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Chapter 5

In-stream itaconic acid recovery from *Aspergillus terreus* fedbatch fermentation

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Abstract

The broad application of itaconic acid in synthetic resins, fibers, plastics, rubbers, surfactants, and oil additives has resulted in an increased demand for this product. As an end step of the bio-based itaconic acid production, a suitable downstream recovery procedure using *Aspergillus terreus* as a study model was analyzed. Here we present preliminary work to integrate fungal fermentation with in-stream product recovery (ISPR) using pertraction for itaconic acid. Based on prior knowledge (Abraham et al. 2000; Schlosser and Marták 2011) and an initial solvent screening, solvent extraction using 2-methyl tetrahydrofuran (2m-THF) was selected as preferred technique for recovering itaconic acid.

Introduction

Itaconic acid is a C5 dicarboxylic acid produced by filamentous fungi *Aspergillus terreus* and *Aspergillus itaconicus* (Tate 1981). This acid is considered as a chemical building block worldwide used for generating co-monomers and further polymers, in the manufacturing of painting materials, rubber, synthetic glass fibers, flexible nylons etc. (Bonnarme et al. 1995; Kautola 1990; Pfeifer et al. 1952; Tate 1981; Willke and Vorlop 2001). Currently, in line with developing a bio-based economy, industrial production of itaconic acid is mainly performed by *Aspergillus terreus* via sub-merged fermentation processes (Okabe et al. 2009).

Here we present preliminary work to integrate itaconic acid production with product separation, aiming at increasing the production level and reducing the process costs. The current industrial down-stream process for recovering itaconic acid is mainly focused on its extraction from the aqueous broth phase after completing the fermentation using techniques such as liquid-liquid extraction, precipitation, absorption, reverse osmosis etc (Hogle et al. 2002;Zhang et al. 2009).

Given the fact that product inhibition of itaconic acid already occurs at 20 g/L in *A. terreus* (Yahiro 1995), we assume that the production of itaconic acid could be stimulated by removing this acid from the bio-reactor during the fermentation process. Therefore, an in-stream product removal (ISPR) method was explored for continuously removing itaconic acid throughout the production phase. Several techniques have been used for in-stream dicarboxylic acids recovery including co-crystallization (Urbanus et al. 2012), adsorption (Davison et al. 2004), solvent impregnated resins (Juang et al. 1995), cooling crystallization (Urbanus et al. 2012) amongst others. In our case, pertraction was selected as an advanced technique to extract itaconic acid from the cultivation broth. Similar to liquid-liquid extraction, pertraction is a membrane extraction process utilizing the features of interactions between two different solvents which are separated by a membrane. The aqueous phase containing the solute can interact with the organic phase at the surface of a hydrophobic membrane and transfer the solute to the organic solvent (Schlosser and Marták 2011).

In this study, to achieve the in stream extraction of itaconic acid, three steps were addressed. First, a suitable solvent had to be selected for the extraction of itaconic acid from the fermentation broth in the pertraction module. The solvent selection criteria include (i) an optimal difference in solubility of itaconic acid between solvent and water phase, (ii) low cost, (iii) easy regeneration and (iv) low solubility/miscibility with water. The

difference in solubility of itaconic acid between solvent phase and water phase is depending on the so called partition coefficient. (Abraham et al. 2000).

A next step was to couple the pertraction module to the batch fermentation production process for itaconic acid adapted with proper in and out flow modules. Finally, the whole in stream recovery process was evaluated in an actual fermentation experiment for itaconic acid production.

Materials & Methods

Chemicals

All chemicals used in this study were purchased from Sigma Aldrich in 98-99% purity. The organic solvents selected in this study are: 2-methyl tetrahydrofuran (2m-THF); Cyclohexanone; Dibutyl Sulfoxide (DBSO); methyl iso-butyl ketone (MIBK); N,N-dibutylformamide; Tributyl phosphate (TBP); tri-n-ocylphosphine oxide (TOPO) +octanol; Tricresyl phosphate.

Itaconic acid solvent/water extraction

A saturated itaconic acid solution was prepared by dissolving itaconic acid in water above its solubility (71 g/L at 20℃) and filtering with a microfilter (0.2 μM). The saturated solution of itaconic acid was mixed with organic solvent in equal volumetric ratio (1:1) and was stirred for 2 hours. The mixture was then transferred to a separating funnel and was allowed to settle overnight to form two stable phases based on the density difference of the water and solvent phases. The two phases were separated by gravity in the separating funnel thereby discarding the interphase. The aqueous phase of the mixture was analysed for itaconic acid concentration by titration.

Solvent toxicity test in shake flask culture of *Aspergillus*

To check the inhibition on biomass growth by organic solvents, *A. terreus* NRRL1960 and an alternative host for itaconic acid production, *A. niger* strain N201 CAD (Li et al. 2012) were cultivated in shake flask experiments with the preferred production medium (Li et al., 2012) saturated with the solvents. Three preselected solvents: 2m-THF, MIBK and DBSO were applied in this test. The sterilized production medium (40 mL) was saturated with solvent as described above to obtain a medium-solvent mixture. *Aspergillus niger* and *Aspergillus terreus* was precultured for two days in production medium (10^6 spores/mL). Subsequently, 1.6 ml pre-culture was inoculated to the solvent-saturated medium. The cultivation was followed for 7 days at 37℃. Culture samples (2 ml) were taken at day2, 4 and (10 ml) at day7 for biomass

determination. Biomass was harvested by filtration of the culture through a pre-weight filter and washed with demineralized water. Subsequently, the harvested biomass with the filter was dried in an oven at 110℃ for 24 hours and weighed. The biomass dry weight was established by subtracting the weight of the dry filter.

Pertraction

For pertraction, the membrane module (non-metallic polytetrafluroethylene (PTFE) /Teflon module) was packed with 2 O-rings and a double spacer layer on both sides of the PTFE (120 μm) membrane. The packed membrane was connected to a pertraction vessel through a centrifugal pump and filled with 300 ml of saturated aqueous itaconic acid solution/ filtrated itaconic acid fermentation culture. Another pertraction vessel filled with 300 mL organic solvent was connected to another centrifugal pump. Both of the pumps were switched on and were allowed to stabilize untill no air bubbles were present during pertraction and no leakage.
Before connecting to the organic phase and starting the actual pertraction, the membrane module connected to the aqueous phase was maintained at a higher pressure of 0.1 bar to check leakage for approximately 20 minutes. Flow diagram of pertraction set-up was shown in Figure 1. Samples were collected each 3-4 hours and analyzed for itaconic acid by titration.

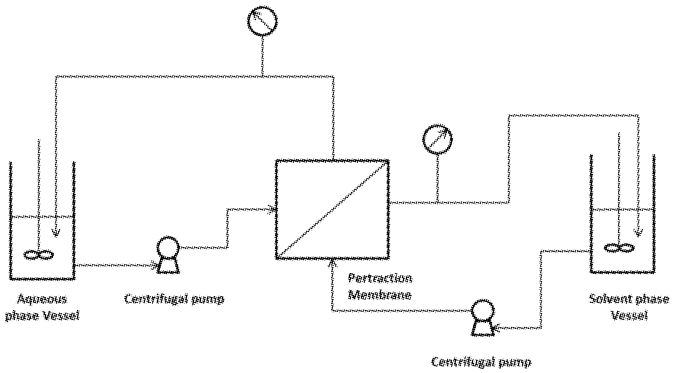

Figure 1. Flow Diagram: Pertraction. The vessel in the middle is the pertraction module, with a pertraction membrane separating the pertraction vessel in two parts. The left part is connected to the aqueous phase vessel, while the right part is connected to the solvent phase vessel. Both parts are circulated via two pumps, one (indicated with an arrow in the circle) is used to pump liquid out of the pertraction vessel and the other (centrifugal pump) pumps liquid phase back to the pertaction vessel.

Solvent Regeneration and itaconic acid recovery

Itaconic acid was recovered from the solvent phase by extraction with 1 M NaOH solution. To determine the required regeneration approach in a model set-up, 100 mL of solvent mixture containing 20 g/L itaconic acid was bubbled from the bottom of the NaOH solution at a speed of 8 mL/min so that small bubbles were formed (Figure 2). Itaconic acid transfer took place and a biphasic system was formed as the bubbles passed through the NaOH phase. Solvent (top phase) was pumped back to the pertraction vessel. From this experiment the suitable concentration of NaOH was determined for regeneration. The selected solvent had a high vapor pressure compared to itaconic acid, adding the possibility of distillation as a regeneration technology. However, continuous regeneration at lab-scale with a base-wash was easier to perform while running the fermentation and pertraction at the same time as well.

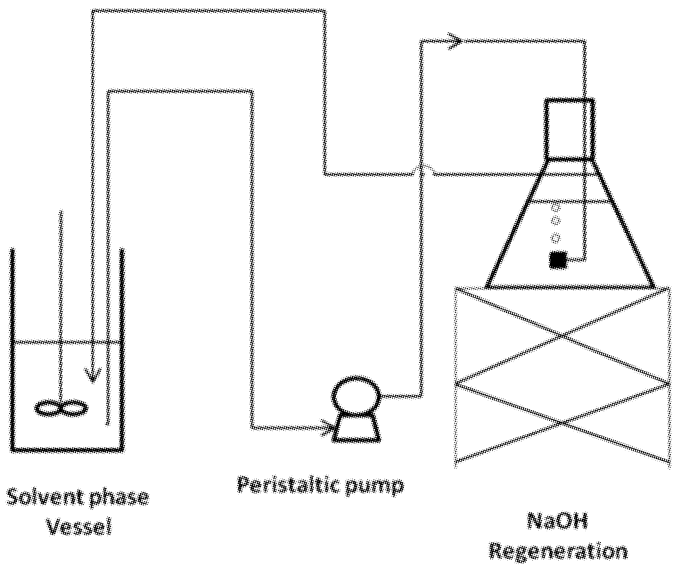

Figure 2. Solvent Regeneration Flow Diagram. The solvent phase (saturated with itaconic acid) was pumped to the NaOH phase for itaconic acid recovery and solvent regeneration. In the NaOH regeneration vessel, solvent phase passed though NaOH phase from the bottom of the vessel. During this step, itaconic acid reacted with NaOH and was recovered from the solvent phase.

In-stream process recovery

An integrated Fermentation-pertraction system for itaconic acid recovery in *A. terreus* fermentation process is shown in Figure 3. In this case, to

prevent fouling of the pertraction membrane and blocking of the tubings by mycelia, a filter membrane (polypropylene membrane, 0.22 μm, Applikon Biotechnology) was inserted into the fermentation broth for harvesting samples and supply itaconic acid culture for pertraction. As indicated in figure 3, the aqueous phase vessel (second from left) was filled with 300 ml filtrated itaconic acid culture broth. Pertraction was initiated after 48 hours of cultivation. The outflow of fermenter was connected to an accumulation vessel which in turn provided feed to the pertraction system.

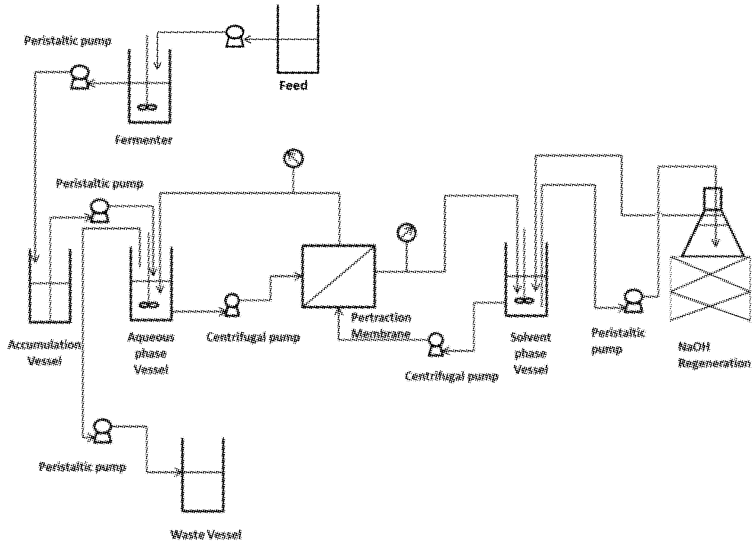

Figure 3. Integrated fermentation-pertraction flow diagram in itaconic acid production process. The filtrated fermentation broth was first accumulated in an accumulation vessel and then continuously pumped into the aqueous phase vessel. As described in figure 1 and 2, aqueous phase vessel is connected to pertraction module and solvent phase vessel is connected with pertraction module and the NaOH regeneration vessel. Meanwhile, to prevent recycling medium broth after pertraction, aqueous phase vessel is also connected to a waste vessel. The decreased volume in fermenter is compensated with feed.

Fermentation set-up, medium conditions and sampling

Aspergillus terreus strain NRRL1960 (Li et al., 2011) was used for a controlled batch fermentation for studying in stream recovery of itaconic acid. The production medium contained per liter 2.36 g of NH_4SO_4, 0.11 g of KH_2PO_4, 2.08 g of $MgSO_4 \cdot 7H_2O$, 0.13 g of $CaCl_2 \cdot 2H_2O$, 0.074 g of NaCl, 0.2 mg of $CuSO_4 \cdot 5H_2O$, 5.5 mg of $Fe_2SO_4 \cdot 7H_2O$ and 1.3 mg of $ZnSO_4 \cdot 7H_2O$ and 0.7 mg of $MnCl_2 \cdot 4H_2O$ and 100 g of glucose as a carbon source. All media were prepared in demineralized water. Before inoculation of the fermenters, *A. terreus* strain was precultured 64 hours in 2x 500 ml

baffled Erlenmeyer flasks with 100 ml of glucose production medium (10^6 spores/ml), 125 rpm, 37℃. Fermentations were performed in 5 liter Benchtop Fermenters (BioFlo 3000, New Brunswick Scientific Co., Inc.) at 37℃ for 7 days. The basic pH regime was initiated at 3. 5 and subsequently fixed at 2.3, by 4M KOH (Base) and 1.5 M H_3PO_4(Acid). In the basic DO (Dissolved oxygen) regime, DO was controlled at 25% . Struktol (Schill & Seilacher) was applied as antifoam agent in all cultures throughout the fermentation.

The feed started from 48 hours was applied for fermentation with a pertraction to compensate the fixed cultivation volume of 5 liter and without extra addition of nitrogen and phosphate source. The feed contained the same ingredients as the production medium, except NH_4SO_4 and KH_2PO_4 which were not added and 50 g/L glucose instead of 100 g/L was included. Biomass of the batch cultivation was determined as described above.

As soon as the aqueous phase vessel (Figure 3) was filled with cell free fermentation broth, the pretraction module was set-up with PTFE membrane (120 μm) attached to both the organic and aqueous pertraction vessels. 2m-THF was used as solvent in the solvent phase vessel (600 ml) and 4N NaOH was filled in the regenerating vessel (900 ml). Pertraction was started around 48 h corresponding to about 30 g/L itaconic acid production. Samples were collected from: A.) the broth leaving the fermenter; B.) the aquous stream leaving pertraction; C.) the NaOH regeneration vessel.

Analytical Methods

Itaconic acid analysis by titration or HPLC analysis

For quantifying the amount of itaconic acid in the solvent screening experiments, titration was carried out as follows: 200 μL of the samples were diluted 100 times to 20 mL and were analyzed with a titrator (Titralab 965). Aqueous phase samples were titrated with 0.1 M KOH with end point at pH 8.0 while the samples from NaOH phase were titrated with 0.1 M HCl till pH 3.0 as end point. The amount of itaconic acid in the samples was determined using a calibration curve.

For quantifying the amount of itaconic acid in samples collected from fermentation cultures and pertraction HPLC analysis was performed Dionex ICS 3000 equipment from Thermo Fisher was used for analyzing organic acids using an organic acids column IonPac® ICE AS6, with 1.6 mM Heptafluorobutyric acid as eluent and a detector of suppressed conductivity CD25.

Results

Solvent selection and inhibition test on fungal growth

To efficiently extract itaconic acid from the fermentation broth, the preferred solvent was preselected based on partition coefficients. A selected group of solvents were experimentally tested using a saturated solution of itaconic acid (1:1 ratio) by liquid /liquid extraction. The partition coefficients were determined by measuring the concentration of itaconic acid in different solvents when interacting with water. Besides the Partition coefficient also low solubility in water and low toxicity were used as selection criterion. In Table 1 an overview of these parameters for the various solvents is given. Tricresyl phosphate, Tributyl phosphate and TOPO+octanol were not considered due to their low partition coefficient (below 3). Although N, N-dibutylformamide and DBSO showed higher partition coefficients, their high price may limit their use for further studies. Overall 2m-THF seems preferable due to its partition coefficient and relative low price.

Table 1. Partition Coefficient comparison of solvents. The level of solubilitiy and price of the solvents were indicated with '+ , + + , + + + , + + + + ' (the higher the more '+ ' is). DBSO and N,N-dibutylformamide were marked in yellow due to their high price.

Solvents	Partition Coefficient	Water Solubility (Mol)	Price
Dibutyl Sulfoxide (DBSO)	15.5	+	+ + +
2 methyl tetrahydrofuron (2m-THF)	**4.4**	**+ +**	**+ +**
N,N-dibutylformamide	22.6	+	+ + +
Cyclohexanone	3.0	+ +	+ +
methyl iso-butyl ketone (MIBK)	0.4	+ +	+
Tricresyl phosphate	0.07	+ + +	+ +
Tributyl phosphate	2.4	+	+ +
TOPO + octanol	2.4	+ + +	+ +

Before using 2m-THF to the pertraction set-up, growth inhibition tests were carried out by cultivating *A. terreus* and *A. niger* in solvent saturated media. Beside 2m-THF, MIBK and DBSO were tested. As shown in Table 2, the biomass growth of two strains was indeed affected for 2m-THF and DBSO and much less/not for MIBK. However, still considerable growth was observed in all cases.

Table 2. Growth inhibition test of *A. niger* and *A. terreus* in the production medium saturated with three different solvents.

Solvent		g/kg (dry biomass)	
		A. niger	*A. terreus*
MIBK	Day 2	2.31	6.23
	Day 4	3.19	7.64
	Day 7	6.99	8.25
2m-THF	Day 2	0.58	1.03
	Day 4	1.08	1.79
	Day 7	2.35	2.98
DBSO	Day 2	1.45	1.63
	Day 4	1.77	2.19
	Day 7	2.02	2.36
No solvent	Day 2	5.13	7.73
	Day 4	8.78	10.09
	Day 7	12.69	10.12

Solvent test on itaconic acid recovery

To test the in situ capability of 2m-THF in extracting itaconic acid, an integrated pertraction regeneration experiment was performed with NaOH as a regeneration phase. As shown in Figure 4, the aqueous phase showed a decrease while the regeneration phase (NaOH) showed an increase in the concentration of itaconic acid, demonstrating successful extraction. Three hours later (180 min), regeneration phase became saturated. As calculated on the slope, 2m-THF had a maximal itaconic acid transfer rate of (0.19 g/L/min).

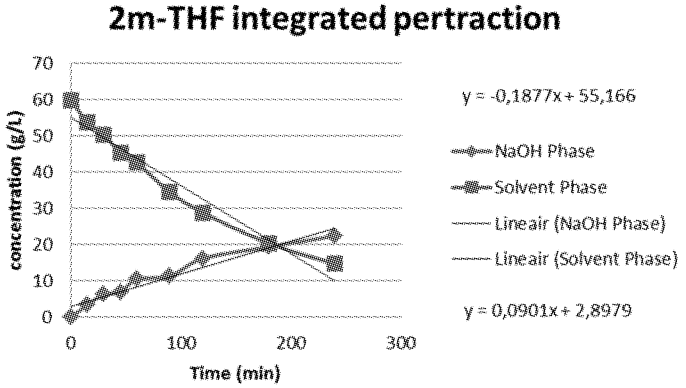

Figure 4. Integrated pertraction with 2m-THF. The aquous phase described the amount of itaconic acid in the solvent (2m-THF). The NaOH phase indicated the amount of itaconic acid in the NaOH solution. The trend lines for both phases were determined by curvefitting.

In order to determine the theoretical recovery into the NaOH phase, the result of 2m-THF integrated pertraction fitted curve was used (y = 0. 0901x + 2.8924; y represents NaOH (g/L), x represents time (min)). Based on this accumulation of itaconic acid in NaOH phase was calculated (Figure 5) using 1M NaOH. As indicated, this would require renewal of the recovery solution several times during fermentation. To avoid this, we decided to use a higher concentration of 4 M NaOH for regeneration.

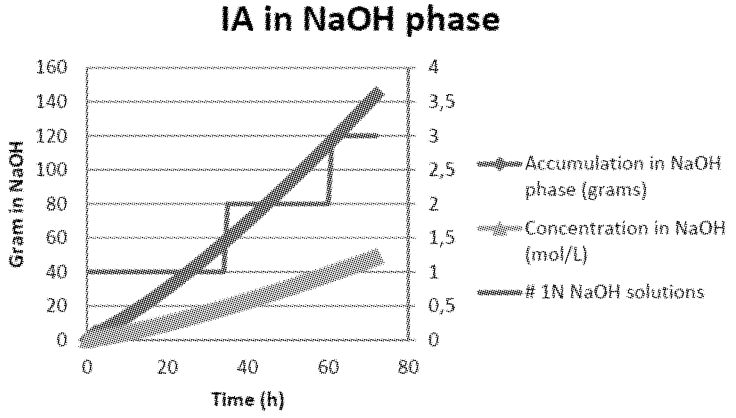

Figure 5. Itaconic acid accumulation calculated in NaOH phase (calculated based on equation y = 0.0901x + 2.8924 in figure 4).

In-stream itaconic acid recovery

After selection of 2m-THF as preferred solvent, we connected the whole pertraction regeneration model to the fermentation module (Figure 3). In this initial stage to prevent fungal growth inhibition by solvent inside fermenter, the outlet stream from fermentation was not recirculated after pertraction. Instead, the extra volume accumulated in the aqueous vessel was transferred to a waste vessel. The volume thus removed from the fermenter was compensated by addition of fresh culture medium.

In the ISPR-coupled fermentation, the in (feed) and out flow rate will influence the concentration of itaconic acid inside fermenter, the accumulation vessel and pertraction vessel (Figure 3). To set-up a proper in and out flow rate, the effect of flow rate on itaconic acid concentration inside the fermenter was estimated. The production rate of itaconic acid was estimated to be 1 g/L/hour based on earlier reference fermentations from *A. terreus* (Li et al. 2011). As shown in Figure 6, increased flow rate would result in decreased concentration of itaconic acid inside the fermenter, and vice versa. A lower itaconic acid concentration inside the fermenter would cause reduction of the concentration gradient across the pertraction membrane. Assuming

about 30 g/l itaconic acid in the fermenter, a flow rate of 3.5 mL/min was favorable. Based on this, a model was developed to predict the concentration of itaconic acid in fermenter, where the concentration inside the pertraction vessel was calculated based on the equation (y = -0.1877x + 55.166; y represents itaconic acid (g/L), x represents time (min)) generated from 2m-THF pertraction experiment described in Figure 4. The calculated concentration of itaconic acid inside the pertraction vessel was about 50% of the concentration inside the fermenter vessel.

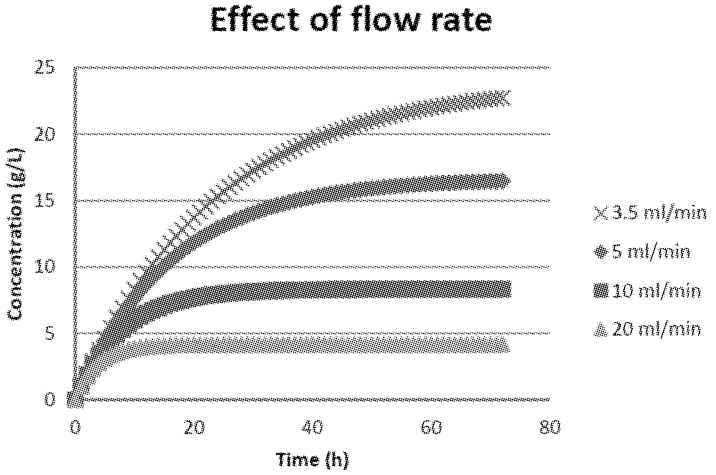

Figure 6. The effect of flow rate on concentration of itaconic acid inside the fermenter [In (g/hour) - Out(g/hour) + production(g/hour) = accumulation(g/hour)].

The amount of itaconic acid produced from ISPR fermentation was also compared with the reference. As present in Figure 7, compared to the reference, the ISPR one accumulated similar amount of itaconic acid per biomass at the end of the fermentation (140 hours). Other metabolites such as citric acid and oxalic acid were not accumulated in both fermentations. It should be noted that the biomass growth of *A. terreus* strain was higher in the ISPR fermentation (Figure 8), probably due to compensating feed edition during pertraction.

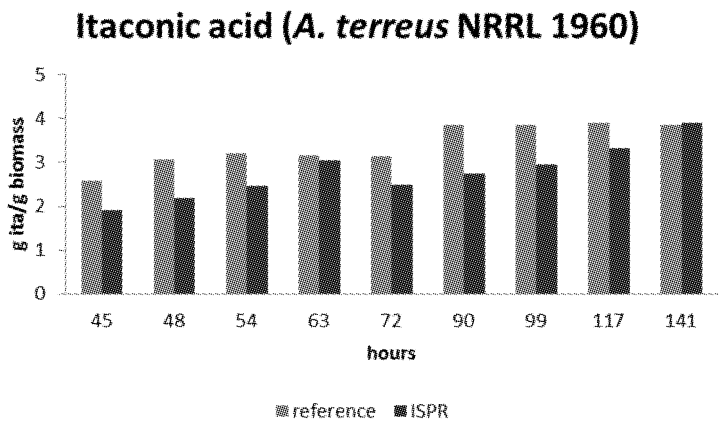

Figure 7. Itaconic acid production of *A. terreus* strain NRRL 1960 in batch fermentations.

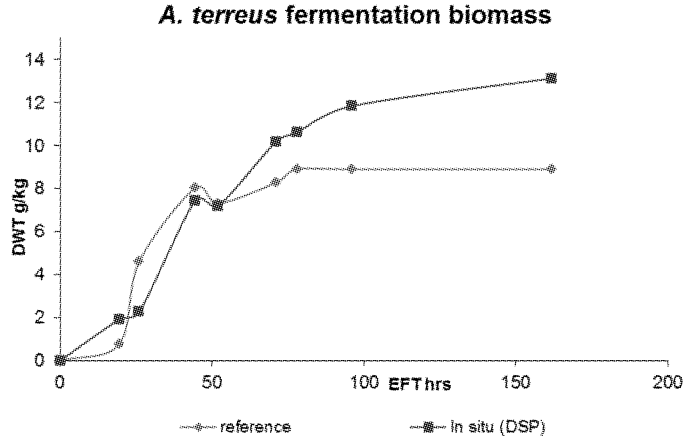

Figure 8. Biomass growth of *A. terreus* strain NRRL 1960 in batch fermentations. (◆— reference fermentation; ■—fermentation with DSP itaconic acid recovery.)

As explained above the initial flow rate of the pertraction module was set at 3.5 mL/min. However, due to the strong fouling of the outer surface of the membrane, both in and out let flow rate was reduced to 2.8 mL/min. Samples were collected at three locations: A: the broth leaving fermenter; B: the stream leaving pertraction; C: the NaOH phase. The amount of itaconic acid determined in these fractions is given in Figure 9. In average, the amount of itaconic acid in sample B was approximately 50% of the amount in sample A, which was expected based on our calculation. As expected, the amount of itaconic acid in sample C steadily increased, eventually higher than the level present in sample B.

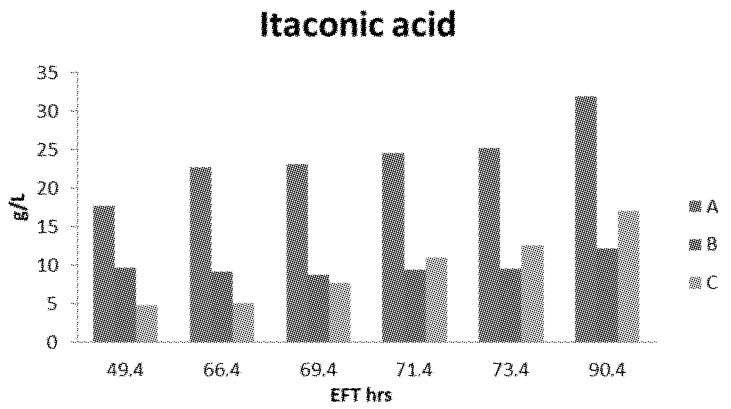

Figure 9. Itaconic acid from samples collected in three different out flow locations (recalculated to the original volume). A.) the broth leaving fermenter; B.) from stream leaving pertraction; C.) from NaOH phase.

Discussion

To evaluate the potential benefits of an in stream recovery process for improving itaconic acid production, we've explored the potential of using an pertraction based ISPR method coupled to the fermenter module. In comparison with the Co-crystallization methods commonly used in industrial itaconic acid recovery (Okabe et al. 2009), the pertraction (solvent extraction) method is more specific and requires simpler equipment. However, most of the organic solvents are toxic, thus requiring specific containment conditions. Moreover, itaconic acid recovery from the solvent and solvent regeneration may limit the use of pertraction. From a small list of potential solvents 2-methyl tetrahydrofuran (2m-THF) was selected as preferred solvent.

Approximately 50% of itaconic acid from ISPR was extracted by the pertraction module (Figure 9). Moreover, efficient itaconic acid recovery and organic solvent regeneration was accomplished

To ensure a high efficiency of itaconic acid extraction, in our set-up an in-stream (inside fermenter) microfiltration step was include prior to the actual pertraction, which prevented mycelial fragments fouling the PTEF hydrophobic membrane. However, due to fouling of the microfiltration unit, the flow rate out of the fermentation broth could not reach 3.5 mL/min as was calculated to be optimal. To solve this problem other filter membranes with less fouling may be used. However, a critical point for membrane selection may be that the membrane should be low pH resistant (pH 1.5-2.5). Recently, a study group from Japan developed a novel PVDF based membrane-integrated fermentation module allowing

filtration for more than 300 hours without fouling (Hideki 2011).
In the initial set-up we developed, to prevent the inhibition of biomass growth by the solvent, the extracted broth was not returned back into the fermenter. However, the solvent test showed the capability of *A. terreus* to grow in solvent saturated medium, second generation ISPR set-up may include to recirculate the broth after extraction back into the fermenter. Also other organic solvent with even less solubility, higher partition coefficient and lower toxicity than 2m-THF may be selected.

Conclusion

This work resulted in an interesting concept to optimize itaconic acid production in fed-batch fermentation coupled to in stream production recovery (ISPR). The designed pertraction module could accurately reach the predicted solvent extraction efficiency. The capability of *Aspergillus* to grow in solvent saturated medium may even provide an opportunity of recirculating the outlet flow back into the fermenter which could eventually lead to a more or less continuous production and separation process.

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