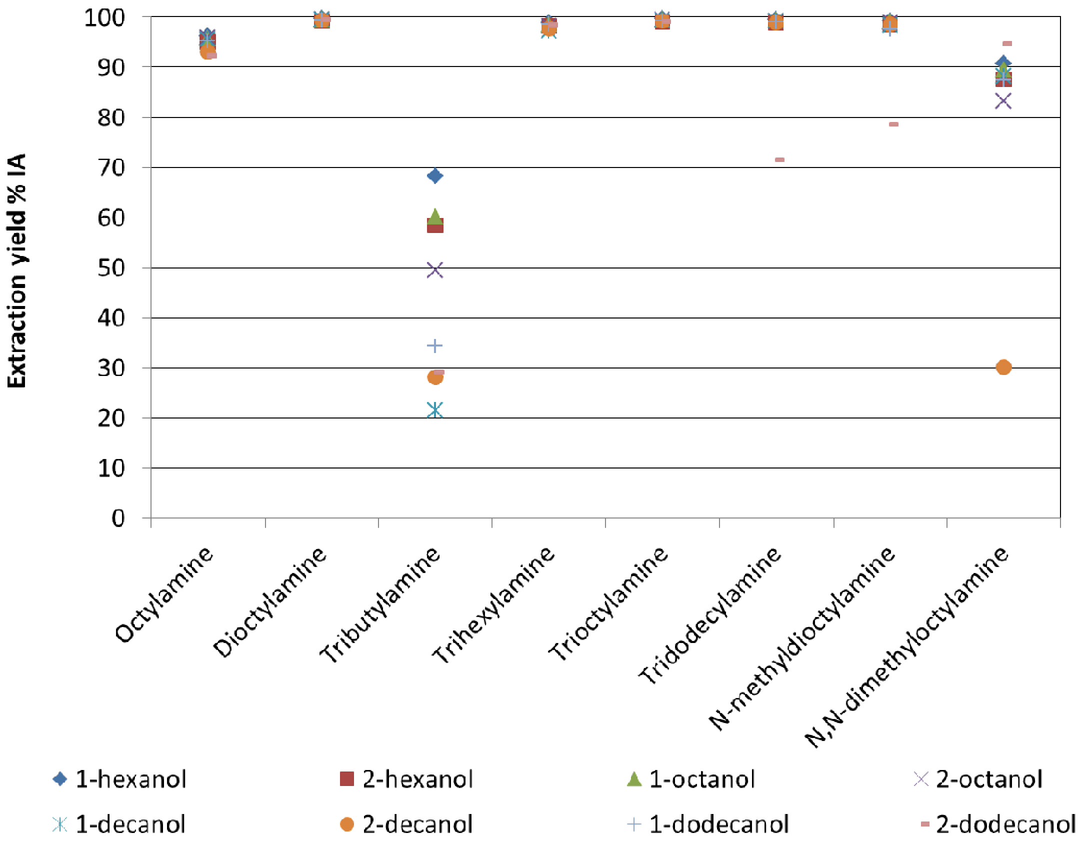


Fig. 1: IA Extraction yields for reactive extraction systems comprising amines and alcohols

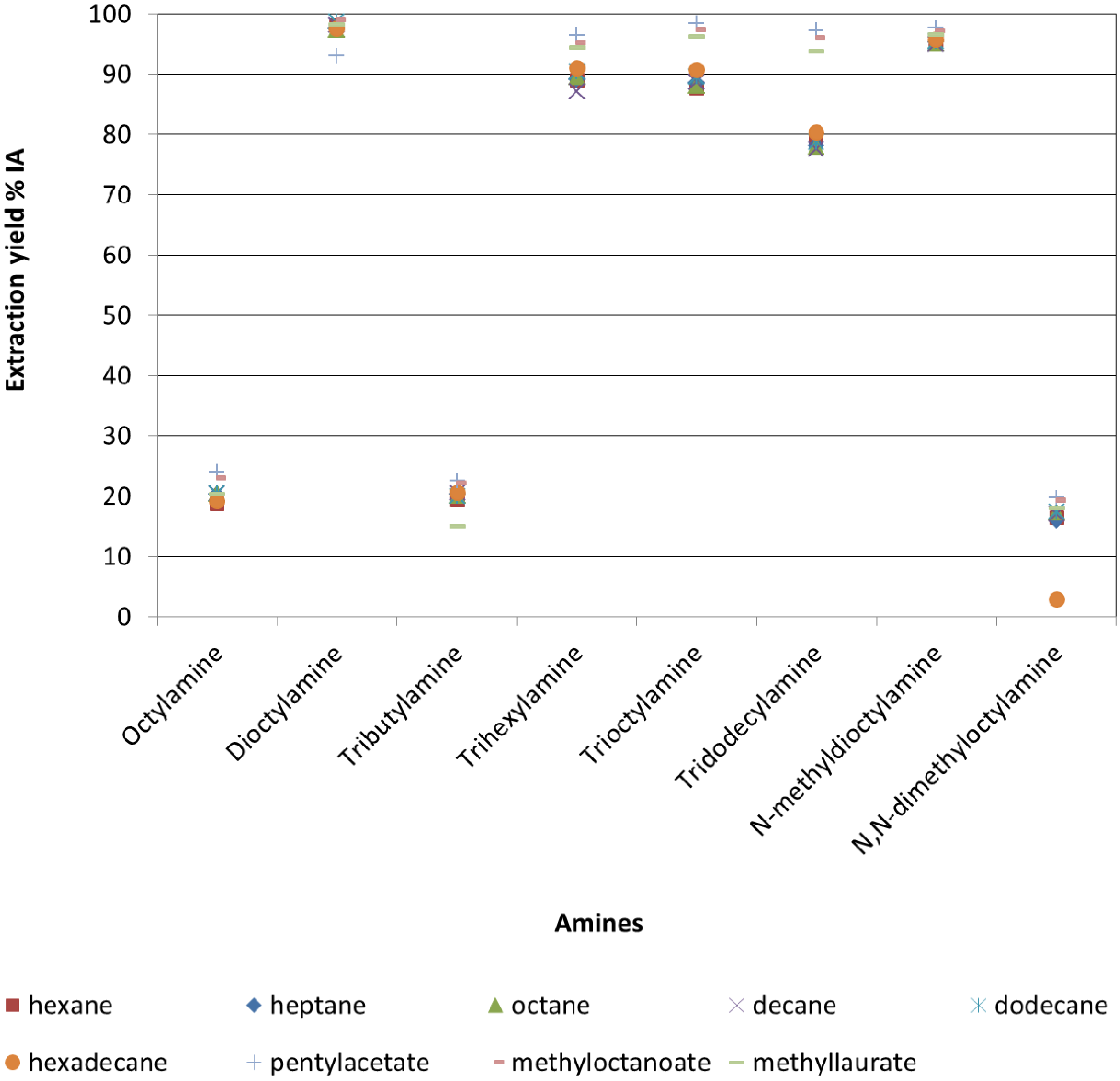
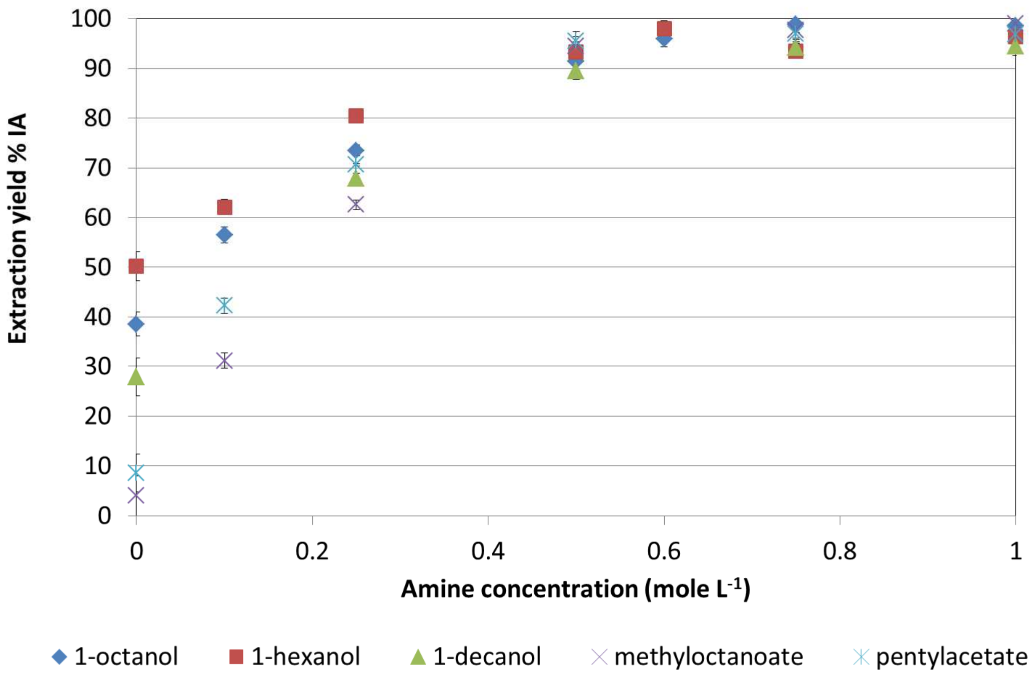
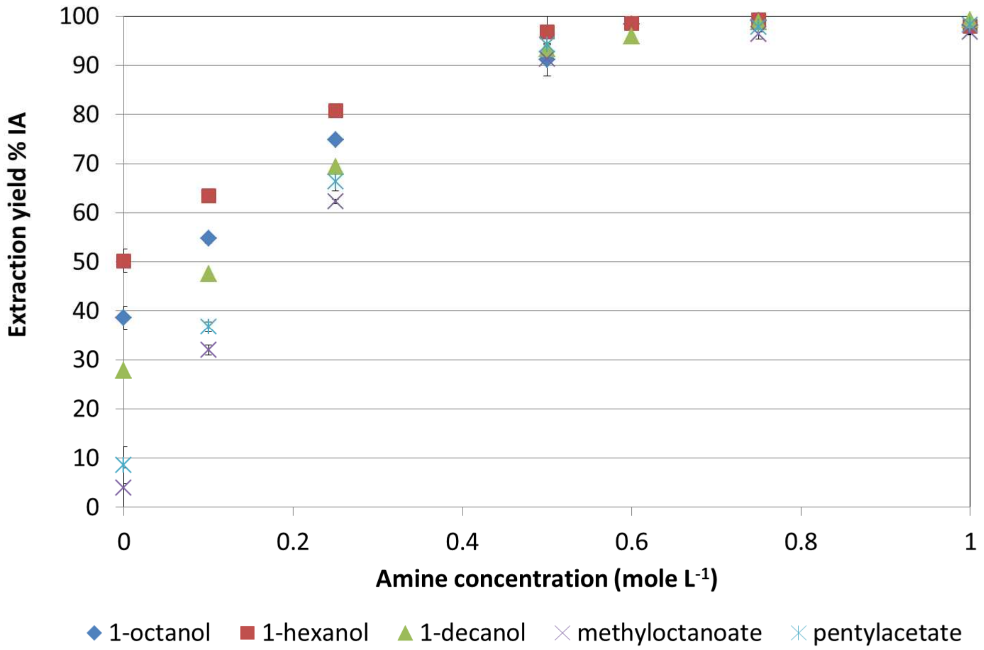


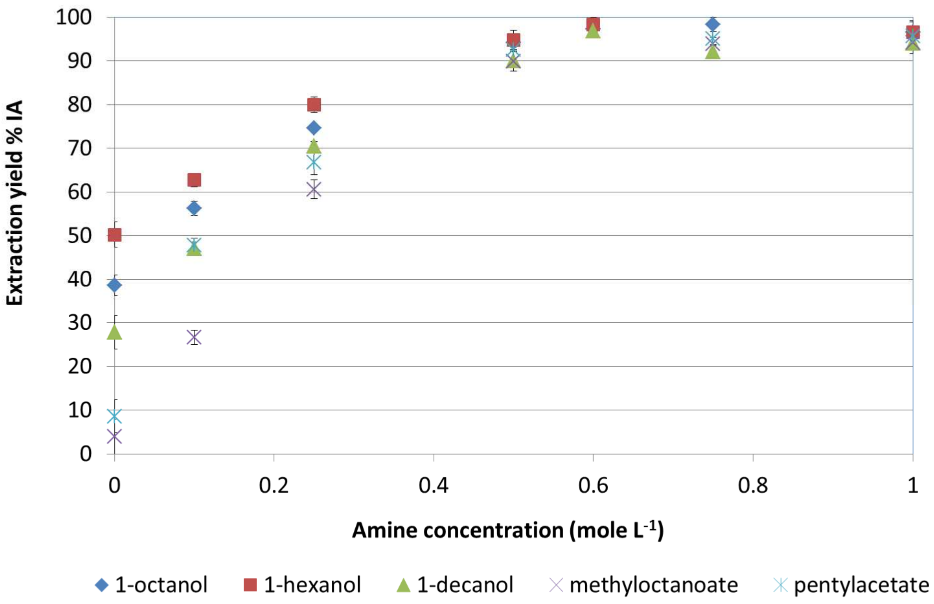
Fig. 2: IA Extraction yields for reactive extraction systems comprising amines and alkanes/ esters


Extraction yield % IA
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
Amine concentration (mole L^{-1})
◆ 1-octanol
■ 1-hexanol
▲ 1-decanol
× methyloctanoate
✳ pentylacetate

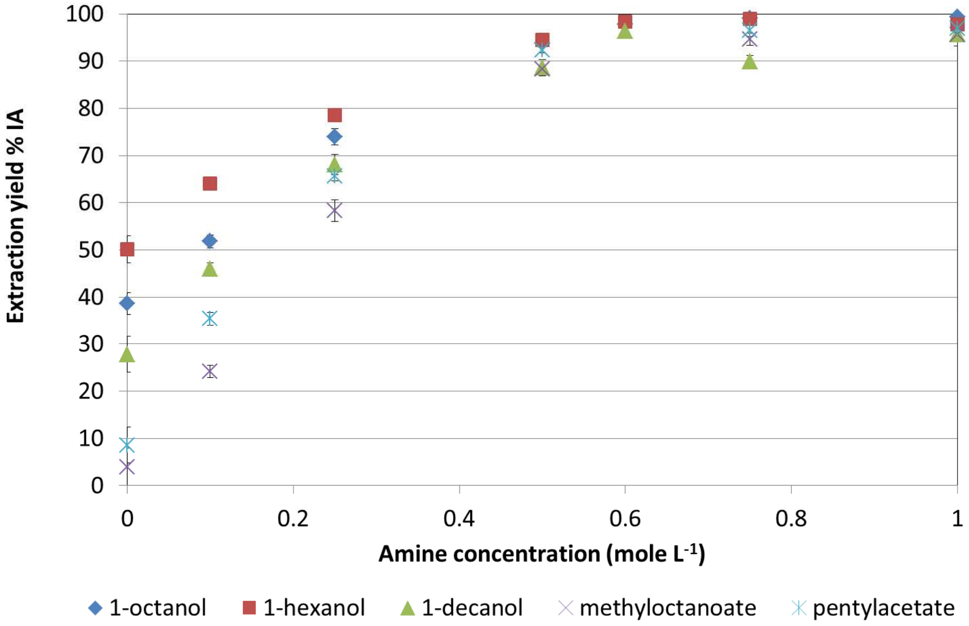
(a)


Extraction yield % IA
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
Amine concentration (mole L^{-1})
◆ 1-octanol
■ 1-hexanol
▲ 1-decanol
× methyloctanoate
✳ pentylacetate

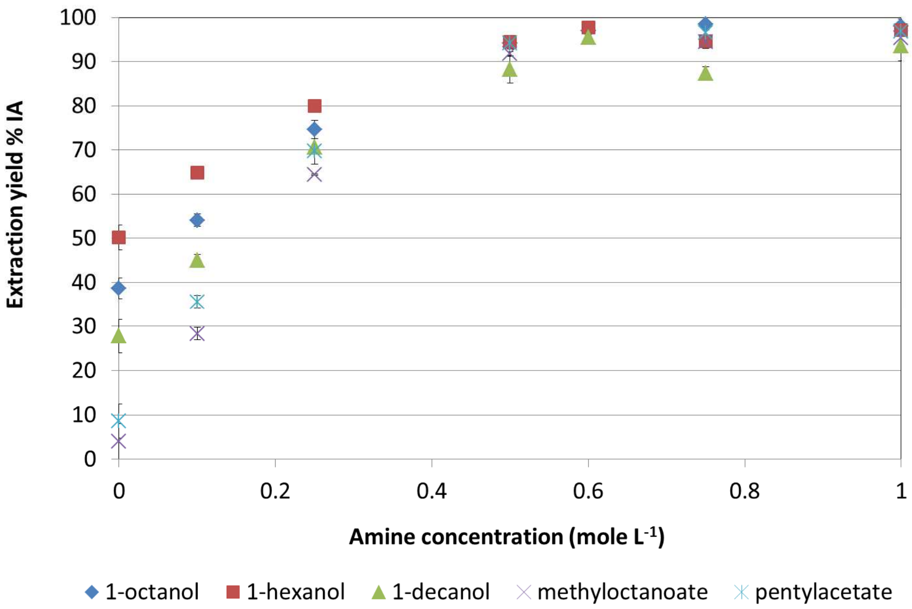
(b)


Extraction yield % IA
0
10
20
30
40
50
60
70
80
90
100
0
0.2
0.4
0.6
0.8
1
Amine concentration (mole L^{-1})
1-octanol
1-hexanol
1-decanol
methyloctanoate
pentylacetate

(c)

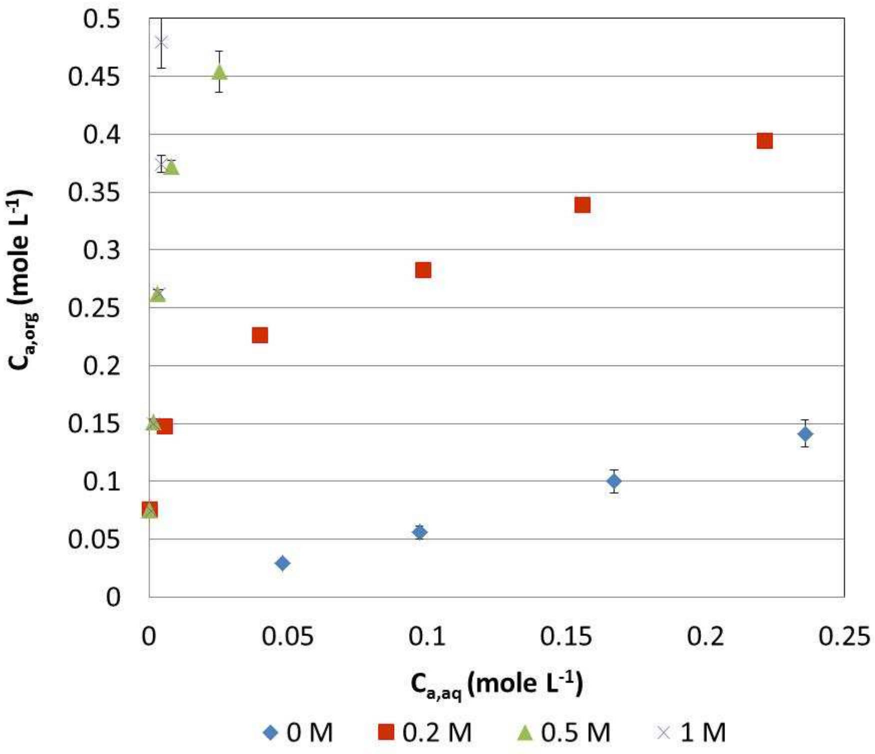

Extraction yield % IA
0
10
20
30
40
50
60
70
80
90
100
0
0.2
0.4
0.6
0.8
1
Amine concentration (mole L^{-1})
1-octanol
1-hexanol
1-decanol
methyloctanoate
pentylacetate

(d)

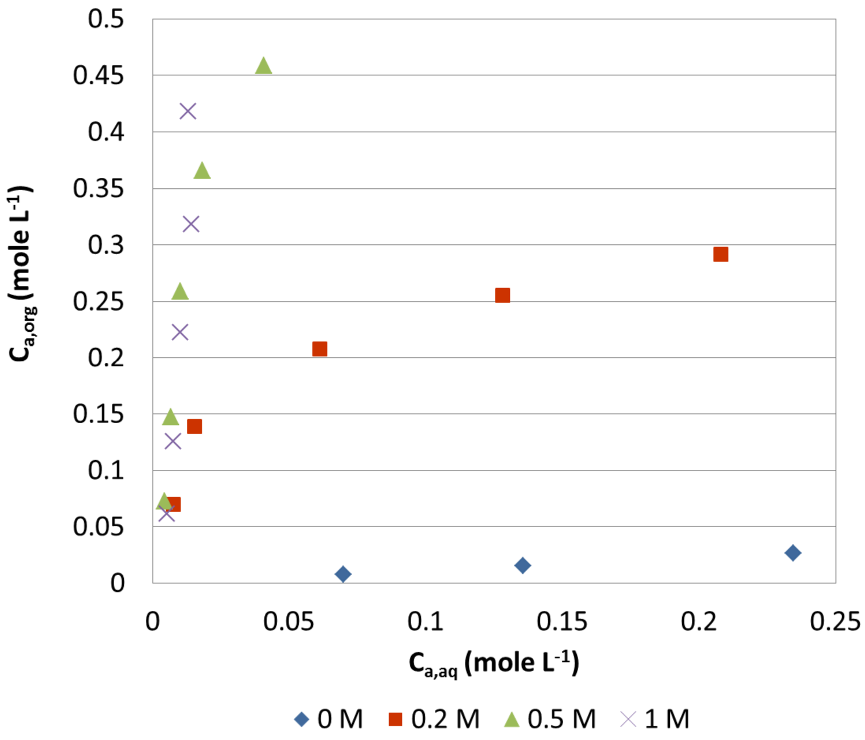


(e)

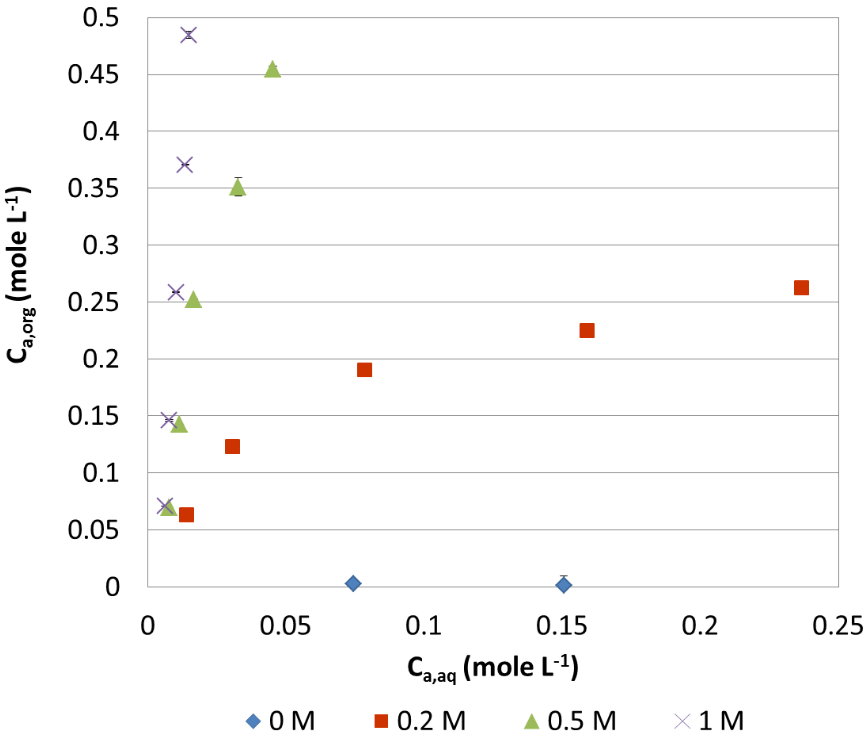
Fig. 3: Effect of varying amine concentration on extraction yield of IA (a) Dioctylamine (b) Trioctylamine (c) Trihexylamine (d) Tridodecylamine (e) N-methyldioctylamine. Average values are reported. The mean and the standard deviation from the replicates are given.


0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
$C_{a,org}$ (mole L^{-1})
0
0.05
0.1
0.15
0.2
0.25
$C_{a,aq}$ (mole L^{-1})
◆ 0 M
■ 0.2 M
▲ 0.5 M
× 1 M

(a)

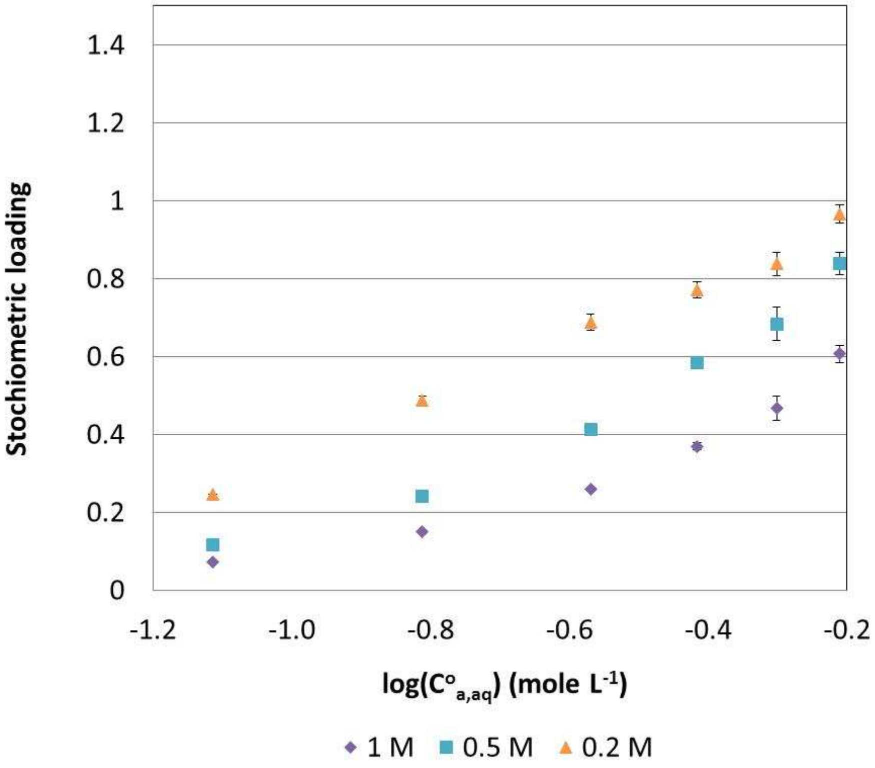

0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
$C_{a,org}$ (mole L^{-1})
0
0.05
0.1
0.15
0.2
0.25
$C_{a,aq}$ (mole L^{-1})
◆ 0 M
■ 0.2 M
▲ 0.5 M
× 1 M

(b)

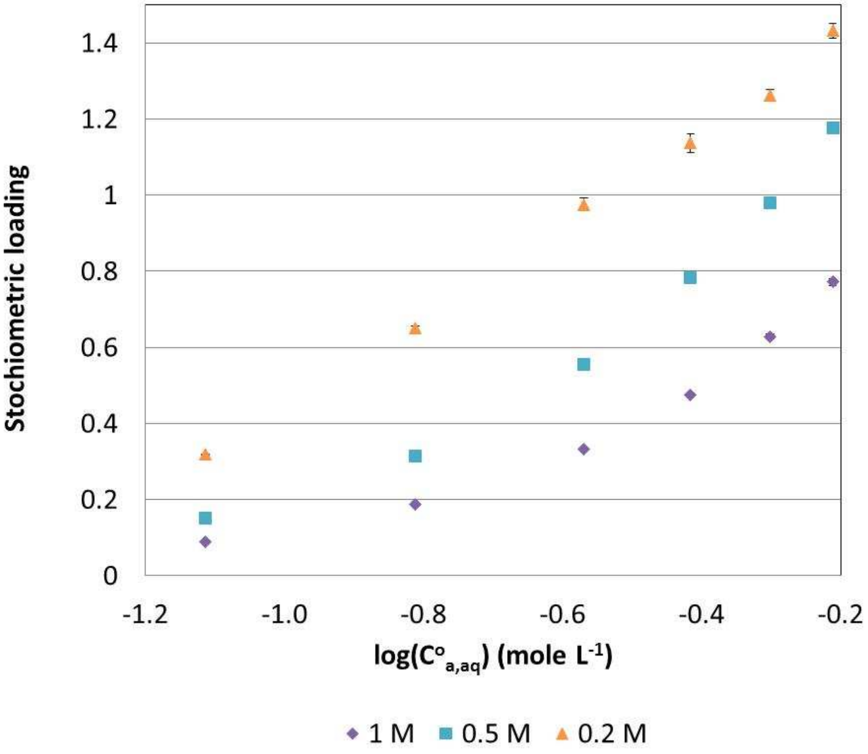


(c)

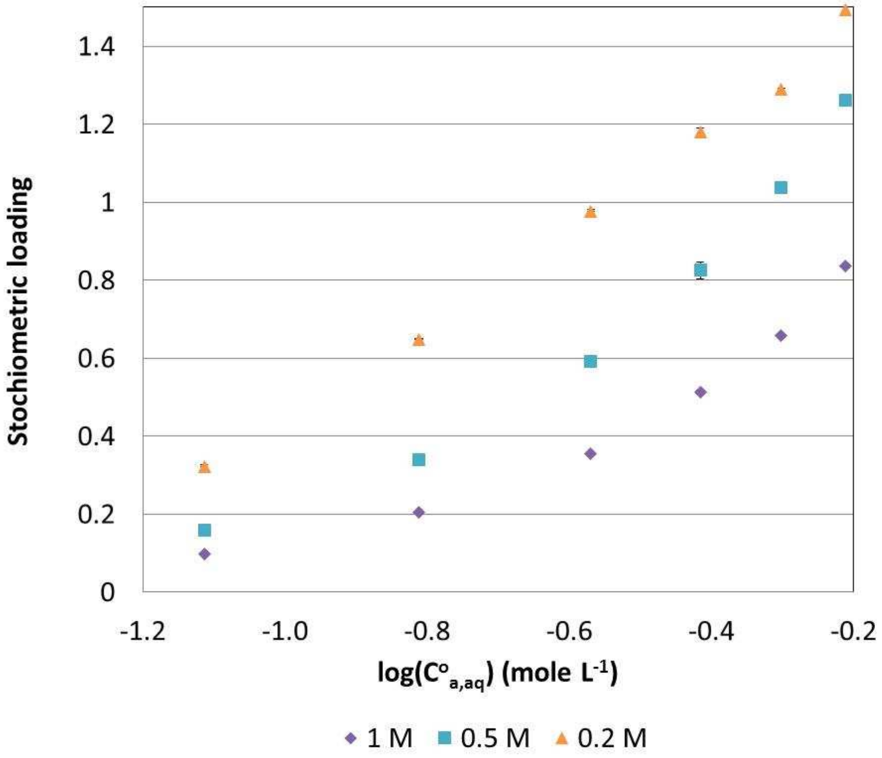
Fig. 4: Chemical equilibria for the extraction of IA using various concentrations of amines (pure diluent, 0.2, 0.5 and 1 mole L^{-1}). (a) Experiments with trioctylamine/1-octanol (b) Experiments with trioctylamine/pentylacetate (c) Experiments with trioctylamine/methyloctanoate. The mean and the standard deviation from the replicates are given.


Stochiometric loading
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-1.2
-1.0
-0.8
-0.6
-0.4
-0.2
log(C°a,aq) (mole L-1)
◆ 1 M
■ 0.5 M
▲ 0.2 M

(a)

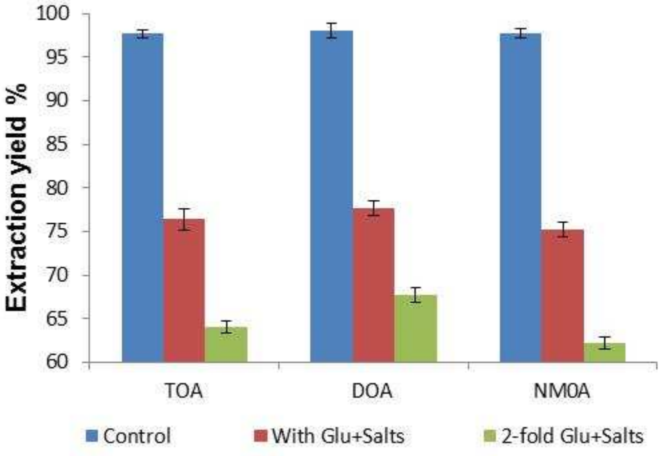

Stochiometric loading
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-1.2
-1.0
-0.8
-0.6
-0.4
-0.2
log(C°a,aq) (mole L-1)
◆ 1 M
■ 0.5 M
▲ 0.2 M

(b)



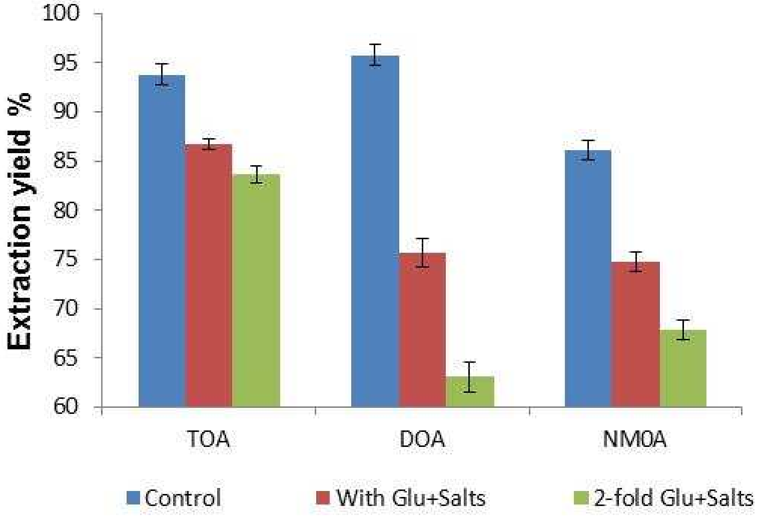
(c)

Fig. 5: Stochiometric loading as a function of IA concentration in the aqueous phase for the various concentrations of amines (0.2, 0.5 and 1 mole L^{-1}). (a) Experiments with trioctylamine/1-octanol (b) Experiments with trioctylamine/pentylacetate (c) Experiments with trioctylamine/methyloctanoate. The mean and the standard deviation from the replicates are given.



Extraction yield %
100
95
90
85
80
75
70
65
60
TOA
DOA
NM0A
Control
With Glu+Salts
2-fold Glu+Salts

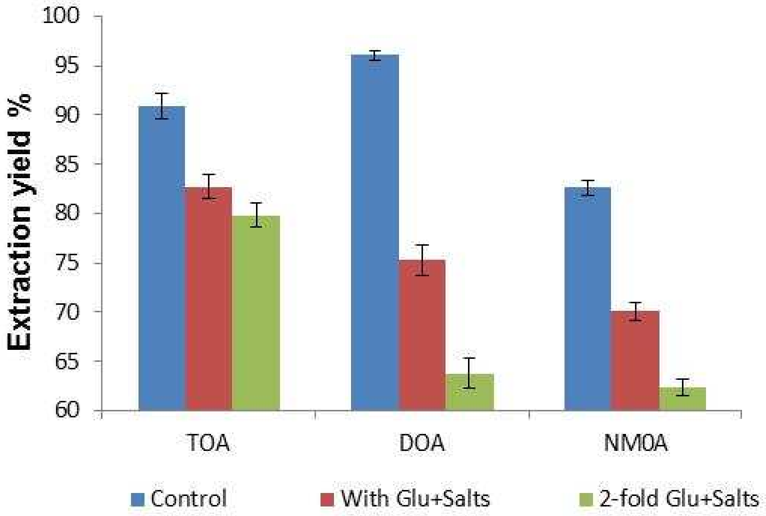
(a)



Extraction yield %
100
95
90
85
80
75
70
65
60
TOA
DOA
NM0A
Control
With Glu+Salts
2-fold Glu+Salts

(b)

(b)



(c)

Fig. 6: Effect of presence of medium components on extraction yield of IA (10 g L^{-1} aqueous solutions) (a) Experiments with 1-octanol (b) Experiments with pentylacetate (c) Experiments with methyloctanoate. TOA- trioctylamine, DOA- dioctylamine, NMOA- N-Methyldioctylamine. Control- Base case with only IA and no glucose and salts. The mean and the standard deviation from the replicates are given.

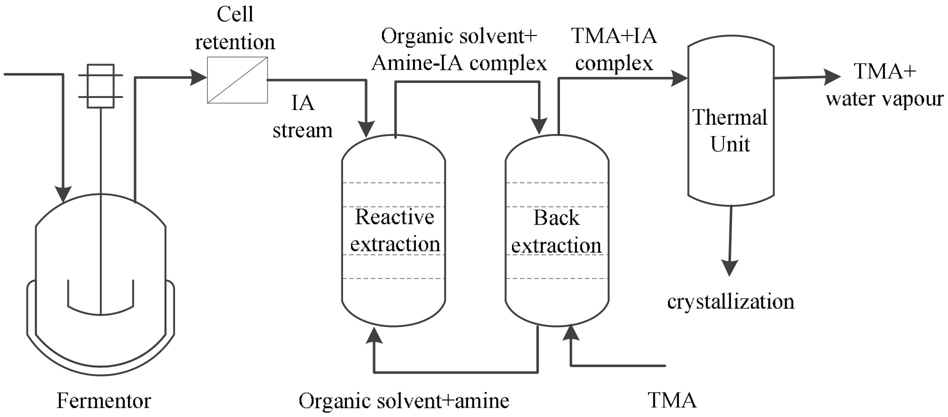


Fig. 7: Process flow design of ISPR-coupled IA fermentation

Table 1 Partition coefficient (K_{dil}) and extraction yield ($E\%_{dil}$) for the (physical) extraction with the pure diluents.

	1-hexanol	2-hexanol	1-octanol	2-octanol	1-decanol	2-decanol	1-dodecanol	2-dodecanol	pentylacetate	methyloctanoate	methyllaurate	hexane	heptane	octane	decane	dodecane	hexadecane
K_{dil}	1	1.5	0.63	0.74	0.35	0.49	0.21	0.27	0.13	0.05	0.11	0.09	0.09	0.09	0.10	0.10	0.10
$E\%_{dil}$	**50.6**	**60.0**	**38.7**	**42.7**	**25.7**	**32.8**	**17.6**	**21.1**	11.2	**4.1**	10	8.5	8.5	8.7	9.3	8.7	8.7

Table 2 Partition coefficient (K) values for 136 reactive extraction systems. **Bold-** amine-diluent combinations with high K values. NMOA- N-methyldioctylamine, N,N-DMOA- N,N-dimethyloctylamine. Grey- amine-diluent combinations for which a third layer was observed indicative for aggregation.

	1-hexanol	2-hexanol	1-octanol	2-octanol	1-decanol	2-decanol	1-dodecanol	2-dodecanol	pentylacetate	methyloctanoate	methyllaurate	hexane	heptane	octane	decane	dodecane	hexadecane
octylamine	26	19	23	24	21	14	20	12	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.2
dioctylamine	**337**	**146**	**283**	**273**	**241**	**183**	**185**	**167**	13	**104**	**53**	51	39	37	51	74	40
tributylamine	2.1	1.4	1.5	1.0	0.3	0.4	0.5	0.4	0.3	0.3	0.2	0.2	0.3	0.3	0.3	0.3	0.3
trihexylamine	**84**	**55**	**67**	**57**	**35**	**43**	**68**	**61**	**26**	20	16	8	8	9	7	9	10
trioctylamine	**294**	**107**	**222**	**218**	**164**	**133**	**125**	**110**	**60**	**36**	24	7	8	7	8	9	10
tridodecylamine	**277**	**86**	**189**	**132**	**135**	**89**	**101**	3	**36**	23	15	4	4	3	3	4	4
NMOA	**123**	**70**	**101**	**84**	**60**	**70**	**41**	4	**43**	**34**	26	20	20	20	19	21	22
N,N-DMOA	10	7	9	5	8	.4	7	17	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	.03