Separation and Purification Technology, Volume 388, April 2026, Article number 136685
DOI: 10.1016/j.seppur.2025.136685

Integrated cost-efficient itaconic acid production from waste potatoes via a holistic upstream-to-downstream approach

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The final published version (version of record) is available online at DOI:10.1016/j.seppur.2025.136685. Please refer to any applicable publisher terms of use.

Abstract

Itaconic acid (IA) holds enormous potential in the polymer industry as a green substitute for several valuable chemicals derived from fossil fuels. However, its full potential is limited by high production costs, resulting from the use of expensive feedstocks and downstream processing approaches. In this context, this study strategizes an inexpensive approach for the efficient production and recovery of IA using damaged/defective waste potatoes, a major food waste across the globe. Initially, the optimized acidic saccharification process (1.5% HCl, 20% w/v solid loading) yielded 170 g glucose/kg potato. The fermentation of activated charcoal-treated potato hydrolysate by *Aspergillus terreus* DSMZ 23081 resulted in 32 g/L IA with a yield and productivity of 0.21 g_{IA}/g_{Glu} (36.2 g_{IA}/kg_{WPB}) and 0.17 g/L/h, respectively. The downstream processing was performed by implementing a salting-out extraction approach. Among various solvent-salt combinations, maximum IA extraction efficiency was observed when 25% (w/v) Na_2SO_4 was used as a salting-out agent with *sec*-butanol as the extractant. Optimizing the physicochemical parameters (pH, solvent volume, shaking time, etc.) led to 92% recovery from pure IA solution. Finally, when the process was applied to potato-based fermentation broth, 80.5% IA was recovered with 85% purity. By integrating valorisation of a low-cost agro-industrial waste with an optimized salting-out extraction strategy that overcomes key limitations of conventional IA purification, the developed bioprocess demonstrates a simple and economically feasible end-to-end strategy for sustainable IA production.

Keywords

Itaconic acid; Waste potatoes; Separate hydrolysis and fermentation; Salting-out extraction; Downstream processing

Nomenclature

Abbreviation	Description
IA	Itaconic acid
WPB	Waste potato biomass
WPAH	Waste potato acid hydrolysate
SHF	Separate hydrolysis and fermentation
DCW	Dry cell weight
SOE	Salting out extraction
DSP	Downstream processing
HMF	Hydroxy methyl furfural
E	Extraction efficiency
K	Partition coefficient
AC	Activated charcoal
V_0	Initial volume of IA solution/fermentation broth
$V_{org.}$	Volume of the organic layer after separation
$V_{aq.}$	Volume of the aqueous layer after separation
M_T	Total mass of IA
$M_{org.}$	Mass of IA in the organic layer
$M_{aq.}$	Mass of IA in the aqueous layer
D_m	Mass distribution ratio
$\%w_{org.}$	% Mass fraction of IA in organic layer
$\%w_{aq..}$	% Mass fraction of IA in aqueous layer
P	Phase ratio

1. Introduction

In the global pursuit of carbon neutrality and a net-zero economy, products derived from microbial fermentation are gaining importance to reduce reliance on fossil-based processes [1]. These are eco-friendly processes with low energy requirements, producing biodegradable products using renewable resources [2–4]. Itaconic acid (IA) or 2-methylidenebutanedioic acid is a bio-based platform chemical bearing substantial value. It is an unsaturated dicarboxylic acid that could serve as a versatile building block in the production of fibres, resins, lattices, plastics, detergents, rubber, paints, surfactants, lubricants, and bioactive compounds [5,6]. IA holds promise as a sustainable alternative to fossil-derived chemicals such as acrylic acid, acetone cyanohydrin, maleic anhydride, and sodium tripolyphosphate [7,8]. Filamentous fungi, *Aspergillus terreus,* possess a specialized gene cluster for IA biosynthesis. The fermentation process involving *A. terreus*, is the primary route for industrial IA production [3]. Despite its high industrial applicability, the large-scale commercialization of IA remains constrained by economic barriers, mainly arising from the use of costly feedstocks such as pure glucose and molasses, along with tedious and energy-intensive downstream processing (DSP) techniques [9–11]. The IA could fully replace petroleum-based polyacrylic acid, tapping into a global multi $billion market if its current market price drops significantly. The current market price of IA (~1.5 USD/kg) must be reduced to 0.5–1.0 USD/kg through inexpensive biomanufacturing strategies in order to efficiently compete with its petrochemical counterparts [12].

Feedstocks and other raw materials account for 40-50% of the bioprocess cost. Utilization of low-cost, non-edible waste biomass as a feedstock offers a promising approach to curtail these expenses and enhance the competitiveness of IA [13]. Lignocellulosic wastes are heterogeneous, recalcitrant, and produce fermentation inhibitors; thus, extensive and expensive pretreatment and processing steps are required [9,14]. In contrast, starchy feedstocks

could be more conveniently saccharified due to their homogenous and amorphous nature as compared to crystalline cellulose [15]. Further, the glucose-rich hydrolysate obtained is rich in high-quality food-grade sugars and devoid of fermentation inhibitors [16,17]. Potato (*Solanum tuberosum*) is the world's fourth most important food crop. In addition to being consumed fresh, potatoes are widely processed industrially to produce items such as starch and chips. This processing generates a vast amount of waste (damaged/defective potatoes, peels, scraps, waste effluent, etc.) across the supply chain. As the market for potatoes and processed potato products continues to expand, so does the volume of waste produced [18]. A significant portion of this waste is either landfilled, leading to serious environmental and economic concerns, or used as low-value animal feed. However, due to their high carbohydrate and sugar content combined with low lignin levels, these waste streams represent promising feedstocks for fermentative conversion into valuable biochemicals, offering a potential shift in the economic landscape of waste utilization [18,19]. Several reports have demonstrated using starch-rich wastes from potato industries as fermentation feedstock [15,18,20,21]. Among different enzymatically saccharified starch-rich industrial residues, starch derived from wastewater coming from the potato industry led to maximum IA production [15]. India is the second-largest producer of potatoes, generating around 50-60 metric tonnes of potatoes annually. Approximately 16% of the total potato produced is discarded as waste due to post-harvest mishandling, damage, spoilage, diseases, cosmetic defects, and mismanaged oversupply [19,22]. Potatoes are typically composed of 75-80% water, 15-20% starch, 1-1.5% fibres, 1.5-2% protein, and less than 1% fat and ash [23,24]. The damaged/defective potatoes, also known as "Cull potatoes", represent an underexploited starch-rich feedstock with high global abundance, suitable for cost-effective IA production [18].

Currently, IA industries rely on DSP approaches, which are energy-intensive, unsustainable, and expensive, contributing to 30-40% of production cost [25]. Studies have

explored various techniques like adsorption, membrane separation, electrodialysis, reactive extraction, precipitation, and crystallization for IA recovery from fermentation broth [10,26–29]. However, each technique has certain drawbacks, such as complexity, high equipment costs, high energy requirements, multistage processing, low purity, toxic byproducts, etc. [10,30]. This necessitates the search for newer downstream strategies suitable for industrial scalability. The salting out extraction (SOE) technique is a simple strategy for effective product extraction where the dissolution of an inorganic salt into the aqueous solution of a compound decreases its water solubility (salting-out), and increases the partitioning effect of the organic solvent [31,32]. It is widely exploited for analysing and extracting drugs, pesticides, proteins, and other organic compounds from complex matrices [32,33]. Several studies have demonstrated use of SOE for the recovery of succinic acid, lactic acid, and butyric acid [31,34–36]. This technique offers several advantages, such as mild operation conditions, simple processing, high selectivity and purity, high extraction efficiency, ease of recovery and reuse, etc., rendering it suitable for industrial scalability [32,33,37]. Given its success with other organic compounds, this technique holds promise for the efficient recovery of IA from fermentation broth, which has not been investigated previously.

In the present study, a comprehensive and integrated upstream-to-downstream approach for the low-cost production and recovery of IA using waste potatoes was developed. Acidic saccharification, a cost-effective strategy, was applied to generate glucose-rich hydrolysate from waste potato biomass (WPB). The hydrolysate obtained was used as a carbon source for IA production by *Aspergillus terreus*, a well-known IA producer. The bioprocess was optimized to maximize IA production from glucose-rich hydrolysate. The SOE technique was applied for efficient recovery of accumulated IA from the broth with a systematic screening and investigation of solvent-salt interactions, pH control, and physicochemical variations**.** Through process optimization at multiple stages, saccharification, fermentation, and

downstream recovery, the study aims to demonstrate a scalable and economically viable bioprocess that could contribute to sustainable IA manufacturing.

2. Materials and methods

2.1 Materials

All the media components, salts/solvents, and reagents were purchased from Himedia Laboratories, Sigma-Aldrich, and Fischer Scientific, respectively. IA (with ≥99% purity) was procured from Sigma-Aldrich. All the salts and solvents used in this study were of analytical and HPLC grade. Damaged/defective 'Chipsona' potatoes were procured from the local market in New Delhi, India.

2.2 Acidic saccharification of waste potatoes

Damaged/defective potatoes were used as feedstock for IA production using a separate hydrolysis and fermentation (SHF) approach. Starch in the potatoes was hydrolyzed into glucose using an acidic saccharification approach, as described in our previous study [38]. Briefly, HCl was added to the WPB slurry and autoclaved under standard sterilization conditions (20 min; 121°C; 15 psi). The acid loading (0.5-3% v/v HCl) and solid loading (5-30% w/v) were optimized to ensure maximum glucose release with the minimum generation of fermentation inhibitors, hydroxymethyl furfural (HMF), and furfural. The resultant waste potato acid hydrolysate (WPAH) was separated from the unhydrolyzed residue by centrifugation (9200 x g X 15 min).

2.3 Microorganism and culture conditions for itaconic acid production

A. terreus DSMZ 23081 was procured from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Germany. It was preserved in 50% glycerol stocks at -80°C and maintained on malt extract agar plates and slants at 30°C. The spores were harvested from malt extract agar plates cultivated for 5 days using 0.9% w/v NaCl

151 solution with 0.05% Tween 20. The concentration of spores in the suspension was determined
152 using a Neubauer chamber.

153 Fermentation for IA production was conducted in synthetic glucose media and WPAH
154 media [39]. A primary culture medium consisting of 5 g/L yeast extract and 30 g/L glucose was
155 prepared in a 250 mL flask with a working volume of 50 mL, and initial pH was adjusted to
156 2.5 with 1 M H_2SO_4. After autoclaving, the primary culture was inoculated with 10^6 spores/mL
157 from the spore suspension and incubated at 30°C at a shaking speed of 150 rpm. Fermentation
158 was carried out in 50 mL standard glucose media in 250 mL flasks. The media composed of
159 100 g/L glucose, 0.1 g/L KH_2PO_4, 3 g/L NH_4NO_3, 1 g/L $MgSO_4.7H_2O$, 5 g/L $CaCl_2.2H_2O$, 1.67
160 mg/L $FeCl_3.6H_2O$, 8 mg/L $ZnSO_4.7H_2O$, and 15 mg/L $CuSO_4.5H_2O$. The pH of the glucose
161 solution was adjusted to 3.4 before autoclaving. The salt solutions were filter sterilized using a
162 0.2 μm nylon filter and added after autoclaving rest of the media. The media was inoculated
163 with 5% v/v inoculum from 48 h old primary culture and incubated at 30°C with a shaking
164 speed of 200 rpm.

165 The IA production from WPAH was carried out with varying concentrations of glucose
166 (50, 100, and 150 g/L) in the hydrolysate, and pH was adjusted to 3.4, followed by filter
167 sterilization and fermentation. For detoxification, the hydrolysate was treated with 1, 2, 5, and
168 10% w/v activated charcoal (AC) by shaking for 2 h at 30°C, followed by filtration using
169 Whatmann paper 1. Further process parameters like shaking speed, inoculum age, and
170 inoculum size were optimized to improve the conversion efficiency.

171 **2.4 Optimization of the salting-out extraction process using pure IA**

172 Preliminary screening of various solvents for their partitioning behaviour for IA and the
173 salting-out effect of different salts was carried out using a solution of pure IA (50 g/L) in
174 distilled water. Both water-immiscible and miscible polar organic solvents were screened.

175 Water-immiscible solvents included *n*-butanol (*n*-But), isobutanol (*sec*-But), methyl ethyl
176 ketone (MEK), methyl isobutyl ketone (MIBK), ethyl acetate (EA), dichloromethane (DCM),
177 and chloroform (CFM). Water-miscible solvents, namely acetonitrile (ACN), tertiary butanol
178 (*tert*-But) *n*-propanol (*n*-Prop), isopropanol (*sec*-Prop), and acetone, were also tested for IA
179 extraction from its salt-saturated aqueous solution. Inorganic salts tested for the salting-out
180 approach included $(NH_4)_2SO_4$, Na_2SO_4, K_2SO_4, $MgSO_4$, KH_2PO_4, NH_4Cl, NaCl, KCl, and
181 $MgCl_2$.

182 Screening experiments were conducted using 20 mL IA solution in 150 mL flasks.
183 Unless stated otherwise, individual salts (10% w/v) were dissolved in the IA solution, and an
184 equivalent volume of organic solvent was added to the solution. For the partitioning of IA into
185 the organic layer, the flasks were kept in shaking conditions at 200 rpm for 3 h at 30°C.
186 Thereafter, the flasks were kept undisturbed overnight for complete separation of aqueous and
187 organic phases. The organic phase was separated with the help of a separating funnel and
188 evaporated in a round-bottom flask using a rotary evaporator (IKA, Breisgau, Germany). After
189 evaporation, the residual solid was dissolved in milliQ water, and the amount of extracted IA
190 was estimated using High Performance Liquid Chromatography (HPLC).

191 The best solvent-salt pair/s resulting in the highest IA recovery was chosen for
192 optimizing other physicochemical parameters to maximize extraction efficiency. Critical
193 factors, viz. salt concentration (0-50% w/v), pH (1.0-5.0), IA concentration (10-80 g/L),
194 extraction/mixing time (1-4 h), initial volume ratio ($V_{0,aq}/V_{0,org.}$), and repeated extraction (1-3
195 times), were investigated.

196 **2.5 Downstream processing of IA from the fermentation broth**

197 After the optimization of conditions for the extraction IA from the aqueous solution,
198 the best solvent-salt combination and other physicochemical parameters were applied for the
199 recovery of IA synthesized in the fermentation broth by *A. terreus* DSMZ 23081 after 10 days

of cultivation on synthetic glucose media and WPAH media. The salt was added directly into the fermentation broth, and the pH was adjusted to an optimum value. Subsequently, the IA extraction was carried out using the chosen organic solvent. The organic phase was manually separated from the aqueous fermentation broth and fungal mycelia by decantation. The upper organic layer was treated for 1 h with AC (2% w/v) by shaking at 30°C for the removal of pigments and impurities from the extract. The AC was removed through filtration, and the filtrate was transferred into a round-bottom flask, followed by rotary evaporation to obtain the dried extract. The condensed solvent collected from the rotary evaporator was reused in the next cycle of extraction. The dried IA extract was analysed for yield and purity.

2.6 Calculation

The saccharification yield of WPB hydrolysis was calculated using Eq. (1):

$$\text{Saccharification yield (\%)} = \frac{\text{Glucose (g) in hydrolysate of 'x' g WPB}}{\text{Starch content in 'x' g WPB} \times 1.11} \text{ X } 100\% \quad (1)$$

The partitioning behaviour of IA into the organic phase under different solvent-salt and physicochemical variations was studied with respect to certain partition parameters, namely mass fraction of IA in organic ($w_{org.}$) and aqueous layer ($w_{aq.}$), mass distribution ratio (D_m), extraction efficiency or IA recovery (E), phase ratio (P), partition coefficient (K) and water content of organic layer ($H_2O_{org.}$). These parameters give a measure of the efficacy of the process, which allows a coherent selection of optimum DSP conditions for IA recovery from fermentation broth. These parameters can be calculated using Eq (3) – Eq. (7) as below:

$$w_{org.} \text{ or } w_{aq.} (\%) = \frac{M_{org.}}{M_T} \text{ X } 100\% \quad \text{or} \quad \frac{M_{aq.}}{M_T} \text{ X } 100\% \quad (2)$$

$$Dm = \frac{M_{org.}}{M_{aq.}} \quad (3)$$

$$E (\%) = \frac{M_{org.}}{M_T} \text{ X } 100\% \quad (4)$$

$$P = \frac{V_{org.}}{V_{aq.}} \quad (5)$$

$$K = \frac{[IA]_{org.}}{[IA]_{aq.}} \quad (6)$$

$$H_2O_{org.}\ (\%) = \frac{(V_o - V_{aq.})}{V_{org.}} \times 100\% \quad (7)$$

where, $M_{org.}$ is mass of IA in organic layer, $M_{aq.}$ is mass of IA in the aqueous layer, M_T is total mass of IA, $V_{org.}$ is volume of organic layer after mixing. $V_{aq.}$ is volume of the aqueous layer, $[IA]_{org.}$ is IA concentration in the organic phase, $[IA]_{aq.}$ is IA concentration in the aqueous phase, and Vo is initial volume of the IA solution.

Finally, to determine the % purity of IA in the final extract, a known amount ($M_{ex.}$) of the final dry extract precipitate was dissolved in Milli-Q water, and IA concentration was analyzed using HPLC. The % purity of extracted IA was calculated using Eq. (8):

$$\%\ \text{Purity of IA} = \frac{[IA]_{Ex.} \times V_{MQ}}{M_{Ex.}} \times 100\% \quad (8)$$

where, M_{Ex} is the mass of dried extract, volume of milli-Q water used to dissolve the extract, and $[IA]_{Ex.}$ is the concentration of IA in the final extract solution.

2.7 Analytical methods

The starch in the WPB was estimated using the method adopted by McCleary *et al*. [40]. To monitor glucose consumption, biomass, and IA production during the fungal fermentation, the samples (1 mL) were withdrawn periodically at every 24 h. The collected samples were centrifuged at 9200 x g for 10 min, and the supernatant was analysed through HPLC for quantification of residual glucose and produced IA. The fungal biomass obtained after centrifugation was dried in an oven to determine dry cell weight (DCW). The IA extracted in the organic layer was estimated after evaporating the solvent and dissolving the total dried extract in Milli-Q water.

244 An HPLC system (Perkin Elmer, Waltham, MA, USA) equipped with a Bio-Rad HPX-
245 87H Aminex ion exclusion column (Bio-Rad Laboratories, Hercules, CA, USA) and a
246 refractive index detector (RID) was used for quantification of glucose and IA. The 5.0 mM
247 H_2SO_4 was used as the mobile phase with a flow rate of 0.6 mL/min. HMF and furfural in the
248 hydrolysate were estimated using a C-18 column and UV Diode array detector (UV-DAD) (270
249 nm wavelength), and a 15 %v/v methanol in water was used as the mobile phase with a flow
250 rate of 0.6 mL/min.

251 Fourier-transform infrared (FT-IR) spectroscopic characterization of the extracted IA
252 and standard IA was conducted to confirm their structural identity. Powdered IA (extract and
253 standard) was mixed with KBr to form a pellet, and the spectrum (4000–400 cm^{-1}) was
254 recorded on an FT-IR spectrometer (Perkin Elmer FT-IR/FIR Frontier, Waltham, MA, USA).
255 For the nuclear magnetic resonance spectroscopy (^1H NMR) characterization, the standard and
256 extracted IA were dissolved in deuterium oxide (D_2O), and spectra were recorded at 25°C on
257 an NMR spectrometer operating at a resonance frequency of 500 MHz (Bruker 500 MHz,
258 Karlsruhe, Germany).

259 **2.8. Statistical analysis**

260 All the experiments in the study were performed in triplicate, and the results presented
261 are the mean ± standard deviation (SD) of triplicate measurements (n =3). Statistical analysis
262 was performed with excel. Statistical analyses among groups were performed using one-way
263 analysis of variance (ANOVA). Differences among groups were declared statistically
264 significant if $p < 0.05$.

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3. Results and discussion

3.1 Acidic saccharification of waste potatoes (WPB)

Waste potatoes (WPB) are a promising choice of feedstock in terms of high glucose content with ease of extraction, achievable through mild acidic or enzymatic processes without extensive pretreatments. Damaged or defective potatoes unsuitable for consumption were used directly for saccharification without drying, minimizing energy consumption. The grated waste potatoes comprised 25% dry matter and 75% moisture content. Solid loading was considered based on the dry matter content of the total WPB, and water was added to achieve the desired reaction volume, taking into account the inherent moisture content (**Supplementary Table 1**). The WPB contained 18.5% w/w starch content, which was hydrolysed to glucose through dilute acid treatment. Initially, the acid loading was optimized by adding 0.5-3% v/v HCl into the WPB slurry with 10% w/v solid content, followed by autoclaving. The effect of HCl concentration was monitored based on the release of glucose (**Fig. 1A**) and fermentation inhibitors, HMF (**Fig. 1B**), and furfural (**Fig. 1C**) production. Starch hydrolysis efficiency increased with rising HCl concentrations up to 1.5% v/v, at which the maximum glucose concentration of 72 g/L was achieved, corresponding to a saccharification yield of 88%. At this acid concentration, minor amounts of HMF (0.6 g/L) and furfural (0.02 g/L) were detected. However, further increases in HCl concentration significantly elevated the formation of HMF and furfural, which could potentially inhibit subsequent fermentation processes. Therefore, 1.5% v/v HCl was chosen as the optimal acid loading for continued experiments.

Following the optimization of acid loading, the next step involved optimizing solid loading. To achieve this, saccharification experiments were conducted using various concentrations of WPB solid (5, 10, 15, 20, 25, and 30% w/v). For the preparation of glucose-rich slurry, 1.5% v/v HCl was mixed with different amounts of WPB, and the impact of solid loading was assessed. Results showed a linear increase in glucose concentration, reaching up

to 136 g/L, with a consistent saccharification efficiency exceeding 80% up to a solid loading of 20% w/v (equivalent to 80% WPB and 20% water) **(Fig. 1D)**. However, increasing the WPB concentration beyond this point significantly decreased saccharification efficiency, likely due to reduced HCl accessibility to starch in the denser slurry. Similar observations have been made in previous studies on biomass hydrolysis, where high solid loading resulted in reduced saccharification efficiency due to limited free water, elevated viscosity, and hindered mixing, imposing mass transfer constraints on acid/enzyme diffusion [41,42]. Moreover, higher WPB loadings led to increased formation of HMF **(Fig. 1E)** and furfural **(Fig. 1F)**, with concentrations of 0.9 g/L and 0.06 g/L, respectively, at 20% w/v solid loading.

Acidic saccharification offers a rapid and economically viable approach for glucose extraction from starchy feedstocks, unlike the two-step enzymatic liquefaction and saccharification method, which is time-consuming and incurs additional enzyme-related costs [38,43]. A previous report on cull potatoes showed that acidic (85.2%) and enzymatic (83.2%) treatment yielded comparable results from 4% (w/v) dried potato powder [44]. In another study, dilute acid saccharification using 0.5% H_2SO_4 was employed on waste potatoes, which generated 55.8 g/L glucose without any fermentation inhibitors. This hydrolysate was successfully applied for ethanol and lipid production using *Saccharomyces cerevisiae* and *Rhodotorula mucilaginosa*, respectively, indicating the potential of acidic hydrolysate of potato as a cost-effective feedstock for fermentation [23]. Similarly, in the current study, the optimized acidic hydrolysis parameters (1.5% v/v HCl and 20% w/v solid loading) applied to the bulk hydrolysis of WPB yielded 170 g of glucose per kilogram of WPB and achieved a saccharification efficiency of greater than 80%. The resultant glucose-rich hydrolysate served as a fermentation medium for IA production.

Studies have shown that fermentation inhibitors, HMF and furfural, are also produced by acid-mediated degradation of polysaccharides rich in pentose and hexose sugars [43]. Saha

318 *et al*. reported that a concentration as low as 0.4 g/L of HMF, furfural, and acetic acid strongly
319 inhibited the growth and productivity of *A. terreus* [45]. Another study demonstrated that IA
320 production from an inhibitor-rich wheat straw hydrolysate could be achieved only after a 1000-
321 fold dilution [46]. This underscores the negative influence of these inhibitors on IA productivity
322 and fermentation kinetics [46,47]. The current study demonstrated that optimized conditions
323 led to minimal formation of HMF (0.9 g/L) and furfural (0.06 g/L). The existing research
324 implicates that HMF and/or furfural toxicity is predominantly responsible for inhibiting
325 enzymes in the central carbon metabolism of all microorganisms [47]. This was also evident
326 for IA production in a report involving corn stover hydrolysate, where furfural exhibited a more
327 substantial influence on IA productivity than the same amount of HMF [48]. A similar
328 inhibitory trend of furfural>acetic acid>HMF was reported in a simulated medium for IA
329 production [49]. Since potatoes have low lignocellulosic content (1-1.5% w/w), primarily
330 confined in the peels, furfural production was much below the tolerable limits. Additionally,
331 the potential generation of other toxic inhibitors, which are typically more prevalent in
332 lignocellulosic feedstocks, is eliminated.
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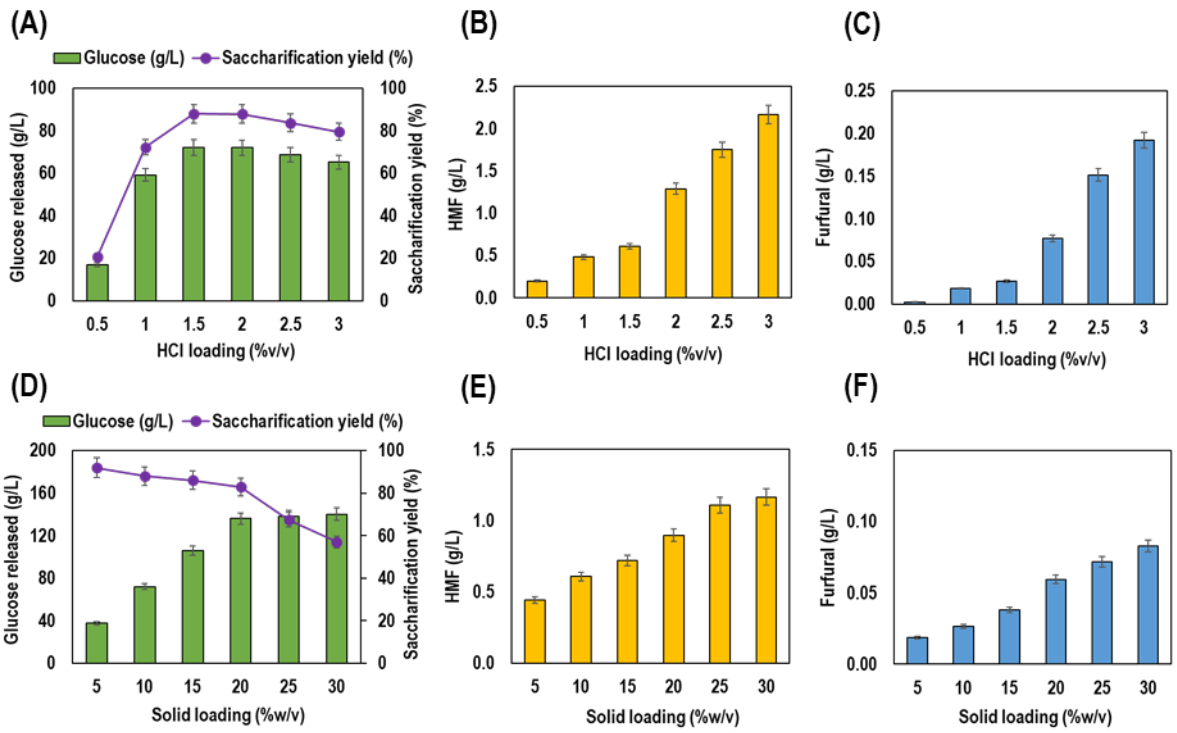


Fig. 1. Optimization of saccharification process for glucose release from waste potatoes. Effect of HCl loading on (A) Glucose release (bar graph) and saccharification yield (Line graph), (B) HMF, and (C) Furfural. Effect of solid loading with fixed acid loading of 1.5% HCl: (D) Glucose release (bar graph) and saccharification yield (Line graph), (E) HMF, and (F) Furfural. The results are presented as the mean ± SD of triplicate measurements. Statistical analysis across acid-loading and solid-loading conditions indicated statistically significant differences between conditions ($p < 0.05$).

3.2 Itaconic acid production in WPAH media

A. terreus DSM 23081 is a commonly utilized strain for IA production in both research and industrial settings. Initially, the fungus was cultivated in a synthetic medium containing 100 g/L of pure glucose as the carbon source. The temporal profiles of residual glucose, dry cell weight (DCW), IA production, and pH are presented in **Supplementary Fig. 1**. After 10 days of fermentation, glucose was fully consumed, yielding a DCW of 17.8 g/L and an IA concentration of 51 g/L. During fermentation, the pH gradually declined, reaching

approximately 1.5 by the end. Despite a substantial yield, the use of pure glucose as a fermentation substrate results in high production costs and needs to be replaced by glucose derived from waste/biomass sources to enhance economic feasibility.

Previous studies have utilized starch or flour derived from sources such as corn, potato, sago, and rice for IA production. However, employing edible feedstocks in biorefineries presents ethical issues [6,50–52]. Therefore, starch-rich waste streams are more appropriate choices for microbial fermentation. In this context, a previous report demonstrated the production of 41 g/L IA with a yield of 0.27 g/g using glucose (150 g/L) obtained from the enzymatic hydrolysis of starchy food waste [53]. In this study, to achieve an inexpensive IA production, the standard glucose media was replaced with glucose-rich WPB hydrolysate (WPAH) and fermentation was carried out at 30°C under similar conditions (pH 3.4; inoculum size-5%v/v; inoculum age-48h; and shaking speed 200 rpm) with no additional supplementation. **Fig. 2** illustrates the time-course profiles for glucose depletion, biomass accumulation, and IA production using WPAH media. Fermentations were performed with varying initial glucose concentrations (50, 100, and 150 g/L). In WPAH, the fungus exhibited a dispersed mycelial morphology. Maximum biomass levels achieved were 13, 21, and 31.5 g/L corresponding to initial glucose concentrations of 50, 100, and 150 g/L, respectively. IA production under these conditions resulted in titers of 6.8, 16.5, and 25 g/L, respectively (**Table 1**). The DCW and IA formation was concomitant with glucose consumption, which was exhausted after 5, 7, and 8 days of fermentation.

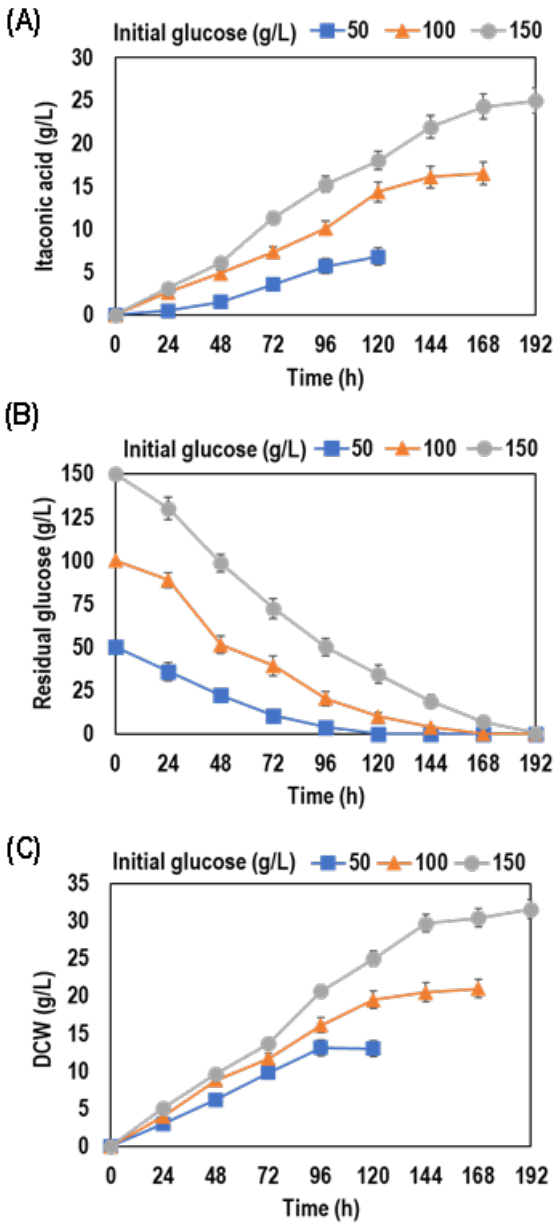


Fig. 2. IA production from WPAH by *A. terreus* DSM 23081 varying initial glucose concentrations of 50 g/L, 100 g/L, and 150 g/L. (A) IA production (g/L); (B) residual glucose amount (g/L); and (C) fungal growth-DCW(g/L). Symbols: square (50 g/L), triangle (100 g/L), circle (150 g/L). Values are presented as mean ± SD of triplicate measurements.

Table 1. Summary of IA production from WPAH by *A. terreus* DSM 23081 in shake flask cultures.

Initial glucose (g/L)	**IA Titer (g/L)**	**IA Yield (g_{IA}/g_{Glu})**	**DCW (g/L)**	**Biomass yield (g_{DCW}/g_{Glu})**	**Fermentation time (h)**
50	6.8 ± 0.7	0.136	13.0 ± 0.6	0.26	120
100	16.5 ± 1.1	0.165	21.0 ± 1.3	0.21	168
150	25.0 ± 1.2	0.167	31.5 ± 2.1	0.21	192

Interestingly, 50 g/L glucose resulted in higher biomass yield (0.26 g_{DCW}/g_{Glu}) but lower IA yield (0.136 g_{IA}/g_{Glu}), indicating preferential metabolic flux toward cell growth under low substrate concentration. In contrast, increasing the glucose concentration to 150 g/L resulted in an improved IA yield (0.167 g_{IA}/g_{Glu}) and a reduced biomass yield (0.20 g_{DCW}/g_{Glu}), indicating a metabolic shift toward IA biosynthesis when ample substrate was available. Notably, the dry cell weight (DCW) observed in WPAH was higher than that in standard glucose media, likely due to the presence of proteins, phosphates, and other minerals in the potato biomass, which may have supported enhanced fungal growth and a dispersed morphology. These findings suggest that impurities present in WPAH did not restrict the growth of the fungus; rather, they had a more pronounced impact on IA synthesis. Notably, concentrated WPAH medium with more than 150 g/L glucose resulted in improper growth of the fungus, characterized by clumping, and a substantially low titer (<10 g/L), possibly due to high osmotic stress and a higher concentration of undesired impurities. Based on these results, WPAH with 150 g/L of glucose was selected for further fermentation studies.

3.3 Activated charcoal (AC) detoxification and process optimization

AC treatment is a widely used detoxification method for biomass hydrolysates prior to fermentation. Multiple studies have demonstrated that AC-mediated removal of impurities (e.g., furan derivatives, pigments, glycoalkaloids, phenolics, organic acids, etc.) from complex media improves metabolite production [47,54,55]. For instance, a study by Liu *et al*. demonstrated that treating corn stover hydrolysate with AC enhanced IA production, reaching levels up to 33.6 g/L, while untreated hydrolysate yielded no IA [48]. Aiming to improve the IA production, WPAH was treated with varying amounts (1, 2, 5, 10% w/v) of AC. The hydrolysate treated with 1% and 2% w/v AC appeared partially pigmented, while a higher amount of AC (5% and 10% w/v) led to complete depigmentation (**Fig. 3A**). These AC-treated (1-10%) WPAH, containing 150 g/L glucose, were subsequently used as fermentation medium. Consistent with previous studies, the AC-mediated detoxification significantly improved IA production in WPAH, with the highest yield achieved using 5% AC (**Fig. 3B**). Under this condition, glucose was exhausted after 9 days, and a mixed pelleted morphology was observed with a DCW of 28.5 g/L. The detoxified WPAH (5% w/v AC) demonstrated a 17% improvement in IA production, resulting in a titre of 29.2 g/L and a yield of 0.19 g/g. Additionally, AC treatment triggered a morphological transition from dispersed to a mixed pelleted form, enhancing its suitability for production. This improvement in IA productivity and changed morphology is likely due to AC's ability to adsorb inhibitory compounds, such as HMF, glycoalkaloids, pigments, and metal ions, that would otherwise hinder fungal metabolism and physiology.

Further, optimization of other process parameters, like shaking speed (**Fig. 3C**), inoculum size (**Fig. 3D**), and inoculum age (**Fig. 3E**), was carried out using AC-treated (5%) WPAH. The optimal shaking speed, inoculum age, and size were found to be 200 rpm, 72 h, and 3% v/v, respectively. The fermentation using AC-treated WPAH (150 g/L glucose) with the

optimal combination of shaking speed, inoculum age, and size, resulted in reduced fungal growth (DCW of 27.5 g/L) and enhanced IA production. Moreover, this process allowed for a completely pelleted morphology and a biomass yield (0.18 g/g) similar to that of pure glucose medium. Pellet formation in filamentous fungi is advantageous for industrial fermentation, as it enhances mass transfer (oxygen and nutrient availability), supports secondary metabolite production, and contributes to a low-viscosity fermentation broth [56,57]. Consequently, the final IA titer improved to 32 g/L, corresponding to conversion yield and productivity of 0.21 g/g and 0.15 g/L/h, respectively. The fermentation profile of the optimized system is shown in **Fig. 3F.** Additionally, the WPAH medium exhibited buffering capacity, as indicated by a modest pH decrease to 2.5 despite the accumulation of 32 g/L of itaconic acid, indicating slight regulation of the medium's pH. The final process yielded 36.2 g IA per kg of waste potatoes (36.2 g_{IA}/kg_{WPB}). Previously, starch recovered from potato processing wastewater was successfully used for IA production. Through optimization of several parameters, including pH, inoculum concentration, peptone and salt supplementation, and deionization, a titer of 35.8 g/L was achieved from 180 g/L glucose [20]. Our study reports a comparable yield from WPAH without requiring additional supplementation or deionization steps.

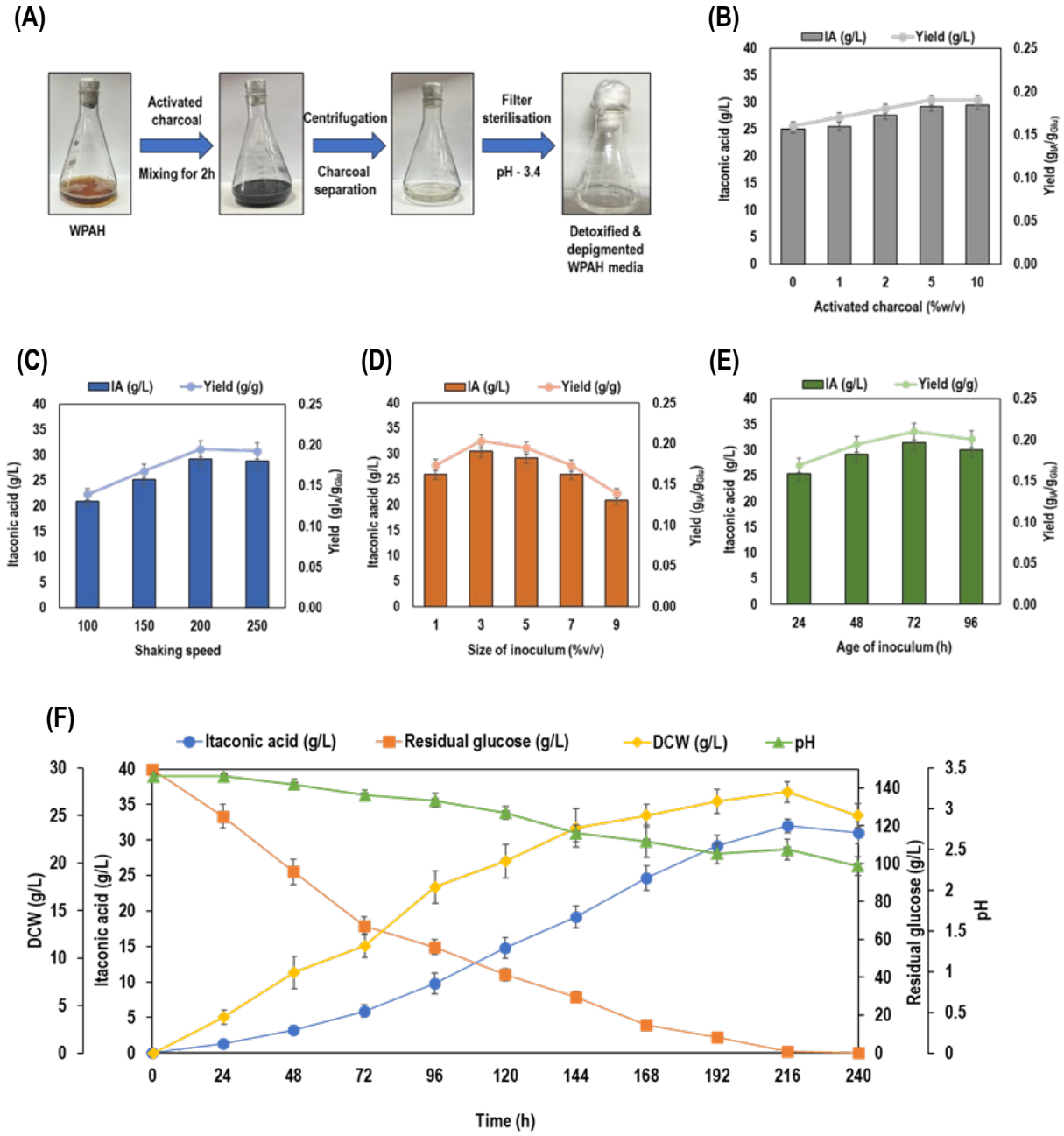


Fig. 3. (A) Detoxification of WPAH by activated charcoal (AC) treatment. (B) Impact of different concentrations of AC on IA production. Optimization of the process parameters for IA production by A. *terreus* DSM 23081 using AC detoxified WPAH hydrolysate: (C) shaking speed; (D) size of inoculum, and (E) age of inoculum. (F) Fermentation profile of itaconic acid (g/L), residual glucose (g/L), DCW (g/L), and pH under optimized conditions. Symbols: circle (itaconic acid), square (residual glucose), rhombus (DCW), and triangle (pH). Values are presented as mean±SD (n = 3), and differences were statistically significant ($p < 0.05$).

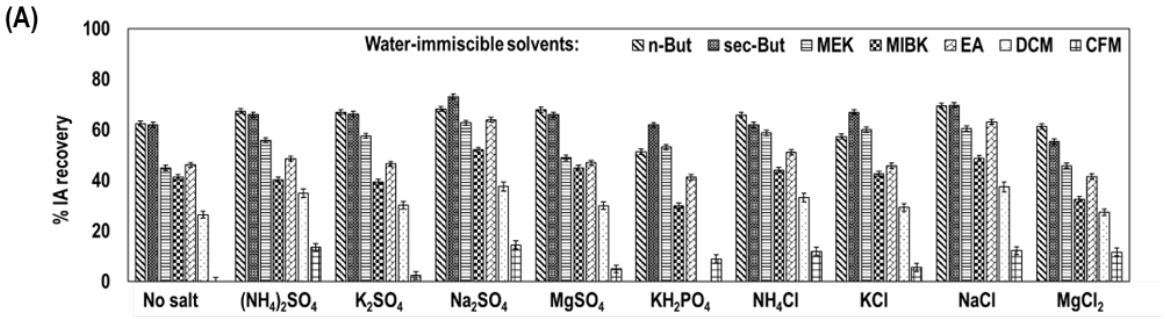
3.4 Screening of solvent and salt combinations for IA extraction

Achieving cost-effective DSP is one of the foremost bottlenecks in IA biomanufacturing [9]. While conventionally used crystallisation methods can yield high recovery in standard medium, they impose significant challenges in complex media like biomass hydrolysate with multiple unwanted components [10]. These methods often necessitate additional separation, concentration, and purification steps, which render the overall process laborious, energy-intensive, and expensive [10,30]. Hence, exploration of alternative, more streamlined approaches is essential. In this context, the SOE was employed for the downstream processing of IA.

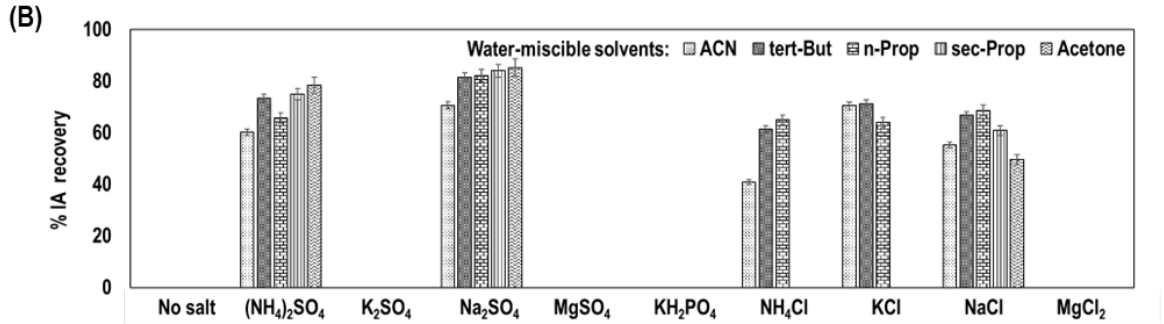
The efficiency of an SOE process hinges on careful selection of salts and solvents and the fine-tuning of process conditions to achieve high purity and yield [37]. Hence, to develop a viable SOE-based DSP technique, various solvent-salt combinations were screened for their IA extraction efficiency. The investigated solvents included water-immiscible and miscible polar organic solvents with high volatility (BP<120°C), which were chosen to efficiently solubilize polar IA and facilitate easy recovery through evaporation **(Supplementary Table 2).** Initially, a simple liquid-liquid extraction (LLE) technique employing water-immiscible solvents was used to extract IA from its pure solution. The extraction efficiency was observed in the following order: *n*-But ≈ *sec*-But > EA > MEK > MIBK > DCM, with no extraction in CFM. The maximum IA recovery of 66% was observed with *n*-butanol and *sec*-butanol **(Supplementary Fig. 2**).

The salting-out process was incorporated by dissolving some common inorganic salts (10% w/v) in the IA solution (**Supplementary Table 3**). The extraction efficiency of water-immiscible solvents was significantly enhanced ($p<0.05$) in salt-saturated IA solutions as compared to IA solutions without salts (**Fig. 4A**). The salt ions bind water molecules via strong

ion-dipole interactions. This reduces the solubility of the less polar IA molecules in the aqueous phase, promoting their transfer into the organic phase. Notably, the addition of certain salts [$(NH_4)_2SO_4$, Na_2SO_4, and NaCl] triggered phase separation when combined with water-miscible solvents, thereby enabling the extraction of IA into the organic layer. Other salts were either precipitated out or failed to induce phase separation in water-miscible solvents (**Fig. 4B**).



	No salt	$(NH_4)_2SO_4$	K_2SO_4	Na_2SO_4	$MgSO_4$	KH_2PO_4	NH_4Cl	KCl	NaCl	$MgCl_2$
n-But	62.8±1.9	67.4±2.0	67.0±2.0	68.3±2.1	68.5±2.0	51.3±1.5	65.9±1.9	57.3±1.7	69.6±2.0	61.4±1.8
sec-But	62.0±1.8	65.9±1.9	66.2±1.9	73.1±2.2	66.2±2.0	61.9±1.8	61.9±1.8	67.0±2.0	69.7±2.1	55.4±1.6
MEK	45.0±1.3	55.9±1.7	57.6±1.7	62.8±1.9	49.1±1.5	53.2±1.6	58.8±1.8	60.1±1.8	60.5±1.8	45.8±1.4
MIBK	41.3±2.4	40.3±2.3	39.4±2.3	52.1±3.1	45.0±2.6	29.9±1.8	44.1±1.3	42.6±2.6	48.8±2.6	32.5±1.9
EA	46.1±2.8	48.6±2.9	46.6±2.8	64.0±3.2	47.1±2.8	41.3±2.4	51.1±1.5	45.8±2.8	63.1±2.7	41.5±2.5
DCM	26.4±1.6	34.8±2.1	30.2±1.8	37.6±2.6	30.7±1.8	Precipitation	33.2±1.9	29.3±1.6	37.5±2.2	27.4±1.6
CFM	0.0±0.0	13.5±0.8	2.4±0.2	14.4±0.8	5.5±0.3	8.9±0.5	11.9±0.5	5.6±0.4	12.2±0.7	11.6±0.7



	No salt	$(NH_4)_2SO_4$	K_2SO_4	Na_2SO_4	$MgSO_4$	KH_2PO_4	NH_4Cl	KCl	NaCl	$MgCl_2$
ACN	Miscible	60.2±3.0	Miscible	70.5±3.5	Miscible	Miscible	40.7±2.0	70.4±3.5	55.2±3.2	Miscible
tert-But	Miscible	73.4±2.2	Miscible	81.5±2.4	Miscible	Miscible	61.3±1.8	71.2±2.1	66.6±2.0	Miscible
n-Prop	Miscible	65.7±2.0	Precipitation	82.1±2.5	Miscible	Miscible	64.9±1.9	63.9±1.9	68.6±2.1	Miscible
sec-Prop	Miscible	74.9±2.2	Precipitation	84.0±2.6	Miscible	Miscible	Miscible	Precipitation	60.8±1.8	Miscible
Acetone	Miscible	78.2±2.4	Precipitation	85.2±3.5	Miscible	Miscible	Miscible	Precipitation	49.6±1.5	Miscible

Fig. 4: Screening of various solvent-salt combinations for IA extraction efficiency from its pure solution. Salting-out extraction using (A) water-immiscible solvents, and (B) water-miscible solvents. Values are shown as mean ± SD (n = 3), and differences between conditions were statistically significant ($p < 0.05$).

548 Many reports have employed SOE with water-miscible solvents, where salt addition
549 induces the formation of an aqueous two-phase system (ATPS), for recovering biochemicals
550 from fermentation broth. For instance, a combination of isopropanol and $(NH_4)_2SO_4$ resulted
551 in 97% recovery of 2,3-butanediol from sugarcane bagasse hydrolysate [58]. This extraction
552 technique has been extensively employed for the recovery of molecules like propanediol and
553 butanediol, but fewer reports exist for organic acids [32,59]. Moreover, partial phase separation
554 in ATPS systems might also risk co-extraction of undesired hydrophilic impurities, potentially
555 compromising product purity [59,60].
556 In the current study, among all screened systems, the salting-out effect of Na_2SO_4
557 resulted in maximum IA extraction efficiency. To investigate this in detail, various partition
558 parameters were evaluated for all the solvents in the presence of 10% Na_2SO_4 (**Table 2**). In the
559 case of water-miscible solvents, although the phase separation occurred upon Na_2SO_4
560 dissolution, the volume distribution remained highly uneven, indicated by the high phase ratio
561 (P>2). In contrast, the phase separation was much more uniform in the case of water-
562 immiscible solvents (P∼1). Among these, *sec*-butanol extracted maximum IA (73.1%), while
563 also retaining 9.1% (v/v) water from the aqueous layer with a phase ratio of 1.2. The extraction
564 efficiency was observed to be fairly high (>80%) in water-miscible solvents; however, a large
565 portion of water molecules was transferred into the organic layer, which may carry other
566 hydrophilic by-products with them. The partition coefficient (K) is considered a more reliable
567 indicator, as it accounts for both the quantity of solute and the volume of each phase. In this
568 study, the highest K values were obtained using *sec*-butanol (2.22) and *tert*-butanol (1.9) as
569 extractants.
570
571

572 **Table 2:** Salting out extraction of IA using a combination of Na_2SO_4 as the salting-out agent
573 with various polar organic solvents (water-miscible and immiscible). Values are expressed as
574 mean ± SD. Overall differences among solvents were statistically significant ($p < 0.05$).

Solvent	Extraction efficiency, E or IA recovery (%)	Mass distribution ratio, D_m	$V_{org.}$ (mL)	$V_{aq.}$ (mL)	Phase ratio, P ($V_{org.}/V_{aq.}$)	Water content in organic layer (%)	$[IA]_{org.}$ (g/L)	$[IA]_{aq.}$ (g/L)	Partition coefficient, K ($[IA]_{org.}/[IA]_{aq.}$)
Water-immiscible solvents									
n-But	68.3±2.1	2.1±0.06	22.0	18.0	1.22	9.09	31.0±0.9	17.6±0.5	1.76±0.05
sec-But	73.1±2.2	2.7±0.08	22.0	18.0	1.22	9.09	33.2±1.0	14.9±0.4	2.23±0.06
MEK	62.7±1.9	1.7±0.05	21.0	19.0	1.11	4.76	29.9±0.9	19.6±0.6	1.53±.0.05
MIBK	52.1±3.1	1.1±0.03	20.5	19.5	1.05	2.44	25.4±0.8	24.5±0.7	1.04±0.03
EA	64.0±3.2	1.8±0.09	22.0	18.0	1.22	9.09	29.1±1.4	20.0±1.0	1.45±0.07
DCM	37.6±2.6	0.6±0.03	17.5	22.5	0.78	0.00	21.5±1.3	27.8±1.6	0.77±0.04
CFM	14.4±0.8	0.2±0.01	20.5	19.5	1.05	2.44	9.0±0.5	43.9±2.6	0.21±0.01
Water-miscible solvents									
ACN	70.5±3.5	30.0±1.5	30.0	10.0	3.00	33.3	23.5±1.2	29.5±1.5	0.80±0.4
tert-But	81.5±2.4	28.0±0.8	28.0	12.0	2.33	28.6	29.1±0.9	15.4±0.5	1.89±0.05
n-Prop	82.1±2.5	32.0±0.9	32.0	8.0	4.00	37.5	25.7±0.8	22.4±0.7	1.15±0.03
sec-Prop	84.0±2.6	33.0±1.0	33.0	7.0	4.71	39.4	25.5±0.8	22.9±0.7	1.11±0.03
Acetone	85.2±3.5	35.0±1.1	35.0	5.0	7.00	42.8	24.3±0.7	29.6±0.8	0.82±0.02

575

576 ### 3.5 *sec*-butanol *v/s tert*-butanol: Effect of Na_2SO_4 concentration

577 The addition of salt increases the density of the aqueous layer and reduces the
578 interactions between water and solvent molecules, thus promoting phase separation. When *sec*-
579 butanol and *tert*-butanol were used with IA solution containing 10% w/v Na_2SO_4, the phase
580 ratio was 1.2 and 2.3, respectively. Complete phase separation (P=1) is favourable to minimize

product contamination with other water-soluble compounds. To achieve this, the effect of change in salt concentration (0-40% w/v) was studied on the partitioning behaviour of *sec*-butanol and *tert*-butanol (**Table 3**). At higher concentrations (>40% w/v), Na_2SO_4 began to precipitate. It was observed that for both solvents, an increment in Na_2SO_4 improved IA, enhanced phase separation, and reduced water content in the upper solvent layer. For *sec*-butanol, increasing Na_2SO_4 to 25% w/v enhanced the extraction efficiency and partition coefficient (K) to 78.2% and 3.6, respectively. A complete phase separation (P=1) was also observed in this condition, and further salt increment did not alter the partition behaviour. In the case of *tert*-butanol, the maximum Na_2SO_4 concentration (40% w/v) increased extraction efficiency to 83.5% with a K of 3.04, however, complete phase separation could not be achieved ($P \neq 1$). With the P value of 1.67, the solvent layer still retained 20% (v/v) water, which might compromise the purity of the final product.

The results indicate *sec*-butanol, a moderately polar solvent with low water miscibility, as an ideal extractant for IA. Multiple studies have supported the effectiveness of sulfate-based salts in facilitating the efficiency of SOE systems [31,34,37]. The salt effectively modified interfacial interactions, resulting in minimal contamination from other impurities. The highly water-soluble Na_2SO_4 with its strongly *kosmotropic* sulphate ions exerts a pronounced salting-out effect, which intensifies as salt concentration is increased [32,37]. The kosmotropic ions form a robust hydration shell with water molecules through strong electrostatic interactions, which significantly reduces the availability of free molecules to solubilize IA, thereby pushing it toward the organic phase. This leads to a notable increase (26%) in the extraction efficiency ($p<0.01$) (**Table 3**). The results indicate that a maximum recovery (78.2%) and complete phase separation (P = 1) were achieved using the sec-butanol-Na2SO4 system, which was chosen for SOE-based recovery of IA from fermentation broth.

606 **Table 3.** Effect of varying Na_2SO_4 concentrations on the salting-out-assisted extraction
607 efficiency of IA using sec-butanol and tert-butanol as extractants. Values reported as mean ±
608 SD. A statistical comparison across different Na_2SO_4 concentrations reveals a significant effect
609 ($p < 0.05$) on IA recovery.

Na_2SO_4 (% w/v)	*sec*-But				*tert*-But			
	IA recovery (%)	**Partition coefficient, K**	**Phase ratio, P**	**Water content in organic layer, $H_2O_{org.}$ (%)**	**IA recovery (%)**	**Partition coefficient, K**	**Phase ratio, P**	**Water content in organic layer, $H_2O_{org.}$ (%)**
0	62.0±2.8	1.10±0.04	1.50	16.7	-	-	-	100
5	69.9±2.5	1.72±0.05	1.35	13.1	84.1±2.3	1.76±0.07	3.00	33.3
10	73.1±2.2	2.23±0.06	1.22	9.1	81.5±2.4	1.89±0.07	2.33	28.6
15	74.1±1.2	2.58±0.04	1.11	4.8	81.8±1.5	2.04±0.08	2.20	27.3
20	76.2±1.1	3.05±0.10	1.05	2.4	82.1±1.7	2.21±0.09	2.08	25.9
25	78.2±2.3	3.60±0.11	1.00	0.0	82.4±2.5	2.39±0.09	1.96	24.5
30	78.2±2.1	3.60±0.10	1.00	0.0	82.7±1.8	2.57±0.10	1.86	23.1
35	78.2±1.9	3.60±0.09	1.00	0.0	83.1±1.2	2.80±0.11	1.76	21.6
40	78.2±2.2	3.60±0.12	1.00	0.0	83.5±1.8	3.04±0.12	1.67	20.0

610

611 ### 3.6 Role of pH in SOE-based IA recovery

612 The efficiency of a solvent extraction process is strongly influenced by the pH of the
613 aqueous medium. IA is a dicarboxylic acid with two acidic protons having dissociation
614 constants of 3.8 (pKa_1) and 5.5 (pKa_2). The ionic state of IA changes with pH variations in
615 accordance with the pKa values (Eq. 9,10), which alters its solubility. In this context, the effect

of pH (0.5-5.0) on extraction efficiency was investigated. The dissolution of salt causes a change in the pH; hence, the media pH was adjusted after salt was added.

$pKa_1 \approx 3.8$ $\quad H_2A \rightleftharpoons HA^- + H^+$ (9)

$pKa_2 \approx 5.5$ $\quad HA^- \rightleftharpoons A^{2-} + H^+$ (10)

Fig. 5A shows variation in log K and %IA recovery with change in pH. The log K values give a measure of the relative affinity of a compound towards water and solvent. A pH of 2.0-3.0 favours the partitioning of IA into the organic layer, which is evident by the positive log K values. The maximum IA recovery (84.65%) was observed at pH 2.0, corresponding to a log K value of 0.74, indicating efficient partitioning. However, beyond pH 3.5, the log K became negative, and the % IA recovery dropped sharply, falling to 53.2% at pH 3.5 and only 5.5% at pH 5.0.

At low pH, the IA molecules predominantly exist in protonated non-ionic form (H_2A), which is easily solubilized in the organic solvent through hydrophobic interactions, thus contributing to the positive value of log K (log $[IA]_{org.}/[IA]_{aq.}$). It is evident from the results that the salting-out effect is operational mainly in acidic medium ($pH<pKa_1$), where non-ionic or unionised IA dominates. In contrast, IA becomes increasingly ionized (HA^-) and hydrophilic at higher pH values, which limits its solubility in the organic phase and reduces extraction efficiency. The charged IA ions prefer to remain in the aqueous layer, resulting in a negative log K. The IA ions compete with the salt ions for ion-dipole interactions with water molecules, diminishing the salting-out effect.

The experimental findings show the critical role of pH in maintaining IA in its unionised form in accordance with the pKa values, which, in synergy with the salting-out effect, significantly improves extraction efficiency. A similar pH-dependent behaviour has been reported in a study investigating multiple carboxylic acids, including lactic, citric, formic,

acetic acids, etc. For all tested acids, the extraction efficiency of an ethanol-$(NH_4)_2SO_4$ system was more pronounced at pH 2-3 [61]. Similarly, an acetone-$(NH_4)_2SO_4$ system for succinic acid showed maximum recovery at pH 3.0 [35]. In line with previous studies, the highest extraction efficiency for IA was observed at pH 2.0 using the developed *sec*-butanol-Na_2SO_4 system (**Fig. 5A**). Interestingly, at extremely acidic conditions (pH<2.0), the %IA recovery is reduced even though the IA exists in its protonated form. This could be attributed to the presence of an excess of protons, which bind with solvent molecules and interfere with their interactions with IA molecules. Hence, pH 2.0, giving the best yield, was selected for further processing.

3.7 Effect of IA concentration and solvent saturation

The optimized SOE system [*sec*-butanol**;** 25% (w/v) Na_2SO_4**;** pH 2.0] was tested across a broad range of initial IA concentrations (10–80 g/L). The extraction efficiency exhibited an inverse relationship with initial IA concentration (**Fig. 5B**). From a low concentration (10 g/L) solution, 93% of IA was extracted**,** with a corresponding K value of 13.2**.** However, as the initial IA concentration increased, a decline in extraction efficiency was observed. At the highest tested concentration of 80 g/L, the IA recovery dropped to 68%, significantly decreasing the K value to 2.1. This trend was also supported by the $[IA]_{org.}$ v/s $[IA]_{aq.}$ plot (Fig.5C). The initial part of the curve is steep and linear, indicating a favourable extraction at low IA concentrations. However, as $[IA]_{aq.}$ increases, the slope gradually declines and ultimately levels off into a plateau beyond $[IA]_{org.}$ of 50 g/L. This behavior suggests a saturation effect, which is likely due to the limited solubility of IA in the organic phase and potential interfacial crowding. The $[IA]_{org.}$ v/s $[IA]_{aq.}$ curve (Fig. 5C) aligns perfectly with the "Langmuir-isotherm" model of adsorption, which describes monolayer adsorption onto a surface with "finite capacity". Analogously, the solvent extraction system showed a similar behavior, where the organic phase shows a "finite capacity" to solvate IA molecules and

becomes saturated when the number of IA molecules is abundant. These findings suggest that the SOE process is highly efficient at low to moderate IA concentrations but faces saturation constraints at higher concentrations (>50 g/L). At elevated solute loads, partitioning is constrained either by thermodynamic saturation or kinetic mass-transfer limitation**,** ultimately reducing the net recovery.

Process modifications like multiple extractions and solvent volume adjustment were investigated to improve yields at high IA concentration. When the volume of *sec*-butanol was doubled, the extraction efficiency increased to 90%. Comparable results were obtained when extraction was repeated with an equal volume of fresh solvent (Fig. 5d). However, a further increase in solvent volume or additional extraction cycle yielded no significant change in %IA recovery ($p>0.05$). Doubling the solvent volume effectively addressed the thermodynamic constraint by increasing the availability of free solvent molecules to solvate IA and improved the process efficiency by 32%. Notably, a shaking/mixing time of 3 h per extraction cycle was optimal for maximum IA recovery, and no improvement was observed through longer extraction times, suggesting an equilibrium state of the system (Supplementary Fig. 3). No yield improvement with prolonged extraction cycles indicated that the mass transfer was no longer the limiting factor in the process.

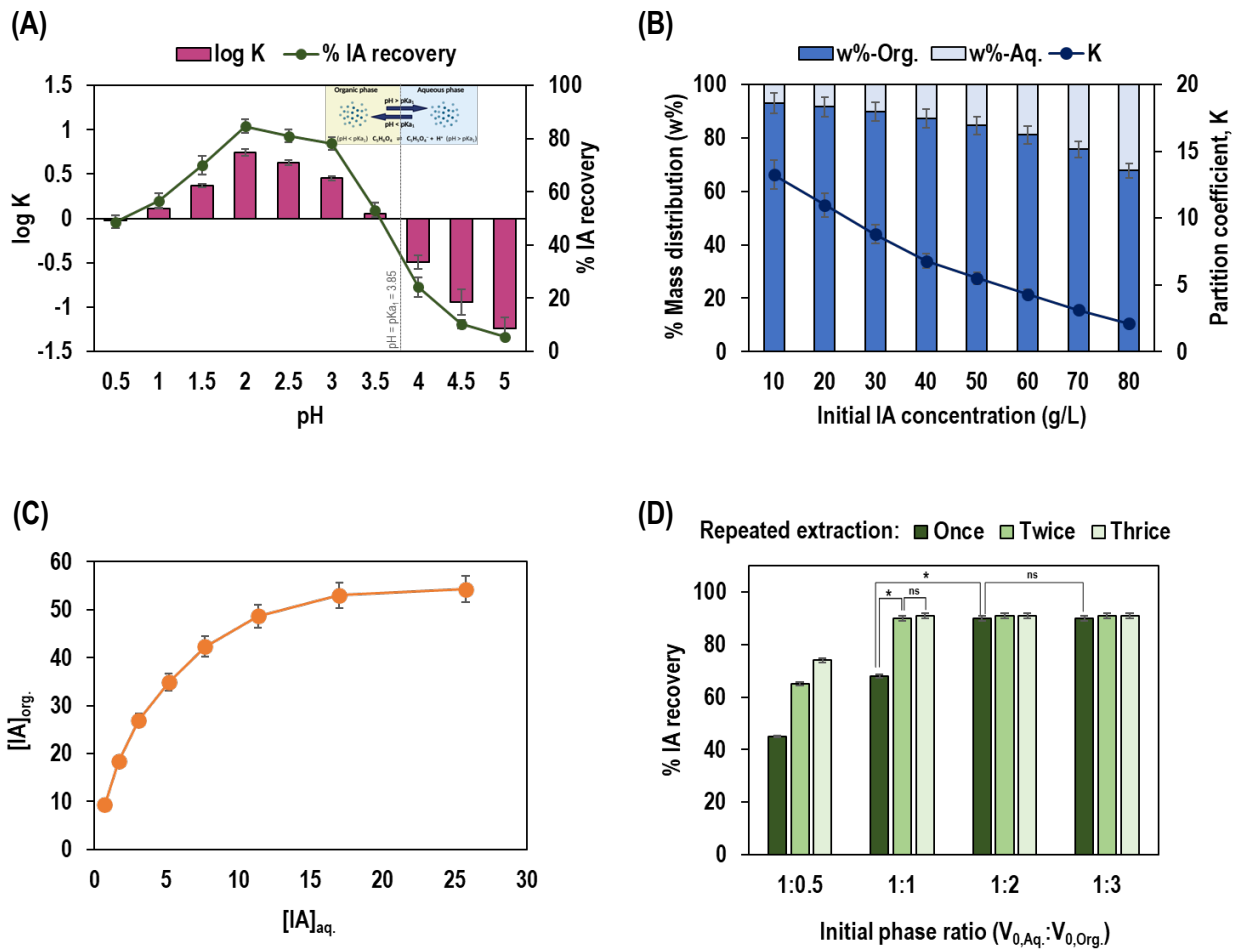


Fig. 5: Impact of various physicochemical parameters on the partition kinetics of *sec*-butanol-Na_2SO_4 (25% w/v) system for IA extraction (A) Effect of initial pH of aqueous solution on log K (bar graph) and % IA recovery (line graph); (B) Impact of IA concentration on %mass distribution (bar graph) and partition coefficient (line graph) of optimized extraction system with pH 2.0; (C) $[IA]_{org.}$v/s $[IA]_{aq.}$ curve with varying IA concentration in the solution; (D) Influence of change in solvent volume (initial phase ratio $V_{0,aq.}$:$V_{0,org.}$) and executing multiple rounds of extraction on %IA recovery.

3.8 Downstream processing of IA from fermentation broth

The developed SOE strategy was implemented for the recovery of IA produced by *A. terreus* using synthetic and potato hydrolysate-based media **(Fig. 6).** After fermentation, 25% w/v Na_2SO_4 was dissolved in the culture broth (containing fungal mycelium), and the pH was adjusted to 2.0 using dilute H_2SO_4. *Sec*-butanol, in an amount twice the volume of the initial mixture, was then added to the culture flask and incubated for 3 h at 200 rpm. The aqueous (broth) and organic (*sec*-butanol) phases were separated by allowing the mixture to stand overnight. Alternatively, the mixture was centrifuged for rapid phase separation. The organic layer acquired the pale orange colour of the pigments produced by the fungus during fermentation, which left behind a pigmented precipitate after evaporation. A treatment with 2% w/v AC prior to evaporation rendered the solvent colourless and resulted in a white extract precipitate. This treatment also enhanced the purity of extracted IA. Finally, 83% of the total IA (51 g/L) was recovered from the IA accumulated on synthetic glucose media with high purity (95%). Implementation of the developed DSP technique on WPAH-based culture containing 32 g/L IA resulted in the recovery of 80.5% IA with 85% purity. The extracted IA was characterized through FT-IR and NMR, and the results were compared to standard IA (**Supplementary Fig. 4**). The overall upstream-to-downstream process of IA production from waste potatoes yielded 29 g IA per kg of waste potatoes.

Solvent extraction techniques, particularly reactive extraction, have been gaining popularity in recent studies due to low energy demand, high yield, and *in situ* extraction [62,63]. It involves a chemical reaction between IA and tertiary/quaternary amine (extractant), forming a complex which shows high affinity to the organic phase (diluent) [62,63]. However, recovery of solid IA, extractant, and diluent is challenging, which adds to the operation cost, complicates scale-up, and leads to environmental concerns [10,30]. In contrast, the developed SOE technique allows easy solvent recovery and reusability since it is based upon physical

partitioning and no chemical reaction. The salt could also be recovered from the broth by precipitating it with cold acetone [31]. The optimized SOE process, when implemented on the *A. terreus* fermentation broth, resulted in substantial recovery (>80%) of solid IA from both synthetic glucose medium (>95% purity) and potato hydrolysate medium (>85% purity) (**Fig. 6**). These outcomes validate the practical applicability of the developed method in both simple and complex fermentation media for achieving a high yield of purified IA in a cost-effective and eco-friendly way. All the experiments performed in the study were performed in shake flasks, and the scalability of the process in bench-top bioreactors and industrially relevant conditions needs to be validated. The next phase of this work will focus on assessing the process efficiency on a larger scale.

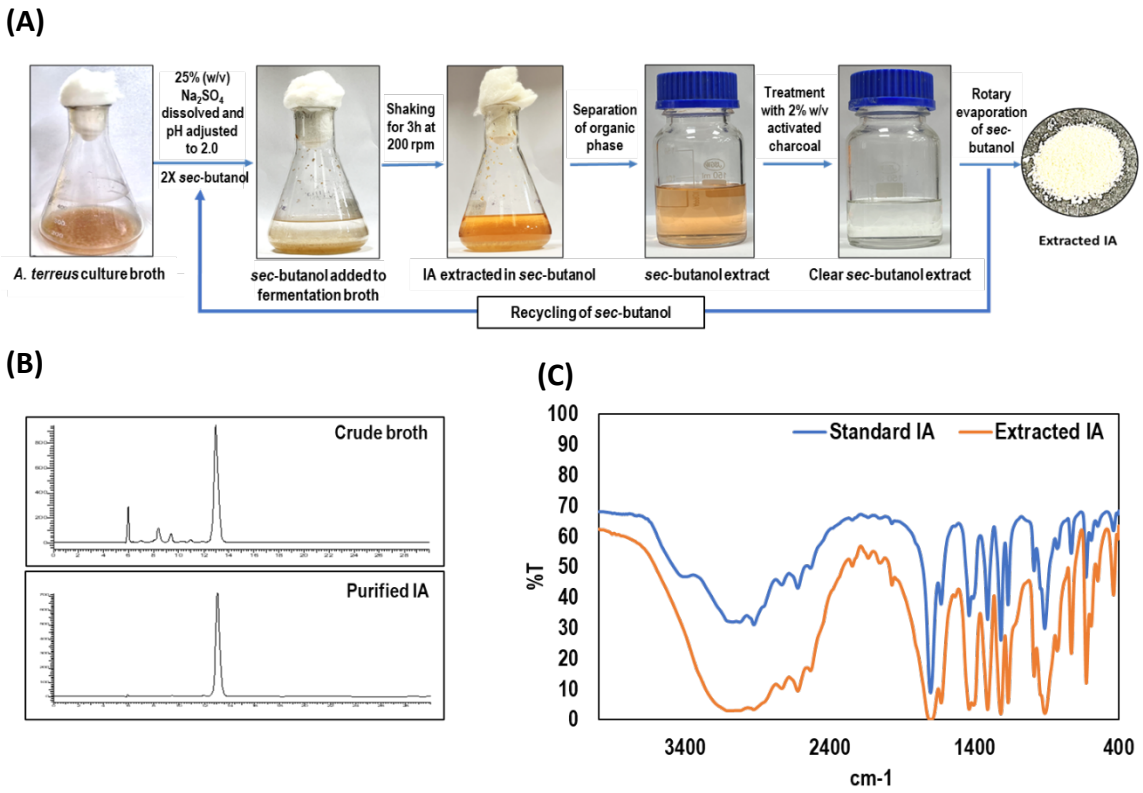


Fig. 6: (A) DSP of IA from fermentation broth using optimized conditions of the SOE system; (B) HPLC chromatogram of crude fermentation broth and purified IA after extraction; (C) FT-IR spectra depicting similarity of characteristic peaks in standard and extracted IA.

4. Conclusions

The current study seeks to dismantle two chief pillars contributing to the high production cost of IA: feedstocks and downstream processes, which impede its widespread applicability. Although existing literature demonstrated bioconversion of waste feedstocks to IA, only a few reports exist where downstream processing techniques have been successfully employed on complex media. By integrating the use of a low-cost starchy waste as substrate with the application of a mild and simple SOE-based downstream processing technique, this workflow presents a novel and comprehensive strategy, offering an effective approach for reducing the overall production cost of IA. This study employed extensive and systematic optimization of parameters influencing the efficiency of each stage of IA biomanufacturing: saccharification, fermentation, and recovery. The developed upstream-to-downstream process allows valorisation of 1 kg of potato waste into 29 g of purified IA with minimal processing steps. This process offers a cost-effective, streamlined, and sustainable alternative to existing bioprocesses for IA production. Further study will focus on process refinement and bioreactor scale-up to improve yields and evaluate the suitability of this strategy for industrial IA manufacturing.

CRediT author statement

Deeksha Gopaliya: Conceptualization, Data curation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Vinod Kumar:** Conceptualization, Supervision, Writing – review & editing. **Sunil Kumar Khare:** Conceptualisation, Supervision, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Deeksha Gopaliya is thankful to the Government of India for the Prime Minister Research Fellowship (PMRF ID: 1401206). The authors are grateful to the Department of Chemistry, IIT Delhi, and the Central Research Facility (CRF) at IIT Delhi for providing the necessary facilities to conduct this research. Suggestions from Dr Nitin Srivastava are greatly acknowledged.

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Integrated cost-efficient itaconic acid production from waste potatoes via a holistic upstream-to-downstream approach

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2026-04-22
Elsevier
https://doi.org/10.1016/j.seppur.2025.136685
Gopaliya D, Kumar V, Khare SK. (2025) Integrated cost-efficient itaconic acid production from waste potatoes via a holistic upstream-to-downstream approach. Separation and Purification Technology, Volume 388, April 2026, Article number 136685

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