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CASE STUDY OF CONTINUOUS ITACONIC ACID FERMENTATION BY *ASPERGILLUS TERREUS* IN A BENCH-SCALE BIOREACTOR

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Itaconic acid (IA) is a highly important bioproduct. The traditional biotechnological production of organic acids generates large quantities of biomass and water streams as waste. Bipolar membrane electrodialysis is a suitable technique for directly recovering IA from clarified fermentation broth whereby itaconic acid is separated without chemical loading. As a result, the remaining diluate stream can be reintroduced into the fermentation process. This membrane operation can be integrated into a continuous fermentation process in order to carry out the process using a high glucose concentration in the effluent, opening up new possibilities for the implementation of continuous itaconic acid fermentation. In this study, a possible highly successful implementation of this technique is presented. The applied gentle stirring technique as well as the low dilution rate of 0.007 s^{-1} is necessary to be complemented by highly efficient oxygenation, thereby promoting itaconic acid generation. With an inlet supplying pure oxygen gas, the acid accumulated and an average of 30.1 $g \cdot L^{-1}$ titer of IA was achieved in the effluent. The volumetric oxygen uptake rate was monitored during the fermentation which fluctuated from 40-88% so further investigation of this may be worthwhile in the future.

Keywords: bipolar membrane electrodialysis; clump morphology; integrated system; oxygen uptake rate

1. Introduction

Itaconic acid (IA) – a five-carbon, unsaturated di-carboxylic metabolite of *Aspergillus terreus (A. terreus)* – has important industrial applications, e.g. as a cross-linking agent in synthetic latex production and as a monomer for unsaturated polyester resins [1]. In addition, much research is being conducted into the potential for further applications of IA in food packaging, pharmaceuticals and environmental protection [2]-[3].

IA is an industrial biotechnological product [4]. One disadvantage of most technological solutions implemented in industry for the production of organic acid via fermentation is the generation of significant waste streams, e.g. massive amounts of biomass and waste water. Bipolar membrane electrodialysis (EDBM) is a promising technique in biotechnology since the separating force is not a chemical reaction based on the addition of a chemical compound but an electric voltage [5]. This separation method results in three valuable material streams: the product in acidic form as well as an alkaline solution generated from alkali metal ions separated from the broth and the diluate – the broth in the absence of small ionic compounds but containing all the residual nutrients.

In the case of IA, the applicability of EDBM was proven in our laboratory not only from model solutions [6]-[7] but also on a real, clarified fermentation broth [8]. In the following step, our goal was to implement a continuous fermentation with a relevant titer of IA in the effluent. In the study on model solutions, it was established that the electrodialysis unit operated more efficiently when the concentration of IA was towards the higher end of the investigated range, namely 5-33 $g \cdot L^{-1}$. The effect of reintroducing the diluate from EDBM back into the fermentation process has not yet been studied.

A few studies, the last of which was published in 2000, have been reported on continuous IA fermentations with *A. terreus* as the producing organism [9]. The achieved acid concentrations, the effective volume and the applied dilution rates are listed in *Table 1*.

The application of the free mycelia of *A. terreus* resulted in a maximum IA concentration of 7.8 $g \cdot L^{-1}$ as reported by Rychtera and Wase [12]. Immobilization [11],[13] by applying a complex reactor design [10] resulted in enhanced titers of IA (see *Table 1*).

The morphology of *A. terreus* is highly important. Clumps (loose pellets) 0.4-0.5 mm in diameter are considered to be optimal for acid production [14], moreover, were found to be controllable by manipulating the degree of mechanical stress.

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Table 1: Continuous IA fermentations with *A. terreus* spp. as the producing microorganism

V_{eff} : effective volume; D: dilution rate; u: flow rate; c_{IA}: itaconic acid concentration in the effluent
*calculated values based on V_{eff} and D; **calculated value at the point of maximum productivity; ***when pH=3.1

Ref.	Strain specification	Technique	V_{eff} [mL]	D [h^{-1}]	u [$mL \cdot h^{-1}$] ($u = D*V_{eff}$)	c_{IA} [$g \cdot L^{-1}$]
[10]	*A. terreus* NRRL 1960	mycelia immobilized in a porous disk	3600	0.017	60	18.2
[11]	*A. terreus* G-026	mycelia immobilized in polyacrylamide gels	100	0.04	4*	15**
[12]	*A. terreus* NRRL 1960	free mycelia	3000	0.04	120*	7.8***
[9]	*A. terreus* NRRL 1963	free mycelia	1800	0.06	108*	0.3
[9]	*A. terreus* NRRL 1963	free mycelia	1800	0.1	180*	0.15
[13]	*A. terreus* NRRL 1960	mycelia immobilized on Celite R-626	14	0.111	1.56*	11

In the case of fermentation with free mycelia, it is apparent that by reducing the dilution rate, the achievable titer of IA in the effluent is enhanced.

Therefore, it can be assumed that by using a well-selected apparatus with a low dilution rate, free-mycelia fermentation with pellet growth can be achieved, producing results similar to when immobilized.

Oxygen is an important substrate for IA formation [15]. During a batch operation, to achieve maximal biomass growth, 2% saturation of dissolved oxygen was shown to be sufficient but IA generation proved significantly better when above 30% [16]. An important factor influencing the production of IA is the variation of dissolved oxygen levels in the fermentation. The monitoring of oxygen uptake during continuous IA fermentation with *A. terreus* has not yet been reported in the scientific literature.

2. Experimental methods

2.1. Microorganism and culture media

In this research, *A. terreus* NRRL 1960, a filamentous fungus, was used as the IA-producing organism maintained on potato glucose agar (Honeywell Fluka™, USA) into which solutions of 20 $g \cdot L^{-1}$ sodium chloride and 10 $g \cdot L^{-1}$ glucose monohydrate were added before sterilization.

A conidial suspension served as the inoculum for the fermentation. The suspension was obtained from a solid-state colony in a Roux culture flask with an agar surface area of 150 cm^2 that was incubated for 7 days at 33 °C. The harvesting solution was 100 mL of a physiological saline solution containing 0.01 w/w % Tween 80.

The composition of 1 L of the continuous fermentation medium: 165.0 g of glucose monohydrate, 0.10 g of KH_2PO_4, 3.0 g of NH_4NO_3, 1.0 g of $MgSO_4*7H_2O$, 5.0 g of $CaCl_2*2H_2O$, $1.67x10^{-3}$ g of $FeCl_3*6H_2O$, $8x10^{-3}$ g of $ZnSO_4*7H_2O$ and $1.5x10^{-2}$ g of $CuSO_4*7H_2O$. The pH was set at 3.0 before sterilization with a 10% (w/w) sulphuric acid solution to prevent the glucose from browning during sterilization. The complete medium was sterilized in the reactor at 121 °C for 45 minutes.

The composition of the refilling medium per liter: 206.25 g of glucose monohydrate, 0.10 g of KH_2PO_4, 2.25 g of NH_4NO_3, 1.0 g of $MgSO_4*7H_2O$, 5.0 g of $CaCl_2*2H_2O$ and $8 \cdot 10^{-3}$ g of $ZnSO_4*7H_2O$, while the pH remained constant.

During the last period of fermentation that lasted two days, the glucose-rich deacidified stream from electrodialysis was reintroduced.

The chemicals applied in this study purchased from Merck were of analytical reagent grade, except for glucose which was supplied by Hungrana Starch and Isosugar Manufacturing and Trading Ltd.

2.2. Fermentation process

The experiment was carried out in a LAMBDA MINIFOR (LAMBDA Instruments GmbH, Switzerland) bench-top autoclavable laboratory fermenter equipped with a 3 liter nominal volume glass vessel. The effective volume was 1.8 L and the medium was tempered at 33 °C.

This instrument was equipped with a special ‘fish-tail’ up-and-down agitator (i.e. non-rotational), which facilitated the efficient transfer of energy in the liquid culture medium. According to its description, this kind of mixing is gentle but efficient in both the horizontal and vertical directions as well as provides optimal oxygenation and gas exchange in the absence of air flooding, which are advantageous properties when working with filamentous microorganisms. This technique was used to achieve clump growth of the productive strain during the continuous IA fermentation.

This morphology proved to be the most efficient IA-producing form of *A. terreus* [14], the formation of which can be influenced by the degree of stirring. The applied up-and-down movement occurred at a rate of 3.0 s^{-1} during the experiment.

The pH was not controlled. A low pH reduces the possibility of microbial contamination. According to Hevekerl et al. [17], the growth rate is reduced when the pH is not controlled but similar IA concentrations can be achieved. Moreover, significantly less biomass may be formed compared to when the pH is controlled. The potential uses of fungal biomass from the production of organic acids are limited and mostly regarded as waste.

At the start of the fermentation, air was supplied at a rate of 1.5 volume gas per volume broth per minute (VVM). After one week, the air supply was replaced by pure oxygen gas at a rate of 0.2 VVM.

6 ml samples were taken daily with a sterile needle and syringe through a silicone septum on top of the fermenter. The instantaneous glucose and IA concentrations in the reactor were determined and the microscopic appearance checked.

The oxygen uptake rate of the mycelia was determined daily.

Continuous operation of the fermenter started on Day 4. The flow rate was 12 $mL \cdot h^{-1}$, corresponding to a dilution rate of 0.007 h^{-1}. The refilling medium was introduced from above through a stainless steel pipe. The end of the pipe was 5 cm above the surface of the broth. The effluent was drained through another stainless steel tube that stretched to the bottom of the reactor vessel. A 2-channel precision peristaltic pump (Masterflex L/S Easy-Load II., Masterflex LLC, US) was used to simultaneously drain and refill. The direction of flow of the two streamlines countered each other.

The effluent container was replaced every 24 hours and the collected effluent volume analyzed for glucose and IA content.

Collected effluent volumes with IA concentrations in excess of 20 $g \cdot L^{-1}$ were combined and clarified by filtration using a glass fibre depth filter disk with a pore size of 1.2 µm (55 $g \cdot m^{-2}$, 270 µm thick). The pH of the clarified broth was adjusted to 5.0 before electrodialysis with a 10% (w/w) solution of sodium hydroxide. IA was recovered in a commercial EDBM unit, resulting in three separate liquid fractions: 1. purified and acidified product stream; 2. alkaline solution; 3. diluate – containing components from the broth which failed to or could not penetrate the ion-exchange membranes in the module, e.g. glucose. The diluate was acidified to pH 3.9 with a 10% (w/w) solution of sulphuric acid. To maintain sterility, it was heat-treated for 30 mins at 90 °C and cooled to room temperature before being used in the continuous fermentation to replace the refilling medium every two days.

2.3. Determination of oxygen uptake rate

To determine the oxygen uptake rate (OUR), the air flow via the inlet was shut off and the rate at which the dissolved oxygen level decreased monitored. An in-line O_2 probe (LAMBDA Instruments GmbH, Switzerland) was connected with a precision amperometer (Metrix MTX 3281, Chauvin Arnoux Group, France), which recorded data every second for 30 seconds. Then the air flow was restarted until an equilibrium was reached again, i.e. when the rate of oxygen consumption was equal to the rate of oxygen dissolution. Three parallel data points were obtained when each measurement was made in $\mu g(O_2) \cdot L^{-1} \cdot s^{-1}$ and averaged. Since after a time, the biomass concentration could not be monitored in the bulk, it was not possible to record data per unit of biomass dry weight (BDW).

2.4. Analytical methods

IA concentration was determined by HPLC on a YL9100-type device (Young Lin Instrument Co., Ltd., South Korea) containing a Hamilton PRP-X300 HPLC column (150 x 4.1 mm, 7 µm) as well as a UV/VIS detector at a detection wavelength of 210 nm. The analytical method employed a gradient elution, where the moving phase was comprised of 'A' (1 mM H_2SO_4) and 'B' (gradient grade methanol, Chem-Lab nv, Belgium) solutions (0 min – 100% A; 1 min – 70% A, 30% B; 6 mins – 70% A, 30% B; 8 mins – 100% A). Before the analysis, the samples were filtered through a 0.2 µm pore-sized Nylon syringe filter (Nantong FilterBio Membrane Co., Ltd) before being diluted a 1000 times using 1 mM sulphuric acid. The injection volume was 100 µL.

The glucose concentration was determined by the DNSA method once the sample had been appropriately diluted [18].

BDW was determined from 3 × 1.5 mL samples until only loose mycelia were present from which point on a homogeneous distribution of biomass was assumed until the 120th hour. The samples were centrifuged at 10,000 rpm for 5 minutes in 1.5 mL Eppendorf tubes with precisely measured weights. The supernatant was removed and the sedimented cells resuspended in 1 mL of sterile water before the centrifugation process was repeated. The washing of wet mycelia was performed three times before the washed mycelia were dried at 80 °C to constant weight.

A ZEISS Primostar microscope (Carl Zeiss AG., Germany) was used to conduct the microscopic investigation using phase contrast equipment at 1000x magnification.

3. Results and Analysis

The continuous fermentation lasted 547 hours (23 days), the final 460 h of which were operated with volume changes. The pH decreased to 1.9 but no IA was detected for a week despite the obvious growth of the fungus. The BDW achieved a concentration of 4 $g \cdot L^{-1}$ and its morphology resembled loose mycelia (*Figure 1*). Maintaining the sterility of the fermentation was challenging because of the significant amount of foam

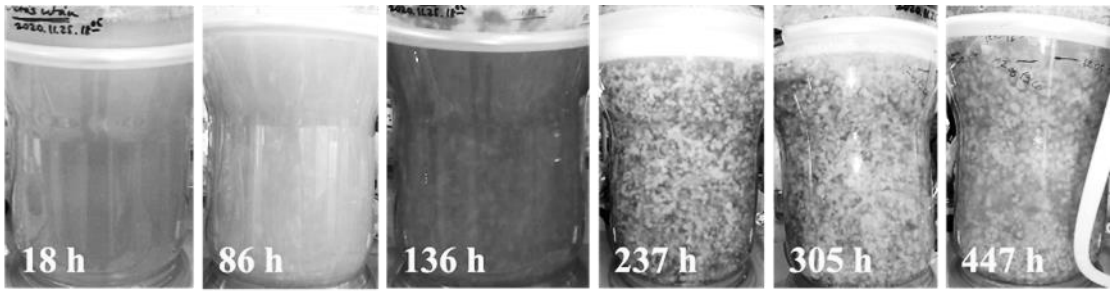

Figure 1: Macro-morphological evolution of *A. terreus* during the continuous IA fermentation

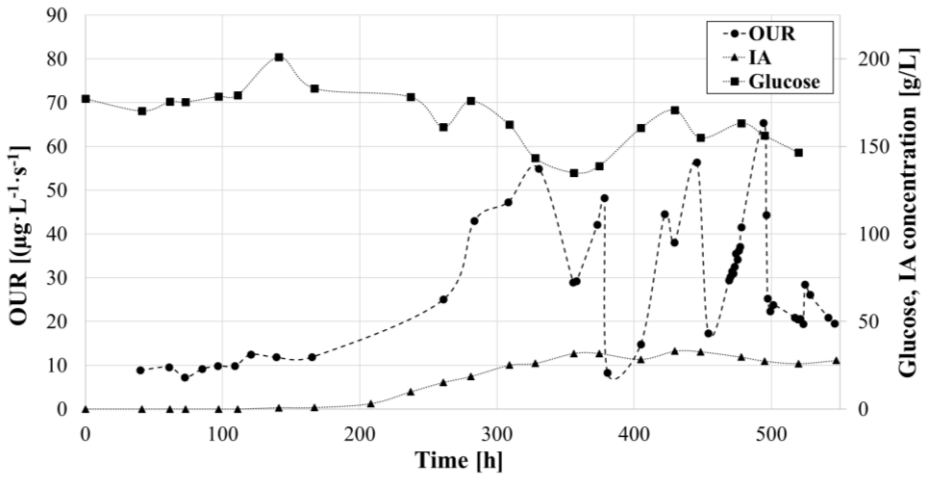

Figure 2: Concentrations of glucose and IA as well as the measured instantaneous values of the oxygen uptake rate (OUR) in the fermenter

that formed. However, no microbial contamination was detected during the experiment.

On Day 4, the continuous operation was switched on at a dilution rate of 0.007 h^{-1}, by which time large amounts of submerged spores had been produced by the fungus. The formation of spores and simultaneous germination was detectable throughout.

Low oxygen availability has been identified as the main parameter hindering the formation of IA. To overcome this, from the 167th hour onwards, pure oxygen was introduced into the fermenter instead of air. Therefore, the equilibrium concentration of oxygen in the medium increased fivefold according to Henry's law compared to that achievable with an air supply, allowing the gas inlet volume to reduce and the formation of foam to be moderated.

The abundant oxygen supply enhanced the production of biomass and the mycelia conglomerated into large clumps with a diameter of 5-8 mm. The extracellular fluid was clear and pale yellow in color. To precisely determine the BDW in such an inhomogeneous broth, a very large sample size would have been ideal but the small effective volume of the bioreactor did not allow this.

After switching to pure oxygenation, IA began to accumulate. Over six days, the IA concentration reached 25 $g \cdot L^{-1}$ and remained above this value until the end of the experiment. A maximum concentration of 35 $g \cdot L^{-1}$ IA was measured in the effluent collected on Days 15, 16 and 18.

The fermentation did not reach a steady state (*Figure 2*). The concentration of glucose in the bioreactor exceeded 120 $g \cdot L^{-1}$, but varied greatly during IA production. The IA concentration, however, did not follow these variations.

The OUR was determined regularly between the 40th and 160th hour. Under these oxygen-limited conditions, the biomass consumed oxygen at an average rate of 10 $\mu g \cdot L^{-1} \cdot s^{-1}$. Nevertheless, measurements could be made since the apparent dissolved oxygen level was 6-7 $mg \cdot L^{-1}$.

After replacing the air with oxygen and noticing that IA had started to accumulate, measurements were resumed from the 260th h onwards. The OUR exhibited unexpectedly large variations periodically (*Figure 2*). A maximum time period of 47 h could be calculated from the discrete data. The maximum and minimum OUR values were between 48-65 and 8-19 $\mu g \cdot L^{-1} \cdot s^{-1}$,

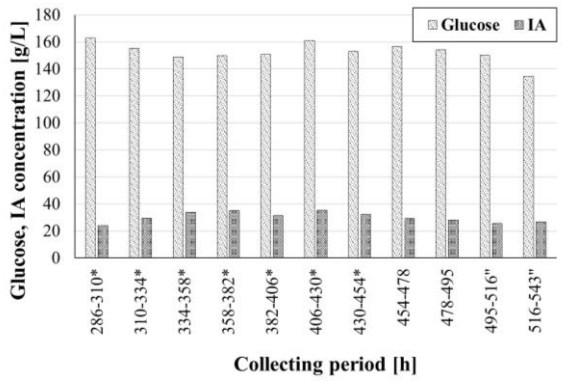

Figure 3: Glucose and IA concentrations in the collected effluent; the portions denoted with (*) were subjected to EDBM product recovery while those labelled with (“) were collected when the diluate was recycled

respectively, resulting in at least a 40% change in this parameter during one period.

The effluent collected from Day 12 until Day 19 with an IA concentration in excess of 20 $g \cdot L^{-1}$ was subjected to EDBM to recover a pure solution of the acid. The glucose and IA concentrations of the effluent collected are presented in *Figure 3*.

The refilling medium was replaced in the 495th hour with this diluate without any supplementation. Once the IA in the EDBM module had been recovered, the glucose and residual IA concentrations in the diluate were 128 and 1.3 $g \cdot L^{-1}$, respectively. Reintroduction of the unsupplemented diluate into the fermentation caused the glucose concentration in the bioreactor to decrease. The IA concentrations were 25.5 and 26.9 $g \cdot L^{-1}$ on the first and second day of diluate dosing, respectively. Replacement of the refilling medium coincided with a sharp decrease in the OUR.

The microscopic morphology shifted to elongated thin filaments from the densely branched thicker form previously present (*Figure 4*).

4. Discussion

In this study, a continuous IA fermentation was performed using *A. terreus* as the producing organism. A special stirring technique was applied along with a high glucose centration and low dilution rate.

It was observed that the oxygen supply is a key parameter to promote IA generation and growth of the microorganism in the preferred clump form. Pure oxygen gas of 0.2 VVM was introduced into the fermenter instead of air.

A maximum product titer of 35 $g \cdot L^{-1}$ was successfully achieved following the 16-day-long IA-producing period, which is higher than those reported in previous continuous fermentations. The average IA concentration in the effluent after 286 hours was 30.1 $g \cdot L^{-1}$, which approximates to the 33 $g \cdot L^{-1}$ concentration proposed for IA recovery by the EDBM technique.

For the first time, the volumetric OUR was measured in the broth during a continuous IA fermentation. The OUR was monitored during oxygen-limited and oxygen-enriched periods of the fermentation. During oxygen-limited periods, the OUR was quite stable at approximately 10 $\mu g \cdot L^{-1} \cdot s^{-1}$. It was found that with an unlimited supply of oxygen and under the applied conditions, the OUR varied periodically with sharply increasing and declining stages, achieving a maximum of 48-65 $\mu g \cdot L^{-1} \cdot s^{-1}$. Unfortunately, the biomass concentration in the reactor could not be monitored once the clumps had grown, since specific data would be more informative. Nevertheless, it can be assumed that the biomass concentration did not change drastically as the OUR increased or decreased during the 8 hours when it was measured more frequently 469 to 477 hours after the fermentation had begun. Therefore, the sharp fluctuation in the OUR is highly important, which varied from 40-88%, and the maximum periodic time was calculated to be 47 hours in duration. It is possible that the anomaly was a consequence of oxygen stress caused by the introduction of pure oxygen into the reactor. However, the formation and germination of spores during fermentation resulted in the presence of different growth kinetics simultaneously, which supposedly had an influence on the OUR. Another uncontrolled parameter during the fermentation was the amount of light the glass fermenter was exposed to. It is known that illumination can influence the metabolic pathways of *Aspergilli* [19]-[21].

Oxygen could also be introduced through special membranes [22], which would possibly further diminish foaming of the broth.

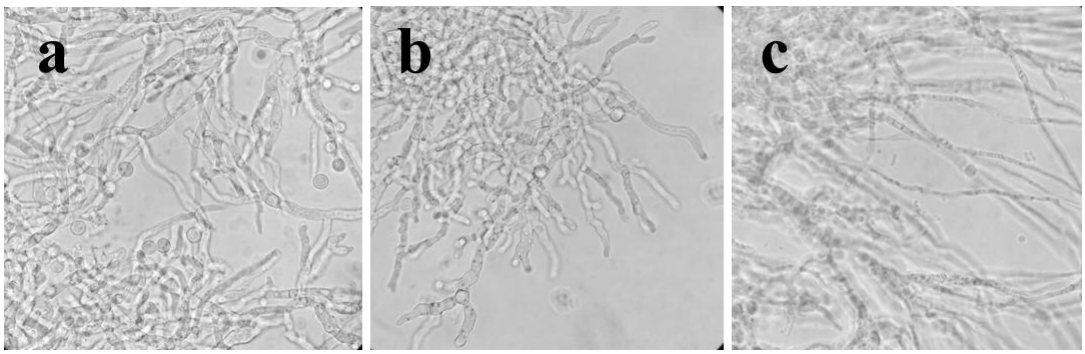

Figure 4: The change in morphology after replacing the refilling medium with diluate from EDBM: a) original morphology; b) morphology 24 h after the replacement; c) filamentous growth after 48 h

To the best of our knowledge, a diluate was recirculated from the EDBM product recovery operation for the first time. The reintroduced diluate contained glucose and residual IA concentrations of 128 and 1.3 $g \cdot L^{-1}$. During electrodialysis, the monovalent metal ions and some of the divalent ones were separated from the alkaline stream. Even though these ions were not supplemented by the diluate, which also contained 37.4% less glucose than the original refilling medium, IA continued to be produced in the reactor. The microscopic morphology of fungus changed into its filamentous form, indicating that only a limited amount of nutrients was available. This case study suggests that after appropriate supplementation, it would be suitable for the diluate to be reintroduced into the IA fermentation. Further experiments shall be conducted in the future while paying special attention to the composition of the diluate from EDBM. More continuous fermentations shall be performed to obtain significant results and collect data on the volume of biomass, possibly using a reactor volume at least one order of magnitude larger.

Although the special mixing technique in the bioreactor contributed to the formation and maintenance of fungal clumps, under oxygen-limited conditions, biomass growth was slow and loose mycelia formed. Oxygen enrichment also seemed to be important to develop the optimal IA-producing morphology.

5. Conclusions

In this study, a possible application of continuous IA fermentation using *A. terreus* was presented. An average IA concentration of the effluent of 30.1 $g \cdot L^{-1}$ was achieved, which to the best of our knowledge is the highest titer obtained by adopting such a process. The availability of oxygen and gentle mixing contributed to the formation of fungal clumps, which are the preferred conditions for IA production. The applied low dilution rate proved to be adequate to achieve a high IA concentration.

Effluent collected from the fermentation was subjected to EDBM in order to recover the IA. The diluate stream of the downstream operation was reintroduced into the same fermentation. IA continued to be produced even though the microscopic morphology of the fungus shifted to thin filaments. Supplementation of the diluate will be an important research area in the future.

In order to account for the periodic changes in the OUR during the productive stage of the fermentation, further research must be carried out. The OUR measurement implemented could be easily automated and by taking frequent measurements, the nature of this periodicity could be determined.

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