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High-efficiency water use and process performance for bioproduction of *trans*-cinnamic acid

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Increasing water scarcity, driven by population growth and climate change, necessitates the development of biomanufacturing processes that maximize water-use efficiency. This report illustrates the potential of biocatalyst and supernatant recycling in a decoupled bioprocess integrated with product removal systems for *trans*-cinnamic production using *Pseudomonas putida* KT2440 expressing RmXAL. With a biocatalyst concentration of 40 g_{DCW} L^{-1} and an L-phenylalanine feed concentration of 35 g_{L-phe} L^{-1} combined with product precipitation, the process achieved a titer of 143.00 g L^{-1}, a production rate of 2.25 g L^{-1} h^{-1}, a yield of 1.50 g *t*CA g glucose^{-1}, and a yield on L-phenylalanine of 0.64 g *t*CA g L-phe^{-1}. Furthermore, this optimized approach substantially reduced water consumption and wastewater generation by 85% compared to standard production processes, promoting the principles of Sustainable Development Goal 6 (SDG) "Clean water and sanitation" and SDG 12 "Responsible production and consumption" thus enabling an industrial bioproduction platform to achieve better environmental and economic sustainability.

Keywords Decoupled recycling bioprocess, Water-use efficiency, Whole-cell biocatalyst, Performance metrics, *In-situ* product recovery

Abbreviations

ISPR	*In-situ* Product recovery
Log (K_d)	Logarithm distribution coefficient
mM9	Modified M9 medium
OpEx	Operating expenses
PAL	Phenylalanine ammonia-lyase
SDG	Sustainable development goal

List of symbols

DCW	Dry cell weight (g L^{-1})
$F(t)$	Feed rate of different time points (L h^{-1})
L-phe	L-phenylalanine (g L^{-1})
Q_p	*t*CA Production rate over the biotransformation time (g *t*CA L^{-1} h^{-1})
Overall Q_p	*t*CA production rate over the total time (g *t*CA L^{-1} h^{-1})
q_p	Specific *t*CA production rate (g *t*CA g $cell^{-1}$ h^{-1})
S_i	Substrate concentration in feed medium (g L^{-1})
t	Time (h)
*t*CA	*trans*-Cinnamic acid (g L^{-1})
$[tCA]_{aq}^{i}$	Initial *trans*-cinnamic acid concentration in the aqueous phase (g L^{-1})
$[tCA]_{aq}^{eq}$	*trans*-Cinnamic acid concentration in the aqueous phase at equilibrium (g L^{-1})
μ	Specific growth rate (h^{-1})
μ_{set}	Specific growth rate for feeding calculation (h^{-1})

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V_0	Fermentation volume after batch phase (L)
X_0	Cell concentration after batch phase (g L^{-1})
$Y_{p/H2O}$	*t*CA yield on water used in the bioprocess (g *t*CA g $water^{-1}$)
$Y_{p/s1}$	*t*CA yield on glucose (g *t*CA g $glucose^{-1}$)
$Y_{p/s2}$	*t*CA yield on L-phenylalanine (g *t*CA g L-phe^{-1})
$Y_{p/x}$	*t*CA yield on biomass (g *t*CA g $cell^{-1}$)

Water is essential for sustaining life, supporting ecosystems, and driving economic activities. It plays a crucial role in agriculture, industry, and energy production. However, population growth and climate change over recent decades have led to inadequate and inconsistent water supply, directly impacting the ecosystem of cities, agricultural yield, industrial productivity, and public health[1,2]. Although efforts to address these issues have been made through water infrastructure, such as wells, reservoirs, and inter-basin diversions, the world's major cities, with a total population of 233 million, are predicted to exhaust their current water resources by 2050[3]. These challenges underscore the urgent need to adopt production processes exhibiting high water efficiency. In biomanufacturing, water is the primary solvent in submerged fermentation[4,5]. Therefore, minimizing water consumption not only conserves resources but also reduces the volume of wastewater generated, which is a critical consideration in industrial applications worldwide[6,7]. Advances in water-efficient bioproduction could contribute meaningfully to resource conservation efforts and reduce industrial strain on water supplies. Moreover, sustainable chemical production processes using whole-cell biocatalysts have gained interest as a means to reduce reliance on traditional chemical synthesis, which often requires extreme conditions[8,9].

trans-Cinnamic acid (*t*CA) is a phenylpropanoid compound that can be naturally synthesized by plants from L-phenylalanine (L-phe) through the activity of phenylalanine ammonia-lyase (PAL)[10–12]. *t*CA and its derivatives are widely used in various industrial sectors, including cosmetics, pharmaceutical, and food additives, because of their biological properties, such as antioxidant, antimicrobial, UV protection, and anticancer[13]. Interestingly, the current purchase price of *t*CA is 152 USD/kg, and the demand for this product is expected to increase significantly over the next ten years. Consequently, green and sustainable production processes for *t*CA production via microbial fermentation have garnered significant interest. Metabolic engineering, a cutting-edge tool, is now being used to increase the *t*CA production capabilities of several production hosts[14,15]. A general strategy begins with controlling carbon flux from central metabolism and expressing feedback inhibition-resistant (fbr) of some enzymes to enhance L-phe production[16–18]. Subsequently, PAL enzymes are expressed to convert L-Phe to *t*CA[19]. Although metabolic engineering enables routes to increase substrate utilization efficiency and limits byproduct formation, it is often insufficient for improving the performance metrics of microbial fermentation processes. Therefore, a decoupled bioprocess, separation between microbial growth and product formation, is an additional technique that can be combined with strain engineering to achieve the goal[20,21]. For the *t*CA production using this strategy, a whole-cell biocatalyst is produced in the first stage under optimal microbial growth and enzyme production conditions. Then, the process is switched to product formation by adjusting the conditions and adding L-phe into the system. During this stage, L-phe is converted to *t*CA by the enzyme within the cells (Fig. 1a).

Recently, Zheng et al.[22] reported that protein engineering could increase the catalytic efficiency of ZmPAL2 expressed in *Escherichia coli*, resulting in *t*CA production at a titer of 31 g L^{-1}. In our previous study, we developed a decoupled bioprocess of recombinant *Pseudomonas putida* KT2440 expressing RmXAL for *t*CA production, and we could also produce a *t*CA of 30 g L^{-1} with a production rate of 6 g L^{-1} h^{-1}[23]. Nevertheless, one of the significant limitations in achieving high-performance metrics for *t*CA production is the product inhibition of PALs. Additionally, the solubility of L-phe also poses a barrier, even though it is highly dependent on pH and temperature[24]. To mitigate these problems, employing production processes with cell recycling or continuous removal of target product from the fermented broth using in-situ product recovery (ISPR) are possible solution strategies[25–27]. Several examples from the literature have documented that these methods can be applied not only for chemical production but also for food additives, antibiotics and some industrially relevant enzymes[28–30]. Moreover, these techniques offer other benefits when applied to fermentation processes. For instance, the number of unit operations and operational expenses (OpEx) related to fermentation process preparation, including cleaning, sterilizing, and preparing inoculum, culture medium, and the bioreactor for the next batch, can be reduced[31,32]. Previously, Son et al.[33] optimized the *t*CA production process using a recycling cell of *Corynebacterium glutamicum*, and it could be produced at 2.1 g L^{-1} with a rate of 0.68 g L^{-1} h^{-1} of each cycle. However, the process did not incorporate supernatant recycling for further production cycles.

To bridge this gap and maximize water efficiency while enhancing production metrics, this study presents the development of a decoupled bioprocess using recombinant *P. putida* KT2440 for *t*CA production by utilizing a cell and supernatant recycling system (called a decouple recycling bioprocess) combined with an ISPR system using precipitation compared to liquid-liquid extraction (Fig. 1b, c). The findings from this research could support the scale-up of *t*CA production, advancing green industry practices by significantly reducing water consumption and minimizing waste. This aligns with global sustainability goals, paving the way for more environmentally friendly and economically viable bioprocesses.

Materials and methods

Microorganism and inoculum Preparation

The recombinant *P. putida* KT2440 expressing PAL from *Rhodotorula mucilaginosa*, called RmXAL, demonstrated the highest potential for *t*CA production in our previous work and was used throughout this study[23]. The gene was expressed under a rhamnose-inducible (*PrhaB*) promoter. The optimal temperature and pH of RmXAL for biotransformation were 37 °C and 8.5, respectively[23]. The cell was grown on LB agar plates with 10 μg mL^{-1} gentamycin at 30 °C for 16–20 h. A primary inoculum was prepared by transferring a single colony to 5 mL of

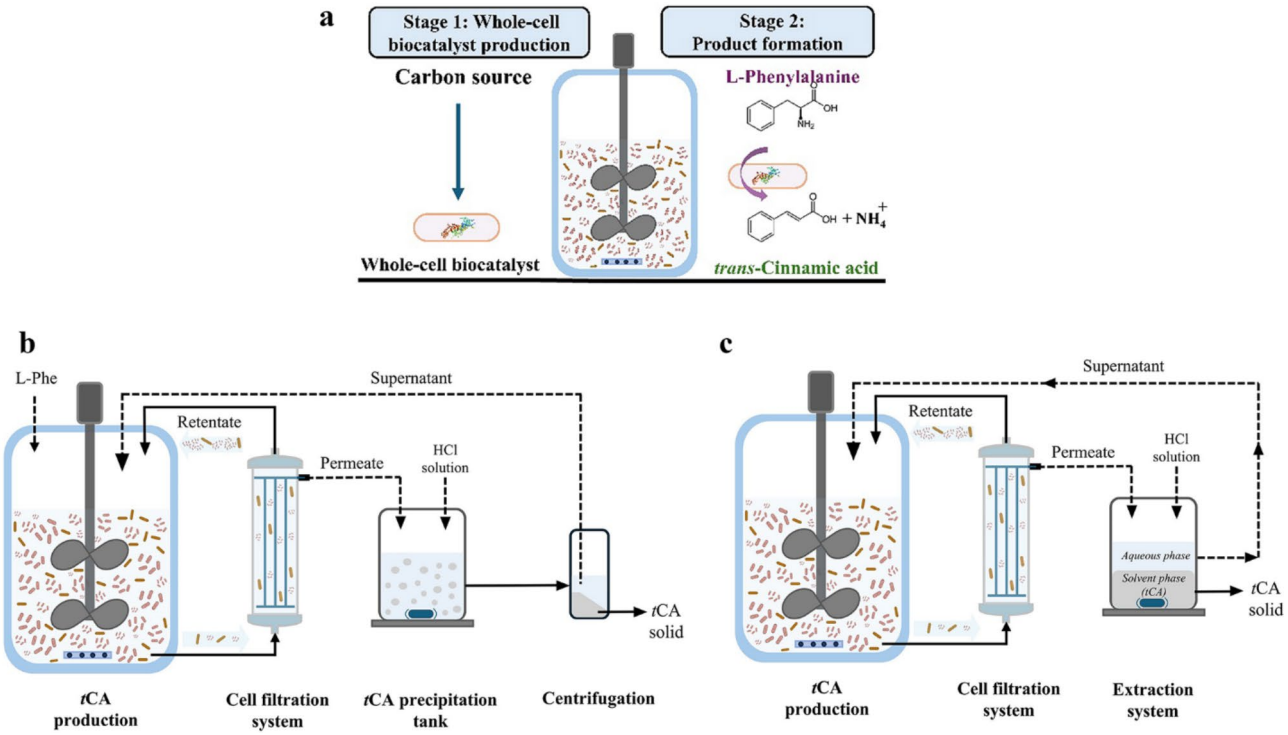

Fig. 1. (**a**) Principle of a decoupled bioprocess for *t*CA production and a schematic diagram of a decoupled recycling bioprocess for *t*CA production integrated with different product removal methods: (**b**) the precipitation system and (**c**) the external-direct liquid-liquid extraction system.

LB broth with 10 μg mL^{-1} gentamycin and cultivated on a shaking incubator at 30 °C, 250 rpm for 6–8 h. The secondary inoculum was carried out by transferring the primary inoculum into a 250 mL baffled flask containing 50 mL of modified M9 medium (mM9) with 10 μg mL^{-1} gentamycin. The initial optical density (OD_{600}) was set to 0.1. mM9 medium was prepared accordingly, which 1 L consisted of 20 g glucose, 3 g $MgSO_4{\cdot}7H_2O$, 3 g KH_2PO_4, 5 g $(NH_4)_2SO_4$, 1 g NaCl, 0.15 g $CaCl_2{\cdot}2H_2O$, 0.05 g $FeSO_4{\cdot}7H_2O$, 1 g of yeast extract and 1.5 mL of Trace Element Solution (TES). The TES in 1 L contained 0.75 g $CoSO_4{\cdot}7H_2O$, 0.5 g H_3BO_3, 12 g $CaCO_3$, 2.5 g $NiSO_4{\cdot}6H_2O$, 2.5 g $CuSO_4{\cdot}5H_2O$, 14.64 g $MnSO_4{\cdot}H_2O$, 15 g $ZnSO_4{\cdot}7H_2O$, 3 g $Na_2MoO_4{\cdot}2H_2O$, and 8 mL HCl. The culture was cultivated under the same conditions as the primary inoculum for 16–18 h.

Fed-batch fermentation of Recombinant *P. putida* KT2440

Whole-cell biocatalysts used throughout this study were obtained by fed-batch fermentation. The process was conducted in a 0.5 L stirred-tank bioreactor (my-Control, Applikon Biotechnology, The Netherlands). The fermentation was initiated by inoculating the secondary inoculum into 250 mL of mM9 medium with 10 μg mL^{-1} gentamycin at the initial $OD_{600} \sim 1$. The fermentation conditions were controlled as follows: temperature of 30 °C, pH 6.8 (maintained using 25% NH_4OH or 2 M HCl solutions), dissolved oxygen tension (DOT) at or above 40% by cascading the aeration (1.0-1.5 vvm) and agitation (800–1200 rpm). After the OD_{600} reached 15, 4.2 mL of 150 mM rhamnose solution was added to induce RmXAL expression. Subsequently, fed-batch fermentation was carried out when the DOT rapidly increased, which indicated that glucose was completely consumed. Feed medium (500 g L^{-1} glucose, 10 g L^{-1} $MgSO_4{\cdot}7H_2O$, 2.5 g L^{-1} rhamnose, and 10 μg mL^{-1} gentamycin) was fed into the bioreactor to increase cell concentration. Feeding strategies were begun with exponential mode and followed by constant mode (8.5 $g_{glucose}$ L^{-1} h^{-1}). The exponential feed profile over a different time $(F_{(t)})$ was calculated using Eq. (1).

$$F_{(t)} = \frac{\mu_{set} X_0 V_0 e^{\mu_{set} t}}{S_i Y_{x/s}} \quad (1)$$

where the μ_{set} is a desired specific growth rate (0.25 h^{-1}), X_0 is cell concentration at the time of feeding start, V_0 is the fermentation volume at the time of feeding start, S_i is the substrate concentration of the feeding medium, $Y_{x/s}$ is the biomass yield on substrate, and t is the time of the fed-batch phase.

When the whole-cell biocatalyst reached 30 and 40 g_{DCW} L^{-1}, the fermentation process was switched to the *t*CA production phase.

Investigation of decoupled recycling system for *t*CA production

To increase high-efficiency water use, enhance performance metrics (titer, rate, and yield), mitigate the effect of product inhibition, and promote the sustainability of the *t*CA production process, cells and supernatants were recycled for use in the subsequent production cycle. After the fed-batch fermentation was completed, the fermentation conditions were adjusted to the optimal condition for whole-cell biotransformation. L-phe was then added into the bioreactor at the final concentration of 25 and 35 g L^{-1}. After the biotransformation operation, the bioreactor was connected to a 0.22 µm crossflow filtration membrane with a filtration area of 35 cm^2 (Lab35, SANI Membranes, Denmark) for cell separation using peristaltic pumps (120s, Watson-Marlow, United Kingdom) at an average permeate flux of 2.43 mL h^{-1} cm^{-2} (Fig. 1b). The retentate line was assembled to the bioreactor, and the permeate line was connected to the *t*CA precipitation tank to precipitate *t*CA by adjusting the pH from 8.5 to 2.5 using a 2 M HCl solution. The resulting white colloid of *t*CA was separated by centrifugation at 6000×*g* for 10 min, and then the supernatant without *t*CA was fed back into the bioreactor to start a new production cycle. After each run, the membrane filter was cleaned with 55ºC of 10 mM NaOH solution for 30 min, followed by flushing with deionized water for 15 min. The membrane filter was then sterilized at 121ºC for 15 min before being used in the next cycle. The initial volume (300 mL) was set consistently for each biotransformation cycle, and total water consumption was quantified upon completion of the production process.

Solvent extraction performance

The solvents used in this study to assess the *t*CA extraction performance were selected based on biocompatibility, environmental friendliness, safety, and price[28,34]. Four solvents (bis(2-ethylhexyl) adipate, ethyl oleate, isopropyl myristate, and tributyrin) were chosen. For this, 2 g L^{-1} of *t*CA was mixed with different solvents at a 1:1 ratio. The final pH was adjusted to 2.5, 4.3, 7.0, and 8.5 using either 2 M HCl or 5 M NaOH solution. Subsequently, the mixture was manually shaken for 2 min and settled at 30 °C for 2 h to reach equilibrium. The aqueous phase was sampled to measure the concentration of *t*CA by high-performance liquid chromatography (HPLC), and then the logarithm distribution coefficient (Log (K_d)) was calculated as Eq. (2).

$$Log\,(K_d) = Log\frac{[tCA]^{i}_{aq} - [tCA]^{eq}_{aq}}{[tCA]^{eq}_{aq}} \tag{2}$$

where $[tCA]^{i}_{aq}$ is the initial *t*CA concentration in the aqueous phase and $[tCA]^{eq}_{aq}$ is the *t*CA concentration in the aqueous phase at equilibrium.

The efficiency of tributyrin for *t*CA in-situ extraction

To investigate the extractant's effectiveness, the pH of the supernatant obtained from whole-cell biotransformation was adjusted to 2.5 by 2 M HCl solution and mixed with varying amounts of tributyrin at volume ratios of 1:1, 1:2, 1:5, 1:10, 1:20, and 1:30 (tributyrin/supernatant). A control experiment was conducted without in-situ extraction. The mixture was settled at 30 °C for 2 h, and then the aqueous phase was sampled to measure the *t*CA concentration by HPLC. The extraction efficiency and supernatant recovery at different ratios were then compared.

Integration of recycling bioprocess and liquid extraction system

The decoupled bioprocess for *t*CA production using a recycling system was integrated into an external-direct ISPR configuration to extract *t*CA from the supernatant, and the potential for supernatant recycling was evaluated. After completing the production process in the first cycle, cells were separated by membrane filtration. The permeate line was connected to the extraction tank (Fig. 1c), and the pH was adjusted to 2.5 using a 2 M HCl solution. Tributyrin was added to the tank at a volume ratio of 1:20. The solution was mixed for 15 min and settled for 60–90 min. The supernatant was pumped back into the bioreactor to initiate a new cycle of biotransformation. The initial volume (300 mL) was set consistently for each biotransformation cycle, and water consumption was quantified upon completing the production process. The total water usage of the liquid extraction method was compared with that of a precipitation-based method and the standard production process without a supernatant recycling system.

The standard bioproduction of *t*CA

The production of *t*CA using a standard operating process without supernatant recycling was carried out in a 0.5 L stirred-tank bioreactor. Fed-batch fermentation was performed to increase whole-cell biocatalyst concentration to 40 g_{DCW} L^{-1}, as described in the fed-batch fermentation of recombinant *P. putida* KT2440 section. Subsequently, L-phe was added to the bioreactor with the final concentration of 35 g $L^{-1,}$ and the fermentation conditions were adjusted to facilitate biotransformation. After the biotransformation operation, cell separation was conducted using a crossflow microfiltration technique, following a similar method described in the investigation of decoupled recycling system for *t*CA production section. The separated cells were fed back into the bioreactor for subsequent production batches. Fresh medium, including 35 g L^{-1} of L-phe, was added to the bioreactor with an initial volume of 300 mL to start the next production cycle. The *t*CA concentration in the supernatant was measured by HPLC.

Analytical procedures

Biomass concentration: Biomass concentration was determined by measuring the optical density (OD) with a spectrophotometer at 600 nm (UV-1800, Shimadzu, Japan). Dry cell weight (DCW) was computed using the

correlation between OD and DCW, which was measured by centrifugation of 2 mL of fermented broth at 5000 × g for 10 min. Cells were washed with distilled water twice and dried at 60 °C in a hot-air oven for 48 h or until a constant weight was obtained.

Residual glucose concentration: Residual glucose concentration in the fermented broth was quantified by HPLC (UHPLC Ultimate 3000, Thermo Fisher Scientific, USA). After centrifuging at 5000 × g for 10 min, the supernatants were filtrated through a 0.2 µm cellulose acetate membrane. The HPLC was performed using a 300 × 7.8 mm Aminex HPX-87 H (Aminex) equipped with a refractive index detector (Refractomax 520; Dataapex). The column temperature was controlled at 60 °C, and a 5 mM H_2SO_4 solution was used as the mobile phase at a flow rate of 0.6 mL min^{-1}.

L-phe and tCA quantifications: Cells were pelleted by centrifugation at 5000 × g for 10 min. The resulting supernatants were then diluted and filtered through 0.2 µm syringe filters. HPLC (UHPLC Ultimate 300, Thermo Fisher Scientific, USA) equipped with a Zorbax Eclipse Plus C18 column (100 × 4.6 mm, 5 µm; Agilent Technology, USA) was used to quantify the concentration of L-phe and *t*CA. The column temperature was maintained at 40ºC. For L-phe analysis, the mobile phase was set at 1 mL min^{-1} for 12 min with 90% of 0.1% (v/v) trifluoroacetic acid (A) in water and 10% of acetonitrile (B). The L-phe was detected using a UV detector at 260 nm. The standard curve of L-phe is shown in Supplementary Fig. 1. The analysis of *t*CA was performed using the same flow rate using a gradient method of mobile phases as follows: (i) equilibrate with 10% B for 1 min, (ii) run gradient from 10 to 70% B for 19 min, (iii) maintaining flow at 70% for 5 min, (iv) run gradient from 70 to 10% B for 3 min, and (v) wash with 10% B for 5 min. The samples were detected using a UV detector at 280 nm. The standard curve of *t*CA is shown in Supplementary Fig. 2.

Statistical analysis of experimental data: All data, except the *t*CA liquid-liquid extraction, were expressed as means ± SD of three biological replicates. One-way analysis of variance (ANOVA) was performed, and Tukey's HSD test was conducted to evaluate statistical differences using OriginPro version 2023 (OriginLab Corporation, USA). Data were considered statistically significant at p-values ≤ 0.05.

Results

Enhancement of *t*CA production performance through decoupled recycling system

The decoupled bioprocess using a whole-cell biocatalyst of recombinant *P. putida* KT2440 for *t*CA production provided a high L-phe conversion, resulting in a high concentration and production rate (Q_p) in the first cycle (Fig. 2a). However, further investigation was needed to optimize performance metrics and fully utilize the cell's high capability to convert substrate into product. In this study, the recycling of whole-cell biocatalysts and supernatants, after removing *t*CA using a precipitation-based technique, was evaluated to initiate the *t*CA biotransformation in subsequent production cycles. The evaluation was conducted using the whole-cell biocatalyst concentrations at 30 and 40 g_{DCW} L^{-1}, along with L-phe concentrations of 25 and 35 g_{L-phe} L^{-1}. As shown in Fig. 2a, the biotransformation process performed by whole-cell biocatalysts of 30 g_{DCW} L^{-1} resulted in similar *t*CA concentrations and production rates for each L-phe concentration across the first four cycles. However, both values decreased continuously by 34.6% for 25 g_{L-phe} L^{-1} and 27.66% for 35 g_{L-phe} L^{-1} from the fifth to the eighth cycle. These results were consistent with the L-phe conversion, which decreased from 95 to 65% conversion (Supplementary Fig. 3). Notably, the *t*CA production process with 40 g_{DCW} L^{-1} biocatalyst showed an insignificant difference in *t*CA concentration and production rate compared to the 30 g_{DCW} L^{-1} with 35 g_{L-phe} L^{-1} in the first four cycles. However, this process maintained a similar L-phe conversion for the first five cycles, followed by a decrease of approximately 27% in the final cycle.

Considering the overall *t*CA production through eight cycles of the decoupled recycling bioprocess to evaluate the overall TRY metrics, increasing whole-cell biocatalyst and L-phe concentration markedly promoted *t*CA production (Fig. 2b). Therefore, the highest accumulated *t*CA concentration of 143 g L^{-1} was achieved with a biocatalyst of 40 g_{DCW} L^{-1} and an L-phe concentration of 35 g_{L-phe} L^{-1}. However, the overall production rate over the total time (overall Q_p) and the overall *t*CA yield on glucose ($Y_{p/s1}$) showed the closed values between the processes using 35 g_{L-phe} L^{-1} of L-phe with biocatalyst concentration of either 30 or 40 g_{DCW} L^{-1}. Consequently, other kinetic parameters related to biocatalyst efficiency and substrate conversion efficiency were considered to determine the most suitable process. As shown in Table 1, the process using a biocatalyst of 40 g_{DCW} L^{-1} demonstrated significantly higher overall L-phe conversion, overall *t*CA yield on L-phe ($Y_{p/s2}$), and overall specific *t*CA yield on biomass ($Y_{p/x}$) than the process using 30 g_{DCW} L^{-1} ($p < 0.05$). Therefore, the decoupled recycling bioprocess with a whole-cell biocatalyst of 40 g_{DCW} L^{-1} and L-phe concentration of 35 g_{L-phe} L^{-1} was selected for further study.

The performance of *t*CA liquid-liquid extraction

*t*CA is a natural product that is toxic to microorganisms and exhibits competitive product inhibition of the PALs enzyme, leading to reduced enzyme activity[11,35]. Although *t*CA could be separated from the supernatant by reducing the pH followed by centrifugation, liquid-liquid extraction is an alternative strategy for extracting *t*CA from the supernatant and mitigating product inhibition. Four biocompatible and environmentally friendly solvents (bis(2-ethylhexyl) adipate, ethyl oleate, isopropyl myristate, and tributyrin) were investigated for *t*CA extraction at different pH. The results showed that reducing the pH from 8.5 (optimal pH for RmXAL activity) to 2.5 significantly enhanced *t*CA distribution into the extractant phase, as indicated by an increase in Log (K_d) (Fig. 3a). Moreover, the negative Log (K_d) values detected at pH 6.8 and 8.5 indicated that *t*CA was predominantly present in the aqueous phase. This might be preliminarily explained by the fact that the carboxylic acid moiety of *t*CA remains deprotonated at these pH levels[34]. Under acidic conditions, tributyrin exhibited Log (K_d) values approximately 1.5-fold higher than the other solvents. Notably, liquid-liquid extraction of *t*CA using tributyrin at pH 2.5 gave the highest Log (K_d) of 2.45 ± 0.11, making it the most efficient solvent for *t*CA extraction at high concentrations.

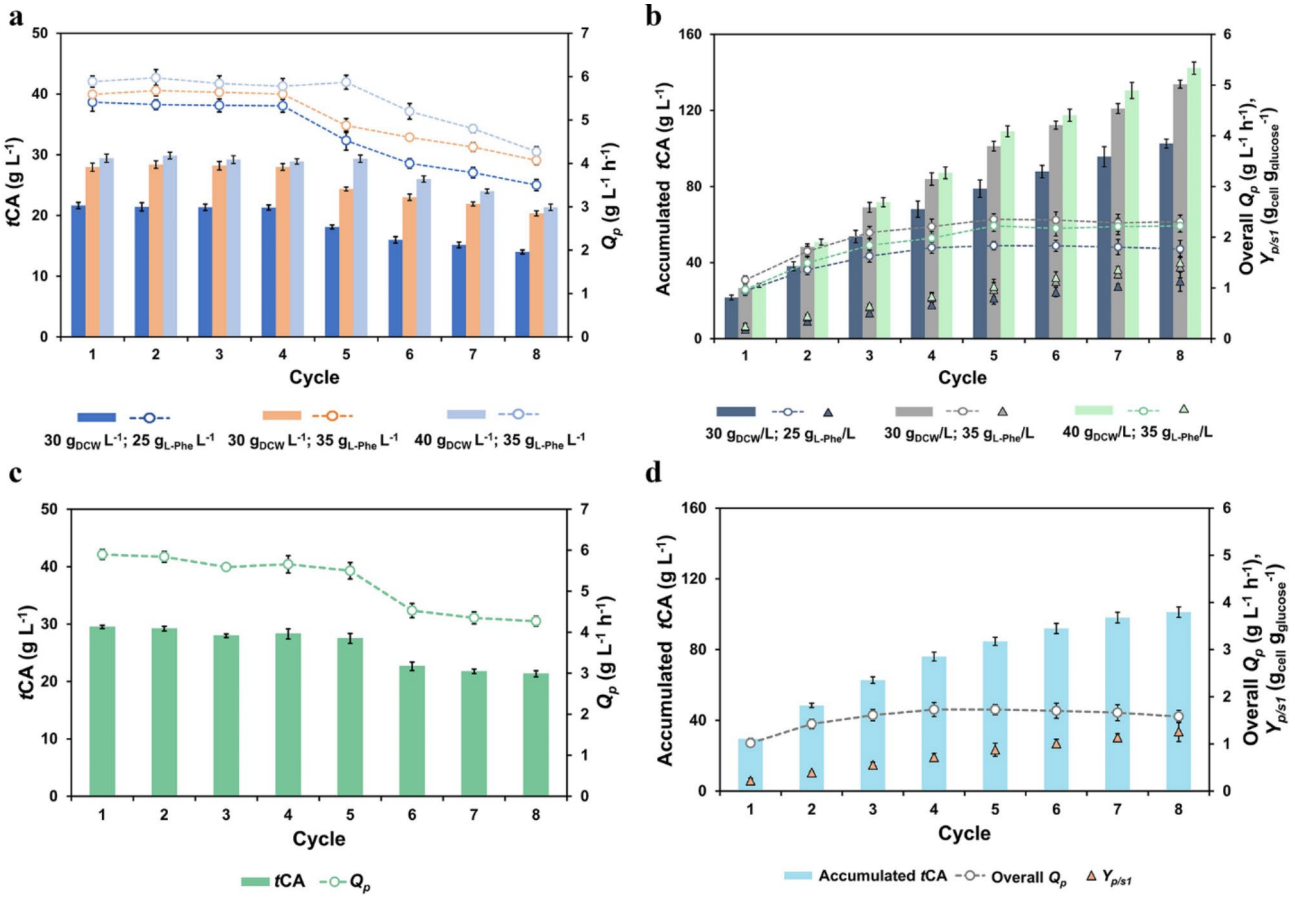

Fig. 2. Decoupled recycling bioprocess for *t*CA production using different product removal strategies. (**a**) *t*CA concentration (bar) and production rate (circle) for each cycle using the precipitation-based method. (**b**) Accumulated *t*CA concentration (bar), overall production rate (overall Q_p; circle) and *t*CA yield on glucose ($Y_{p/s1}$; triangle) of the precipitation-based method. (**c**) *t*CA concentration (bar) and production rate (circle) for each cycle using the liquid-liquid extraction method. (**d**) Accumulated *t*CA concentration (bar), overall Q_p (circle) and $Y_{p/s1}$ (triangle) of the liquid-liquid extraction method. The data represents the mean ± SD, $n = 3$ (biological replicates).

Parameters	Whole-cell biocatalyst concentration (g_{DCW} L^{-1})	
	30	40
Overall L-phe conversion (%)	52.57 ± 1.81^b	71.82 ± 2.65^a
Overall $Y_{p/s2}$ (g *t*CA g L-phe^{-1})	0.44 ± 0.02^b	0.64 ± 0.02^a
Overall $Y_{p/x}$ (g *t*CA g cell^{-1})	4.12 ± 0.18^b	4.51 ± 0.11^a
Overall q_p (g *t*CA g cell^{-1} h^{-1})	0.11 ± 0.00^a	0.11 ± 0.01^a

Table 1. Comparison of whole-cell biotransformation performance with different biocatalyst concentrations using 35 g L^{-1} of L-phenylalanine. All data are presented as means ± SD ($n = 3$ biological replicates). Values marked with different superscript letters within the same row were significantly different ($p < 0.05$). The exact *p*-values for Overall L-phe conversion (%), Overall $Y_{p/s2}$, and Overall $Y_{p/x}$ are 0.0005, 0.0003 and 0.033, respectively.

To identify the optimal ratio of tributyrin/supernatant for *t*CA extraction, aimed at efficiently removing *t*CA from the aqueous phase and maximizing supernatant recovery for use in subsequent *t*CA production cycles, the various ratios of 1:1, 1:2, 1:5, 1:10, 1:20, and 1:30 (tributyrin/supernatant volume) were tested and compared to the control (the sample without in-situ extraction). As shown in Fig. 3b, the residual *t*CA in the aqueous phase increased from 0.5 to 2.6% as the ratio increased from 1:1 to 1:20. Moreover, a significant increase was observed in the ratio of 1:30, where residual *t*CA reached 8.1%. Indeed, the control resulted in the highest residual *t*CA in the aqueous phase despite the potential for *t*CA precipitation at low pH. This approach not only accounts

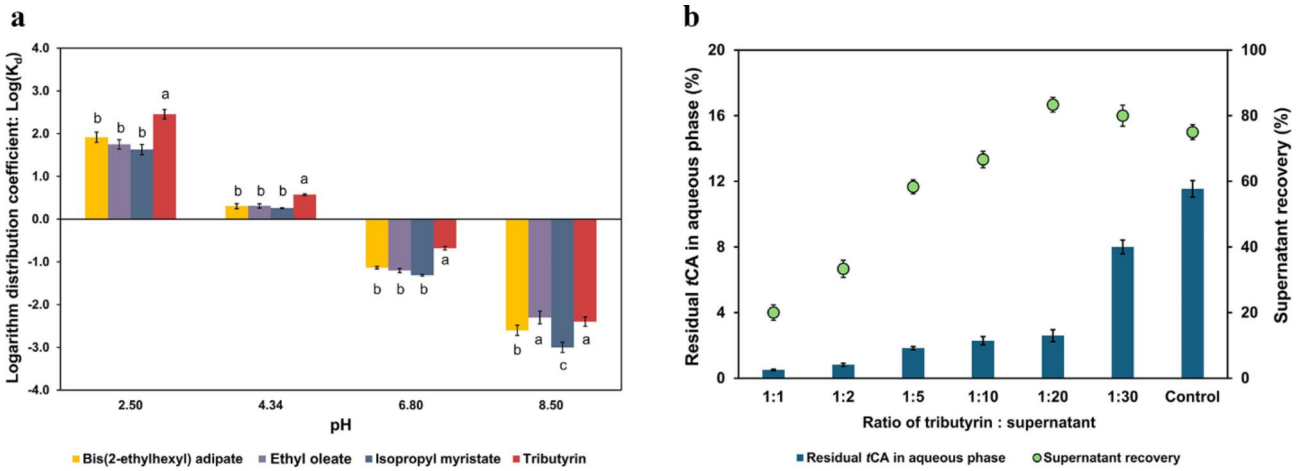

Fig. 3. *t*CA liquid-liquid extraction performance. (**a**) *t*CA distribution coefficient (Log (K_d)) across different solvents and pH. (**b**) the efficiency of tributyrin in *t*CA extraction. All data are presented as mean values with SD ($n = 3$ technical replicates). Statistical significance is indicated with different letters (p-values < 0.05). The exact p-values for the pH of 2.50, 4.34, 6.50, and 8.50 using different extractants are 0.0001, 0.0030, 0.0001, and 0.0002, respectively.

for the low *t*CA concentration in the aqueous phase after extraction, which positively impacts PAL activity by relieving product inhibition in the subsequent cycles, but also maximizes recovers supernatant for reuse in the following cycles, minimizing fresh media added in the production *t*CA process. Increasing the tributyrin/supernatant volume ratio from 1:1 to 1:20 enhanced supernatant recovery by approximately 4.3-fold. However, the supernatant recovery decreased by about 1.1-fold at the ratio of 1:30 and in control (Fig. 3b). Based on these results, a tributyrin/supernatant volume ratio of 1:20 showed low residual *t*CA in the aqueous phase, which did not affect subsequent *t*CA production, and maximized supernatant recovery. Therefore, this ratio was used for the *t*CA extraction produced in the bioreactor.

Integrated decoupled recycling bioprocess and liquid-liquid extraction system for *t*CA production

The production of *t*CA using a cell and supernatant recycling process, where *t*CA was removed from the supernatant by centrifugation, was described in a decoupled recycling system. Alternatively, *t*CA removal can be accomplished through a chemical method, specifically liquid-liquid extraction. Notably, adjusting the pH of the supernatant beneath the pK_a of *t*CA (4.37) promoted efficient *t*CA extraction into the solvent phase. However, *t*CA could shift from the solvent phase to the aqueous phase when the pH was adjusted to 8.5, which is the optimal pH for RmXAL activity, as shown in Supplementary Fig. 4. Consequently, an external-direct product recovery was implemented for *t*CA production in combination with a decoupled recycling system, as illustrated in Fig. 1c. The results showed that *t*CA production proceeded at a consistent production rate and reached a similar concentration across the first five cycles. However, both production values sharply decreased by approximately 23% after the fifth cycle, followed by a slight decline until the production process was completed (Fig. 2c). Considering the overall performance metrics of this process, accumulated *t*CA concentration increased 2.9-fold from the initial cycle to the fifth cycle, aligning with the results from each production cycle, and the accumulated *t*CA concentration reached 101.20 g L^{-1} by the final cycle (Fig. 2d). Interestingly, even though the overall *t*CA yield on glucose ($Y_{p/s1}$) steadily increased throughout the production cycle, the overall production rate (overall Q_p) slightly decreased after the fifth cycle.

Comparative water use efficiency and *t*CA production performance

The *t*CA production using a decoupled recycling bioprocess proved to be the most effective method to enhance key performance metrics, including titer, production rate, and yield. Interestingly, the two different methods for removing *t*CA from the supernatant gave similar *t*CA production patterns across each cycle, as well as the cumulative amount of *t*CA (Fig. 4a). Additionally, the kinetic parameters related to whole-cell biotransformation efficiency, which are overall L-phe conversion, overall product yield on L-phe ($Y_{p/s2}$), overall specific *t*CA yield on biomass ($Y_{p/x}$) and overall specific *t*CA production rate (q_p), showed insignificant difference between the two *t*CA removal techniques (Table 2). These findings suggest that using tributyrin for *t*CA liquid-liquid extraction does not negatively impact RmXAL activity.

However, the accumulated *t*CA concentration derived from the precipitation-based removal technique was 40.5% higher than the liquid extraction system (Fig. 4b). Similarly, the overall *t*CA production rate over the total time (overall Q_p) showed significant differences between the two *t*CA removal methods. The primary reason is that precipitation-based *t*CA removal allows better supernatant recovery compared to the liquid extraction method, reducing the need to add fresh media to maintain the initial volume in each cycle, as shown in Table 2. Consequently, the overall *t*CA yield on water used in the bioprocess (overall $Y_{p/H2O}$) of the production process

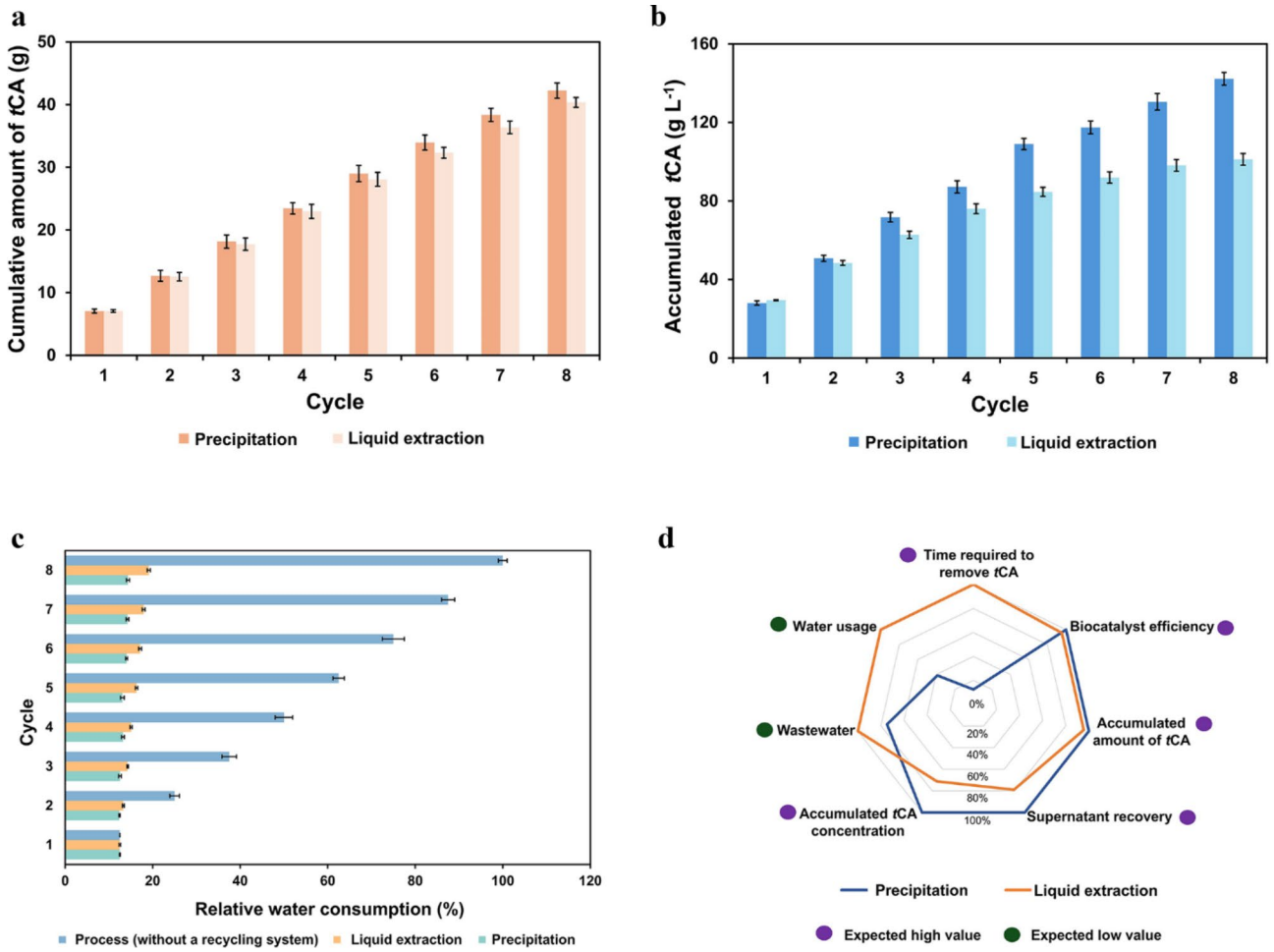

Fig. 4. Comparison of *t*CA production and water consumption using different product removal methods. (**a**) Cumulative amount of *t*CA. (**b**) Accumulated *t*CA concentration. (**c**) relative water consumption of decoupled bioprocess with and without the supernatant recycling system. (**d**) The efficiency metrics of the overall operating process for *t*CA production. The data are presented as means ± standard deviation ($n = 3$ biological replicates for the precipitation method and technical replicates for the liquid extraction method).

Parameters	*t*CA removal technique	
	Precipitation	Liquid-liquid extraction
Overall L-phe conversion (%)	71.82 ± 2.65[a]	68.68 ± 1.68[a]
Overall $Y_{p/s2}$ (g *t*CA g L-phe^{-1})	0.64 ± 0.02[a]	0.62 ± 0.01[a]
Overall $Y_{p/x}$ (g *t*CA g cell^{-1})	4.51 ± 0.11[a]	4.31 ± 0.13[a]
Overall q_p (g *t*CA g cell^{-1} h^{-1})	0.11 ± 0.01[a]	0.11 ± 0.01[a]
Overall $Y_{p/s1}$ (g *t*CA g glucose^{-1})	1.50 ± 0.13[a]	1.26 ± 0.05[a]
Overall Q_p (g *t*CA L^{-1} h^{-1})	2.25 ± 0.10[a]	1.57 ± 0.07[b]
Overall $Y_{p/H2O}$ (g *t*CA g water^{-1})	0.12 ± 0.01[a]	0.09 ± 0.00[a]
Fresh medium added (%)	23.75 ± 2.56[a]	65.83 ± 2.10[b]

Table 2. Comparative production parameters of whole-cell biotransformation by Recombinant *P. putida* KT2440 under decoupled recycling process with different *t*CA removal techniques. All data are presented as means ± SD ($n = 3$ biological replicates). Values marked with different superscript letters within the same row were significantly different ($p < 0.05$). The exact *p*-values for *overall* Q_p and fresh medium added are 0.0009 and 0.0001, respectively.

Production strategies	Fresh medium added per production cycle (mL)*								Total fresh medium added from cycle 2–8 (mL)	Total fresh medium used (mL)	Absolute water-saving (mL)**
	1	2	3	4	5	6	7	8			
A	300	8	9	9	10	11	10	12	69	369	1996
B	300	25	22	23	26	26	26	30	178	478	1887
C	300	295	295	295	295	295	295	295	2065	2365	-

Table 3. Summary of fresh medium added and total volume of fresh medium used in the processes with different *t*CA production strategies. A represents the decoupled bioprocess integrated with a precipitation-based method. B represents the decoupled bioprocess integrated with a liquid extraction-based method. C represents the standard production without a supernatant recycling system. * The initial volume for each biotransformation cycle is 300 mL. ** Absolute water-saving of A and B is compared to C. The values are presented as mean volumes with a standard deviation (SD) of less than 2%.

using precipitation-based *t*CA removal was 33% higher than the liquid extraction method (Table 2). This efficiency in volume control contributes to the higher production process performance compared to the liquid extraction system. Notably, the decoupled recycling bioprocess resulted in a substantial reduction in total water consumption by approximately 85% compared to the *t*CA standard production process without a supernatant recycling system (Fig. 4c; Table 3). This reduction positively impacts freshwater conservation efforts and lessens the burden on wastewater treatment processes. Based on these results, the decoupled recycling bioprocess with precipitation-based *t*CA removal has proven a practical approach for *t*CA production.

Discussion

Enhancing performance metrics while minimizing water consumption of *t*CA production bioprocess through whole-cell biotransformation presents significant challenges compared to traditional chemical synthesis, which ordinarily offers a higher yield and fast production rate. However, there is growing support for more sustainable *t*CA production by whole-cell biocatalysts-based processes due to their lower environmental impact, high catalytic efficiency, renewable resource utilization, and easier handling[36–38]. To establish a viable sustainable *t*CA production platform suitable for industrial applications, this study developed a decoupled recycling bioprocess of recombinant *P. putida* KT2440 expressing RmXAL for *t*CA production by utilizing a dual recycling strategy, reusing both cells and supernatants, combined with a product removal technique.

The optimization of whole-cell biocatalyst concentration, recycling times, and substrate loading was initially evaluated for cell recycling to utilize the cell's high catalytic capability. Concurrently, *t*CA was removed from the supernatant to alleviate product inhibition, enabling its use in subsequent production batches. Therefore, an essential step in this process involved reducing the pH of the supernatant below the pK_a of *t*CA (4.37), resulting in *t*CA precipitation, which could then be effectively separated by centrifugation[39,40]. This aligns with Zang et al.[24], who reported that pH values significantly influenced the *t*CA concentration in the reaction supernatant, and *t*CA solubility was lower when the pH of the solution was close to the pK_a of *t*CA.

The biotransformation using 40 g_{DCW} L^{-1} of whole-cell biocatalysts and 35 g_{L-phe} L^{-1} of L-phe provided the highest efficiency (Fig. 2a, b; Table 1). These results are consistent with Law et al.[41], who observed that increasing biocatalysts led to higher product titers and production rates. However, further increases in biocatalyst concentration did not improve production because of some limitations, such as product inhibition, oxygen availability, and other process constraints[42]. The cell recycling process offers an alternative approach to improve the entire product yield from carbon sources, especially in decoupled bioprocesses because cells consume the carbon source primarily during the growth phase but can be reused for multiple production cycles, leading to more efficient resource utilization. This method significantly reduces time and OpEx, including costs associated with culture medium and inoculum preparation, as well as the number of operational units required. Additionally, it minimizes waste emissions compared to single-batch operations[43–45]. These advantages make the process a more cost-effective and sustainable option for industrial-scale production.

Another technique to remove *t*CA from the supernatant is liquid-liquid extraction. Four organic solvents were compared for *t*CA extraction under varying pH, with tributyrin at pH 2.5 showing the highest Log (K_d). The pH plays an important role not only in microbial growth or enzyme activity but also in extraction performance[37]. The acidic pH promoted *t*CA extraction, which might be explained by the protonation of the *t*CA carboxylic acid moiety[34]. In this case, reducing carbon chain length (from C22 to C15) increased the solvent's capability to extract *t*CA due to the simplicity of forming the solvation shell around *t*CA molecules. The finding is supported by a previous study indicating that pure tributyrin showed an effective extraction of *trans*-cinnamaldehyde, exhibiting a high distribution coefficient. Notably, a tributyrin/supernatant ratio of 1:20 indicated the solvent's high *t*CA extraction efficiency, in which a low concentration of *t*CA was detected in the aqueous phase (data not shown). Furthermore, the benefits of a high ratio of extraction not only reduce the OpEx but do not affect enzyme activity, as observed by a similar production profile of each cycle (Fig. 2a, c) and cumulated amount of *t*CA (Fig. 4a) in alignment with a previous report which showed that tributyrin displayed excellent biocompatibility, as relative optical density maintained above 99% after the ISPR process[28].

Although the external-direct liquid-liquid extraction process was effective and positively impacted biocatalyst performance, in analogy to the precipitation-based method (Table 2), the accumulated *t*CA concentration, overall *t*CA yield on water used in the bioprocess and overall production rate differed significantly. This difference was

primarily due to lower supernatant recovery, which necessitated the addition of fresh medium for subsequent *t*CA production cycles. As a result, water usage increased, and wastewater treatment became more demanding (Fig. 4d), ultimately leading to higher production costs and wastewater management challenges in industrial production processes[4,6,46,47]. The liquid extraction system also required more time to remove *t*CA from the supernatant via precipitation, negatively impacting the overall operation time and elevating liquid waste volume. Additionally, *t*CA products derived from the liquid extraction might need further downstream steps to remove tributyrin, such as vacuum distillation. Despite these challenges, the decoupled recycling bioprocess showed a significant reduction in water consumption compared to the process without the supernatant recycling system (Fig. 4c), indicating highly efficient water usage throughout the production process. By optimizing water utilization, this bioprocess not only supports environmental goals but also sustains high productivity, making it a promising alternative for industrial applications.

Based on these results, the decoupled recycling bioprocess integrated with precipitation-based *t*CA removal shows the most practical approach for a sustainable *t*CA production process to increase performance metrics, high water consumption efficiency, and minimizing the amount of wastewater generated throughout the process. Furthermore, this production strategy aligns with Sustainable Development Goal (SDG) 6 "Clean water and sanitation", substantially increasing water-use efficiency through water recycling, and SDG 12 "Responsible production and consumption", through sustainable management and use of natural resources. Thereby, this approach enables an industrial production platform to achieve better environmental and economic sustainability.

Conclusion

In this study, a decoupled recycling bioprocess was developed to enhance performance metrics, increase water use efficiency, and minimize wastewater generation for *t*CA production. A whole-cell biocatalyst concentration of 40 g_{DCW} L^{-1} of recombinant *P. putida* KT2440 expressing RmXAL and an L-phenylalanine feed concentration of 35 g_{L-phe} L^{-1} integrated with product precipitation showed the best condition. This approach resulted in a titer of 143 g L^{-1}, a production rate of 2.25 g L^{-1} h^{-1}, at a yield of 1.50 g g $glucose^{-1}$ after eight recycles. Moreover, this process significantly reduced freshwater consumption, achieving an 85% reduction compared to a coupled bioprocess system. These advancements align with the principles of SDG 6 "Clean water and sanitation" and SDG 12 "Responsible production and consumption", paving the way for an industrial bioproduction platform that promotes both environmental and economic sustainability.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information.

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Author contributions

S.A., S.I.J., and J.M.W. performed research design, article revision, and approved the submitted version. S.A. conducted fermentation, data analysis, and article writing. J.M.W. contributed the conceptualization and final article completion.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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