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ARTICLE

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Strain evolution and novel downstream processing with integrated catalysis enable highly efficient coproduction of 1,3-propanediol and organic acid esters from crude glycerol

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Abstract

Bioconversion of natural microorganisms generally results in a mixture of various compounds. Downstream processing (DSP) which only targets a single product often lacks economic competitiveness due to incomplete use of raw material and high cost of waste treatment for by-products. Here, we show with the efficient microbial conversion of crude glycerol by an artificially evolved strain and how a catalytic conversion strategy can improve the total products yield and process economy of the DSP. Specifically, *Clostridium pasteurianum* was first adapted to increased concentration of crude glycerol in a novel automatic laboratory evolution system. At m^3 scale bioreactor the strain achieved a simultaneous production of 1,3-propanediol (PDO), acetic and butyric acids at 81.21, 18.72, and 11.09 g/L within only 19 h, respectively, representing the most efficient fermentation of crude glycerol to targeted products. A heterogeneous catalytic step was developed and integrated into the DSP process to obtain high-value methyl esters from acetic and butyric acids at high yields. The coproduction of the esters also greatly simplified the recovery of PDO. For example, a cosmetic grade PDO (96% PDO) was easily obtained by a simple single-stage distillation process (with an overall yield more than 77%). This integrated approach provides an industrially attractive route for the simultaneous production of three appealing products from the crude glycerol fermentation broth, which greatly improve the process economy and ecology.

KEYWORDS

automatic adaptive evolution, *Clostridium pasteurianum*, coproduction, 1,3-propanediol, organic acid esters

Abbreviations: ALE, adaptive laboratory evolution; DSP, downstream processing; HPLC, high performance liquid chromatography; MA, methyl acetate; MB, methyl butyrate; PDO, 1,3-Propanediol.

1 | INTRODUCTION

1,3-propanediol (PDO) is a vital value-added monomer which is wildly used in plastics industry for synthesizing polytrimethylene terephthalate (PTT), polytrimethylene naphthalate (PTN), polytrimethylene isophthalate (PTI), and thermoplastic polyester elastomer (Fokum et al., 2021; Sun, Ren, et al., 2018). It also has extensive applications for manufacturing cosmetics, foods, medicines, detergents, solvents, glues, and resins (Sabra et al., 2015). The global PDO market is expected to reach $776.3 million in 2022, with a compound annual growth rate (CAGR) of 10.4% from 2014 to 2022 (Zabed et al., 2019). Currently, PDO can be produced by both chemical and biological synthesis. Owing to advantages such as mild reaction conditions, and using renewable bio-substrates, the microbial production of PDO has gained growing interest in both academic and industrial fields.

In particular, one promising route for the efficient and safe bio-production of PDO is the anaerobic fermentation of glycerol using the nonpathogenic strain *Clostridium pasteurianum* (Groeger et al., 2016; Jensen et al., 2012; Kaeding et al., 2015). Previously, our group has successfully demonstrated the production of PDO by a newly isolated low-butanol-producing strain *C. pasteurianum* K1. In a fed-batch fermentation of crude glycerol at up to 1 m^3 pilot scale, a final PDO titer of 55 g/L, a yield of 0.52 g/g and an overall productivity of 2.3 g/L/h were achieved (Kaeding et al., 2015). The whole process was done under nonsterile conditions without N_2 aeration and without using yeast extract in the medium, which demonstrated the robustness and cost-effectiveness for the PDO production by *C. pasteurianum*. However, to avoid possible growth inhibition caused by the toxicity of crude glycerol, low initial glycerol concentration (<30 g/L) in the medium and low glycerol feeding rate (<20 g/L/h) were used in the fermentation, which highly limited the final PDO titer and productivity. As a by-product of biodiesel production, crude glycerol is cheap and abundant for the economical bioconversion of PDO (Laura et al., 2020), but it also contains a lot of impurities such as high degree of unsaturated fatty acids and soluble proteins which are reported to have strong inhibitory effect on the cell growth and glycerol utilization of *C. pasteurianum* (Samul et al., 2014; Venkataramanan et al., 2012). The low tolerance of the strain to crude glycerol is a major bottleneck hindering a high-level PDO bio-production. One solution to this bottleneck could be the metabolic engineering for direct strain improvement. However, the lack of adequate genetic engineering tools still limits its application on *C. pasteurianum* (Jensen et al., 2012; Schmitz et al., 2019). As an alternative, adaptive laboratory evolution (ALE) that mimics the natural adaptation of microorganisms to the artificial stress in the lab, could be helpful (Liang et al., 2020). Briefly, ALE subjects targeted strain to repeated or continuous cultivation under stress conditions for many generations to improve tolerance toward inhibiting environmental conditions (Y. Q. Sun, Shen, et al., 2018). In recent years, many ALE experiments have been reported to improve the crude glycerol tolerance of the microbes for enhanced PDO production (Szymanowska-Powałowska, 2015; A. H. Zhang et al., 2019). For example, a highly crude glycerol-tolerant *Clostridium butyricum* was selected after more than 50 repeated cultivations in the crude glycerol containing medium (crude glycerol concentration from 30 to 110 g/L) (A. H. Zhang et al., 2019). Fermentation with the evolved strain led to the overall productivity of PDO increasing from 0.97 to 2.14 g/L/h by 114%. However, an obvious disadvantage of the traditional ALE experiment is that it requires the passaging of cells manually for many generations to enrich favorable genetic changes at the expense of a lot of manpower. Moreover, the timing of cell passage in ALE is usually not well-determined due to the lack of real-time monitoring of cell growth (Alves et al., 2021; A. H. Zhang et al., 2019). Therefore, it is necessary to develop a smart ALE device which can regulate the cell growth and conduct long-term ALE in an automatic manner for achieving better and more stable crude glycerol tolerance of *Clostridium pasteurianum*.

Another bottleneck affecting the process economy of PDO bio-production from crude glycerol by *C. pasteurianum* is that the oxidation and reduction reactions of glycerol will take place simultaneously in the strain to maintain the redox and energy balance (Sabra et al., 2016). As a result, the conversion of glycerol to PDO will be inevitably accompanied by the formation of different types of organic acids, which leads to a theoretical maximum product yield lower than 0.72 mol PDO/mol glycerol (Dietz & Zeng, 2014). Specifically, acetic acid and butyric acid are the major by-products of glycerol metabolism in *C. pasteurianum* (Sabra et al., 2014; C. Zhang et al., 2021). In this case, one strategy that can further improve the process economy could be the coproduction of PDO and organic acids. In fact, many coproduct processes have been developed recently for reducing the production cost of PDO from glycerol fermentation (Groeger et al., 2016; Huang et al., 2017; Suppuram et al., 2019). For instance, Huang et al. constructed a recombinant strain of *Corynebacterium glutamicum* and creatively developed a coproduction process for PDO and glutamate (Huang et al., 2017). The reducing equivalents generated during glutamate fermentation can be recycled for PDO production, which resulted in the maximum yield of 1.0 mol PDO/mol glycerol and 1.0 mol glutamate/mol glucose. Glutamate can be easily separated from PDO by acidification and crystallization in the fermentation broth. Another study done by Groeger et al. reported the coproduction of PDO and butanol by the high-butanol producing strain *C. pasteurianum* DSM 525 (Groeger et al., 2016). With a cosubstrate fermentation strategy (glucose + glycerol) and gas stripping for in situ butanol removal, 53.7 g/L PDO and 39.2 g/L butanol can be simultaneously produced and separated. However, to date, very few studies have focused on the integrated process for the coproduction of PDO and organic acids due to the difficulty of complete acid removal from PDO (C. Zhang et al., 2021). The fiber grade PDO for high-quality polymer synthesis requires a purity > 99.5% without any organic acid residue (Kurian, 2005). To overcome this challenge, several industrially applicable methods have been proposed to separate the organic acids from fermentation broth, such as ion exchange (Adkesson et al., 2011), electro-dialysis (Gong et al., 2006; Wu et al., 2011), and two-phase salting-out extraction (Li et al., 2019; Song et al., 2013). However, these methods produce a large amount

of waste water containing the medium salts and organic acid salts (C. Zhang et al., 2021), which reduces the economic interest to recover the organic acids from the waste water.

Recent research from our group has demonstrated that acetate and butyrate in ammonium form can be completely separated from PDO and partially recovered from fermentation broth without producing any high-salt waste water (C. Zhang et al., 2021). The process involves (1) ultrafiltration to remove cell and protein; (2) evaporation to remove water; (3) vacuum distillation of PDO and organic acids under glycerol suspension, where the free acids are liberated; (4) a second vacuum distillation to recover the free acids from PDO. Finally, alkaline hydrolysis to eliminate the PDO esters impurities, and the rectification of highly pure PDO were performed. Although the obtained PDO purity was over 99.6% with a satisfactory overall yield more than 76%, the total recovery yield of acetic and butyric acid were less than 50%. More than half of the acids were lost in water evaporation step and in the esterification with PDO. Therefore, further smart design of downstream processing (DSP) to improve the recovery yield of both PDO and organic acids from crude glycerol fermentation broth is urgently needed.

In this study, a novel fully automatic ALE system capable of online monitoring of cell growth was firstly developed to perform a long-term crude glycerol adaption of *C. pasteurianum*. This system can significantly increase the number of adaptation cycles during the ALE and thus shorten the time for obtaining a stable improved phenotype compared with the traditional manual adaption process. The improved PDO production by the adapted strain was further confirmed in nonsterilized fed-batch fermentations of crude glycerol using a simple medium without yeast extract. Moreover, a new DSP for the coproduction of PDO, methyl acetate (MA) and methyl butyrate (MB) from the crude glycerol fermentation broth was proposed for the first time. By dissolving the concentrated broth in acidified methanol, followed by adding a heterogeneous catalyst Amberlyst 15, the medium salt can be separated due to salt crystallization, and the free acetic and butyric acid can be esterified with methanol simultaneously. The novelty of this strategy lies on the significant reduction in both the boiling points of the acids and the reactivity of the acids with PDO by converting the free acids to their corresponding methyl esters. The integrated catalytic conversion step is crucial for achieving high recovery yield for both PDO and organic acids from the crude glycerol fermentation broth.

2 | MATERIALS AND METHODS

2.1 | Materials

Crude glycerol was obtained from PT Musim Mas (Indonesia). It contained by weight 80% glycerol, 10.41% water, 3.9% ash, 2.21% nonglycerol organic matter, and 3.54% salts. The pH value of the crude glycerol was 6.8. The acidic ion exchange resin (Amberlyst-15) was purchased from Rohm and Haas Company, which has a structure of macroporous polystyrene crosslinked with divinylbenzene. The resin has a dry weight capacity (min.) of 5.2 eq/kg (H^+ form), moisture retention of 51%–59% (H^+ form), and surface area of 20–40 m^2/g. Pure glycerol (≥98%), standards of 1,3-PDO (≥98%), MA (≥99%), and MB (≥99%), and all other reagents of analytical grade were commercially purchased from Carl Roth (Germany).

2.2 | Microorganism, media, and fermentation conditions

A wild-type strain of *C. pasteurianum* which was previously isolated from an active sludge sample and proven to have significantly low butanol production by our group (Kaeding et al., 2015) was further purified by isolating a single colony from a cyro stock solution maintained at −80°C for several years. We checked the identity of the purified strain using 16 S rDNA analysis again, and the analysis using National Center for Biotechnology Information-The Basic Local Alignment Search Tool showed that it has a high similarity (>99%) to the *C. pasteurianum* type strain DSM 525 (ATCC 6013). This purified strain termed as *C. pasteurianum* C8 was used for the fermentations and adaptive evolution in this study and restored at −80°C in 2-ml cryo vials containing 75% active culture and 25% glycerol (v/v).

Seed cultures for fermentation experiments were prepared in anaerobic bottles as previously described by Kaeding et al. (2015). The medium for all precultivations consisted of (per liter of water): 40 g pure/crude glycerol; 3 g $(NH_4)_2SO_4$; 0.75 g KCl; 2.45 g NaH_2PO_4; 4.58 g Na_2HPO_4; 0.28 g Na_2SO_4; 0.42 g citric acid; 0.2 g L-cysteine; 0.024 mg biotin; 0.015 mg calcium pantothenate; 5 mg $FeSO_4 \cdot 7H_2O$; 0.26 g $MgCl_2 \cdot 6H_2O$; 3 mg $CaCl_2 \cdot 2H_2O$; 2 g/L $CaCO_3$; 2 ml trace element solution (70 mg/L $ZnCl_2$; 100 mg/L $MnCl_2 \cdot 4H_2O$; 200 mg/L $CoCl_2 \cdot 6H_2O$; 20 mg/L $CuCl_2 \cdot 2H_2O$; 35 mg/L $Na_2MoO_4 \cdot 2H_2O$; 25 mg/L $NiCl_2 \cdot 6H_2O$; 60 mg/L H_3BO_3; 0.9 mL/L 37% HCl). After a two-step cultivation procedure (first overnight and second 12 h at 35°C), 100 ml of seed culture with an OD_{600} value over 4.0 were inoculated in a 2-L glass bioreactor (Eppendorf) filled with 1 L unsterilized fermentation medium to initiate the batch or fed-batch fermentations. The compositions of the fermentation medium was (per liter of water): 30–120 g pure/crude glycerol; 3 g $(NH_4)_2SO_4$; 0.5 g KH_2PO_4; 0.5 g K_2HPO_4; 0.42 g citric acid; 0.26 g $MgSO_4 \cdot 7H_2O$; 0.024 mg biotin; 0.015 mg calcium pantothenate; 20 mg $CaCl_2 \cdot 2H_2O$; 0.5 g cysteine hydrochloride$\cdot H_2O$; 100 mg $FeSO_4 \cdot 7H_2O$; 2 g/L $CaCO_3$; 2 ml trace element solution. It is worth mentioning that yeast extract was used neither in the seed culture medium nor in the fermentation medium.

Unless otherwise stated, fermentation experiments were conducted at 35°C with agitation at 200 rpm, and pH was controlled at 6.5 by automatic adjustment with 25% ammonia solution. To achieve anaerobic conditions, the medium was only degassed with 0.6 vvm N_2 for 30 min before the inoculation, and no sparging of N_2 was needed throughout the process after inoculation. The feeding to the fed-batch fermentations was done with a feeding solution containing 50% pure or crude glycerol depending on the experimental purpose. In fed-batch fermentations, continuous feeding was initiated when

the residue glycerol concentration was lower than 20 g/L, and the feeding rate was adjusted to maintain the glycerol concentration within the range of 10–20 g/L. In pulsed fed-batch fermentation, 80 g of 50% glycerol solution was added into the bioreactor three to four times, when the residue glycerol concentration was lower than 20 g/L.

Continuously fed-batch fermentation of crude glycerol using the same fermentation conditions described above was also carried out in a 1 m^3 stainless steel bioreactor (Frings). The initial volume of the fermentation medium containing 80 g/L crude glycerol was 500 L. The process consisted of three seed cultures in anaerobic bottles and two more in 10 L and 100 L fermenters. Finally, 50 L seed culture with an OD reached 6 was inoculated in the 1 m^3 fermenter to initiate the fed-batch fermentation.

2.3 | Initial tolerance test of *C. pasteurianum* C8 to crude glycerol

To evaluate the inhibition of crude glycerol, initial tolerance test was performed in batch culture. A cyro stock culture stored in −80°C was first grown in anaerobic bottle with the preculture medium described above which contained 30 g/L pure glycerol. Then the culture in exponential growth phase was inoculated into the preculture medium containing various concentrations of crude glycerol (10, 15, 20, 25, 30, 40, 60, 80 g/L). A preculture medium with pure glycerol (30 or 80 g/L) was also inoculated and used as control. After anaerobic cultivation at 35°C for 12 h, OD_{600} of the culture was determined and compared. All tests were conducted in triplicates.

2.4 | Automatic ALE

The automatic ALE experiment for high crude glycerol tolerance was carried out continuously in a novel system developed by our group. The working principle of the system is shown in Figure 1, and a photo of the whole instrument can be seen in Figure S1. After precultivation in pure glycerol medium, an active culture was inoculated into an anaerobic bottle with a starting crude glycerol concentration of 30 g/L. The initial OD was set at 0.5 by adjusting the inoculum size. Cultivation was maintained at 35°C with a constant stirring at 50 rpm using a magnetic stirring bar. To realize a real-time measurement of OD_{600}, 2 ml of the culture in the anaerobic bottle were automatically transferred to a photometer (Ultrospec 10, Biochrom) for OD detection every 2 min. After the OD value was recorded by the computer, the culture in the detection cuvette was transferred back to the anaerobic bottle to maintain a constant working volume of 50 ml. Once the OD_{600} reached 1.5, the inlet flow of fresh medium (with the same crude glycerol concentration) and the outlet flow (waste) of culture were initiated simultaneously to dilute the culture until the OD value was reduced back to 0.5, and then the next cycle of adaptation continued. The time interval (△T) for each adaptation cycle was recorded, and the concentration of crude glycerol in the fresh medium was increased by 10 g/L each time when the

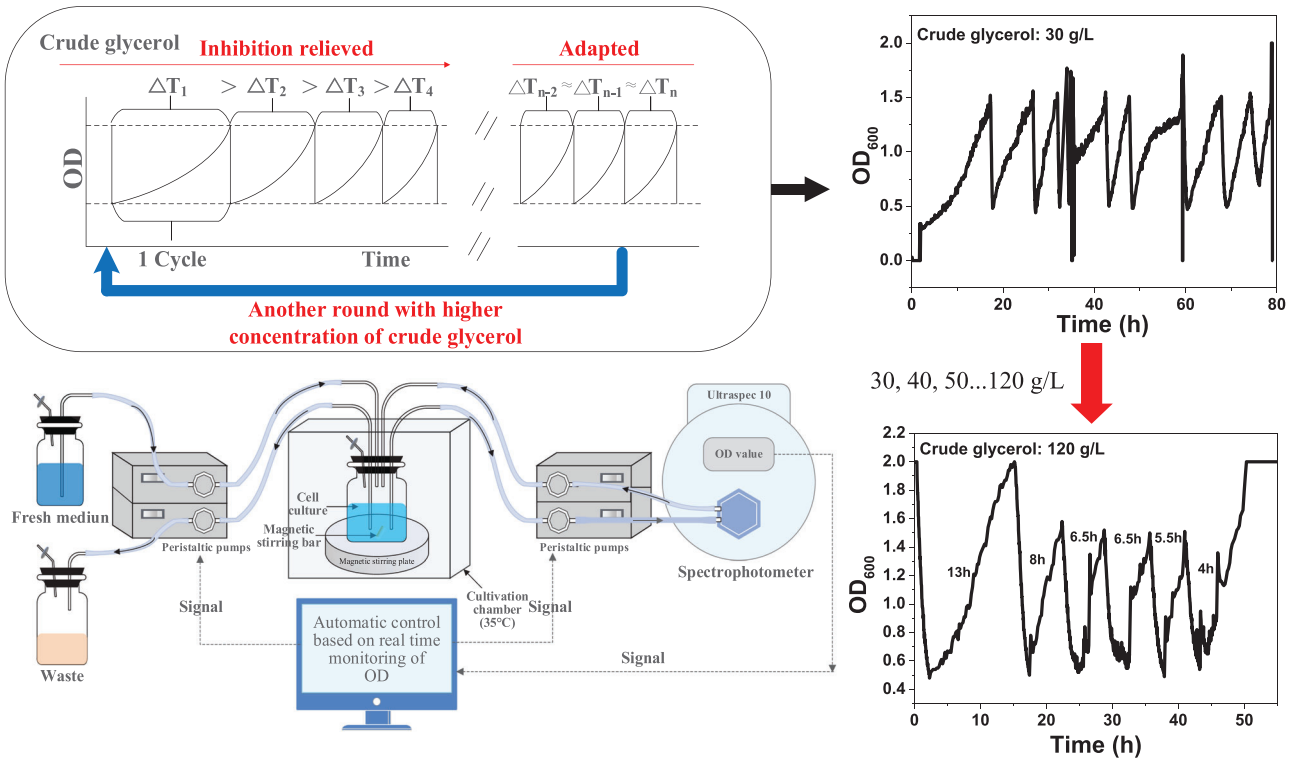

FIGURE 1 Schematic diagram of an automatic ALE system and typical results of the continuous adaptive evolution for high crude glycerol tolerance. ALE, adaptive laboratory evolution

△T maintained constant for more than 10 cycles. After more than 100 adaptation cycles from 30 to 120 g/L crude glycerol, an end-point adapted strain (termed as *C. pasteurianum* G8) which was fast-growing without any lag phase in 120 g/L crude glycerol, was selected for further fermentation experiments.

2.5 | Esterification experiments using the synthetic broth

All batch esterification reactions were carried out in 200 ml round-bottomed flask. The two necked flask was equipped with a sampling port, and a spiral coil reflux condenser in the middle to recycle back liquid after vapors condensed during reaction runs. Oil bath fitted with temperature control was used to maintain the targeted reaction temperature. The whole reaction system was placed on a magnetic stirring plate for controlling the constant stirring of a magnetic stirring bar in the flask.

In a typical experiment, a mixture of methanol, PDO, glycerol, and catalyst (Amberlyst-15) were fed into the flask, and the heating was started with condenser water on and with a constant stirring speed of 600 rpm. After the targeted temperature was reached, a mixture of acetic acid and butyric acid was injected into the flask via syringe to initiate the esterification reaction. A total of 0.5 ml sample was withdrawn every 30 min for analyzing the reaction conversion of acetic acid and butyric acid. The initial mass ratio of methanol, PDO, glycerol, acetic acid, and butyric acid for all experiments was kept constant at 27.6: 6.2: 3.6: 1.6: 1. This ratio was set to mimic the real composition of crude glycerol fermentation broth after ultrafiltration, concentration, and methanol precipitation treatments as described in Section 2.6. The total quantity of all reactants without catalyst was fixed at 100 g.

The esterification of acetic acid and butyric acid with methanol catalyzed by Amberlyst-15 in the synthetic broth was examined under the following different reaction conditions: temperature (25, 40, 50, and 70°C), catalyst loading (0%, 5%, 10%, and 20% (w/w)), and water content (0%, 2.5%, 5% and 10% (w/w)). For different alcohols, same molar ratio of ethanol and 1-propanol were also tested to react with the acids. After the reaction was finished, the reaction mixture was filtered to separate the sample solution from the used solid catalyst. All results shown are averages with standard deviations of experiments conducted in triplicates.

2.6 | Downstream processing for recovering 1,3-PDO and organic acid esters from the crude glycerol fermentation broth

2.6.1 | Ultrafiltration and concentration of the fermentation broth

Fermentation broth from the 1 m^3 crude glycerol fermentation in this study was used to evaluate the DSP. Removal of biomass and protein was performed by ultrafiltration described by C. Zhang et al. (2021). The pH of the permeate from ultrafiltration was then adjusted to 9 by adding 50% NaOH water solution, and the glycerol content in the permeate was adjusted to 4% by adding 80% crude glycerol. After the adjustment of pH and glycerol content, the fermentation broth was fed to a rotary evaporator (IKA) for concentration. In the first stage, 85% of the water was evaporated under a heating temperature of 80°C and a vacuum pressure of 50 mbar. In the second stage, the heating temperature was increased to 120°C for the complete water removal.

2.6.2 | Alcohol precipitation to remove salts and esterification of acetic and butyric acid with methanol

The concentrated broth was mixed with anhydrous methanol at a mass ratio of 1: 2.5, and the pH of the mixture was adjusted to 2 by adding 37% HCl solution. After standing still overnight, the precipitated inorganic salts were removed by centrifugation, and the methanol solution was fed to a similar reaction system as described in Section 2.5 for the esterification of acetic and butyric acid, except that the volume of the round-bottomed flask was 2 L. The reaction was carried out at 70°C, with an Amberlyst-15 loading of 10% (w/w) for 2.5 h. The mixture was filtered to separate the methanol solution from the used catalyst after the reaction was finished. Subsequently, methanol, MA, and MB were together separated from the esterified broth at the heating temperature of 60°C and the vacuum pressure of 200 mbar in a rotary evaporator.

2.6.3 | Final rectification of PDO

The residue after separating the methanol and methyl esters was mixed with water at a mass ratio of 2:1. Subsequently, 1% active carbon (w/w) was added, and 50% NaOH solution was used to adjust the pH to 9. The mixture was then heated up to 60°C with stirring for 1 h. The active carbon was removed by micro-filtration and the obtained permeate was fed to the rotary evaporator for complete water removal as described in Section 2.6.1. Finally, colorless and odorless PDO was evaporated and collected under the heating temperature of 145°C and vacuum pressure 20 mbar.

2.7 | Analytical methods and calculations

The cell density was measured spectrophotometrically as optical density (OD) at 600 nm and transformed to biomass concentration (g/L) using a linear relationship between cell dry weight (CDW, after cells dehydration at 80°C/3 days) and OD_{600} (Equation 1). OD_{600} was measured using a Genesys 10 UV Scanning photometer (Thermo Scientific) with 1-mm cuvettes. For samples with OD > 0.8, the sample was diluted with 0.1% HCl milli-Q water solution. The specific

growth rate (μ in h^{-1}) was calculated using Equation (2), where X_1 and X_2 are the biomass concentrations at time points t_1 and t_2, respectively.

$$CDW(g/L) = 0.44 \times 600;\ r^2 = 0.987, \quad (1)$$

$$\mu = \frac{LnX_2 - LnX_1}{t_2 - t_1} \times 100\%. \quad (2)$$

Glycerol, PDO, butanol, methanol, ethanol, 1-propanol, acetate, butyrate, formate, lactate, pyruvate, MA, and MB were quantified using high-performance liquid chromatography (HPLC, Kontron Instruments) with an Aminex HPX-87H 300 x 7.8 mm column (Bio-Rad Laboratories) as the separation column at an operation temperature of 30°C. The eluent for the HPLC detection was 0.1% trifluoroacetate and the flow rate was maintained at 0.6 ml/min. The detection was assessed by a UV-detector (Shimadzu) at a wavelength of 210 nm and a differential refractometer RI-detector (Kontron Instruments). The volumetric productivity of PDO (Q_{PDO}, in g/L/h) was calculated using Equation (3):

$$Q_{PDO} = \frac{C_2 - C_1}{t_2 - t_1} \times 100\%, \quad (3)$$

where C_1 and C_2 are the PDO concentrations at time points t_1 and t_2, respectively.

From the HPLC data, the conversion of acetic acid (X_A) or butyric acid (X_B) was calculated as follow:

$$X = \frac{C_0 - C_e}{C_0} \times 100\%, \quad (4)$$

where C_0 is the initial concentration of acetic acid or butyric acid; C_e is the concentration of acetic acid or butyric acid after esterification.

The content as weight fraction in percentage (wt %) of each component (C) in each purification step was calculated from Equation (5):

$$C = \frac{c \times d \times v}{w} \times 100\%, \quad (5)$$

where c is the concentration (g/L) determined by HPLC, d the dilution factor, v the volume (L), and w the weight (g) of the sample taken for analysis.

3 | RESULT AND DISCUSSION

3.1 | Fed-batch fermentation of pure and crude glycerol by *C. pasteurianum* C8

To evaluate the PDO production capacity of *C. pasteurianum* C8, fed-batch fermentation using pure glycerol as substrate was first carried out with pulse feeding strategy. As shown in Figure 2a and Table 1, no lag phase of cell growth was observed after inoculation for the fermentation. A maximum specific cell growth rate (μ_{max}) of

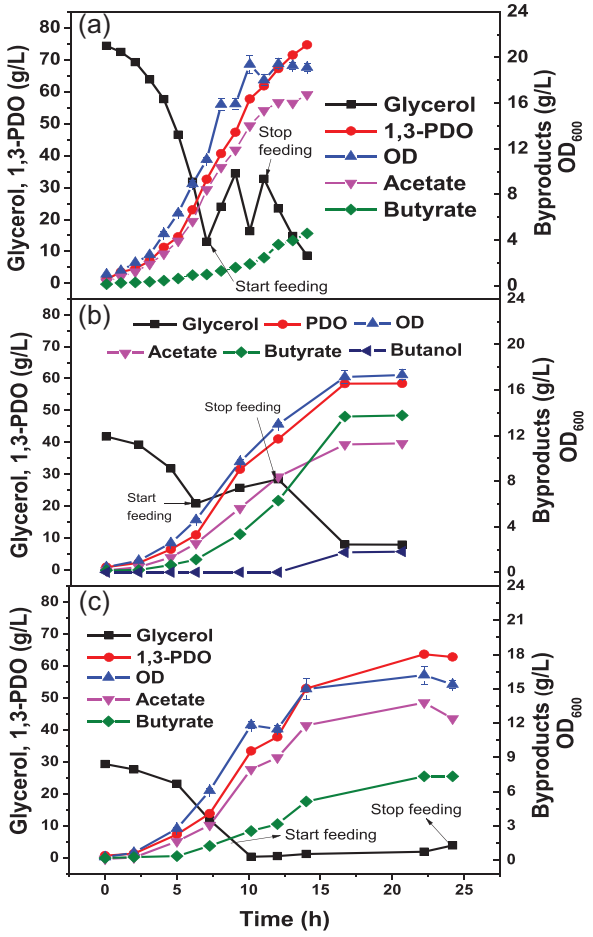

FIGURE 2 Fed-batch fermentations of *Clostridium pasteurianum* C8 using pure glycerol and crude glycerol as substrates. (a) pure glycerol + pulse feeding; (b) crude glycerol + pulse feeding; (c) crude glycerol + continuous feeding

$0.52 \pm 0.04\ h^{-1}$ was reached, and glycerol consumption and PDO production increased rapidly along with the fast accumulation of biomass. The concentration of glycerol decreased from initially 80 g/L to less than 20 g/L within 7 h of fermentation, and then the feeding of 50% glycerol was initiated. The maximum glycerol consumption rate of 19.18 g/L/h and PDO production rate of 10.44 g/L/h both occurred in this feeding period. The final PDO concentration and overall productivity reached 74.65 g/L and 5.33 g/L/h with a high PDO yield of 0.54 g_{PDO}/g_{Gly}. Furthermore, no butanol was produced during the whole process of pure glycerol fed-batch fermentation, which indicates a high metabolic selectivity and a high production efficiency of *C. pasteurianum* C8 for PDO.

Compared with pure glycerol, crude glycerol from biodiesel production has a better potential to serve as a cheap and abundant carbon source for a cost-effective bio-production of PDO. However, a lot of studies have showed that the impurities such as free fatty acids in crude glycerol may inhibit the growth of *Clostridium* species (Samul et al., 2014; Tan et al., 2018; Venkataramanan et al., 2012). To evaluate the possibility of using crude glycerol in the fed-batch

TABLE 1 Comparison of parameters of fed-batch fermentations by different *Clostridium pasteurianum* strains

Group	C8 (1 L)			G8 (1 L)			G8 (1 m^3)	
Initial glycerol concentration (g/L)	80	40	30	80	80	120	80	
Glycerol type	Pure	Crude	Crude	Crude	Crude	Crude	Crude	
Feeding strategy	Pulse	Pulse	Continuous	Pulse	Continuous	Continuous	Continuous	
Fermentation time (h)	14	21	24	18	14	20	14	19
Biomass (g/L)	8.41 ± 0.15	7.54 ± 0.24	6.77 ± 0.14	9.56 ± 0.12	6.76 ± 0.10	8.95 ± 0.45	7.63 ± 0.29	6.88 ± 0.09
μ_{max} (h^{-1})	0.52 ± 0.04	0.41 ± 0.01	0.48 ± 0.01	0.52 ± 0.02	0.59 ± 0.02	0.28 ± 0.01	0.59 ± 0.10	
PDO (g/L)	74.65	58.36	62.78	71.47	74.23	64.60	74.53	81.21
Acetate (g/L)	16.75	11.21	12.39	17.82	17.62	12.44	18.10	18.72
Butyrate (g/L)	4.58	13.66	7.34	11.62	8.13	15.32	10.27	11.09
Butanol (g/L)	0	1.75	0.56	1.49	0.97	0.93	0.84	2.47
Yield PDO/gly (g/g)	0.54	0.46	0.54	0.49	0.52	0.45	0.53	0.49
Q_{PDO} (g/L/h)	5.33	3.43	2.59	3.97	5.30	3.59	5.32	4.27

Abbreviation: PDO, 1,3-propanediol.

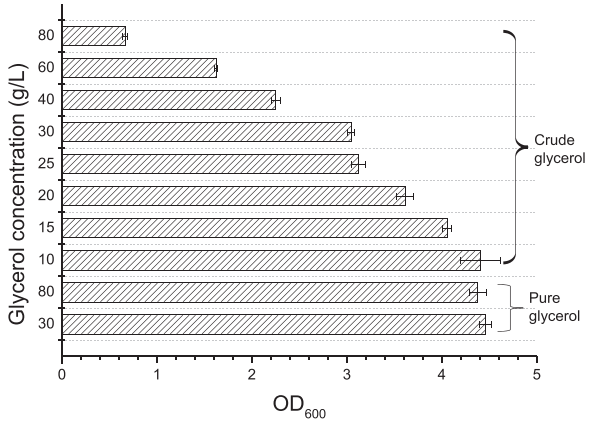

FIGURE 3 Initial tolerance test of *Clostridium pasteurianum* C8 to crude glycerol

fermentation for PDO production, an initial tolerance test for *C. pasteurianum* C8 was conducted. It can be seen that the growth of the strain decreased by more than 50% as the concentration of crude glycerol exceeded 60 g/L (Figure 3). After evaluating the tolerance, a crude glycerol concentration of 40 g/L, where the strain can still maintain 50% growth in the tolerance test, was set as the initial glycerol concentration in the fed-batch fermentation using the same three-pulse feeding strategy. As shown in Figure 2b and Table 1, although no lag phase of growth was observed after inoculating the strain into the crude glycerol containing medium, the μ_{max} (0.41 ± 0.01 h^{-1}), maximum glycerol consumption (12.13 g/L/h), and PDO production rate (6.68 g/L/h) were much lower than those achieved with pure glycerol. Finally, PDO production stopped at 58.36 g/L with significantly lower overall productivity and yield of 3.43 g/L/h and 0.46 g_{PDO}/g_{Gly}, respectively. To our surprise, 1.75 g/L butanol was produced at the end of the fed-batch fermentation. It is possible that high PDO concentration and the accumulation of crude glycerol impurities together evoked a stress environment, forcing the strain to produce butanol as a stress release for cell maintenance. Moreover, the butyrate production was also much higher (13.66 g/L with crude glycerol vs. 4.58 g/L with pure glycerol) in the crude glycerol fermentation. It is well known that acetic acid production can provide more NADH and leads to the theoretical maximum PDO yield of 0.72 mol/mol glycerol, while the butyric acid production can provide more ATP for cell growth and maintenance (Zeng, 1996). When the strain was in a highly stressful condition, higher butyrate production was more preferable for the strain to produce more energy for supporting its survival. This also led to more carbon loss in the oxidation pathway and thus reduced PDO yield from glycerol. Therefore, apart from the specific growth rate and the butanol production, the acetate/butyrate ratio can be also considered as an indicator for evaluating the tolerance of *C. pasteurianum* to crude glycerol.

To further avoid toxicity from crude glycerol, a lower initial glycerol concentration of 30 g/L, and a continuous feeding of 50% crude glycerol with a feed rate of 15 g/L/h were used in the fed-bath fermentation. The most significant improvement observed was a reduced production of butyrate (7.34 g/L) and butanol (0.56 g/L), which led to a sharp increase of PDO yield from 0.46 to 0.54 g_{PDO}/g_{Gly} by 17%. At the same time, the final PDO titer increased from 58.36 to 62.78 g/L (Figure 2c and Table 1). In addition, the acetate/butyrate ratio also increased from 0.82 to 1.69, indicating a reduced stress of *C. pasteurianum* C8 in the continuous fed-batch fermentation. Although low initial glycerol concentration and low feeding rate can effectively avoid stress response of the strain to crude glycerol toxicity, and as a result

improved the PDO yield significantly, the PDO productivity was greatly reduced due to the longer time of feeding. The maximum and overall PDO production rate were only 7.54 and 2.59 g/L/h in the continuous fed-batch fermentation of crude glycerol, which were 28% and 51% lower than those in the pulse fed-batch fermentation of pure glycerol, respectively. The results from pure and crude glycerol fed-batch fermentations indicate that *C. pasteurianum* C8 has potential as an efficient PDO producer, but its tolerance to crude glycerol should be improved to fully exploit the production capacity of this strain.

3.2 | Automatic long-term adaptive evolution of *C. pasteurianum* C8 for crude glycerol tolerance

ALE is widely used to obtain robust microbes with high tolerance for different environmental stresses. However, the achievement of a stably improved phenotype which can fully withstand a specific inhibitor usually requires a long-term ALE with great efforts (Alves et al., 2021; A. H. Zhang et al., 2019). To increase the efficiency, long-term ALE of *C. pasteurianum* C8 was conducted in a home-made automatic evolutionary system integrated with the real-time monitoring and control of cell growth in this study.

As shown in Figure 1, the OD_{600} of the strain during the repeated batch cultivations was automatically controlled between 0.5 and 1.5 by the ALE system. This was realized by adding of fresh medium into the cultivation bottle whenever the OD_{600} reached 1.5 and the addition was stopped when the OD_{600} reached 0.5 again. There are many advantages to keep the biomass sufficiently low in the long-term automatic ALE experiment. First, lower biomass can prevent bio-fouling in the tube connecting the cultivation bottle and the OD detector. Second, the pH of the culture can be maintained at 6.3–6.5 without external pH control owing to the negligible acids production by the limited biomass. It is well known that pH lower than 5.0 leads to significant growth inhibition to *C. pasteurianum* (Dabrock et al., 1992), which may affect the efficiency of the ALE experiment. However, the integration of pH control with probes and peristaltic pumps for acid/base solution requires a bigger size of bioreactor, and thus increases the working volume and medium consumption for the long-term ALE experiment. Thus the strategy employed here makes the system as simple as possible and highly efficient as well. Last but most importantly, the time needed for 1 adaptation cycle (OD_{600} from 0.5 to 1.5) is significantly reduced compared with those reported in the manual ALE experiment (>12 h for 1 cycle) (Alves et al., 2021; Liang et al., 2020; A. H. Zhang et al., 2019). An example of the automatic ALE process for 30 g/L crude glycerol is presented in Figure 1. Totally 10 adaptation cycles were conducted within 80 h. The immediate dilution of the culture with fresh medium when the OD_{600} reached 1.5 not only reduced the time interval between two adaptation cycles but also ensured that the next adaptation cycle was initiated with cells in the exponential growth phase. Undoubtedly, inoculum in good growth state is beneficial to avoiding lag phase, reducing infection risk and therefore increasing the number of adaptation cycles in the automatic ALE experiment. Based on the above-mentioned advantages, *C. pasteurianum* C8 was successfully adapted to each concentration of crude glycerol (30–120 g/L), and finally adapted to 120 g/L crude glycerol after more than 40 days of automatic ALE with 106 adaptation cycles. The $\triangle T$ for one adaptation cycle progressively decreased during the repeated cultivations, and a reduction to 4 h which was the same with pure glycerol, was achieved in the last cycle with 120 g/L (Figure 1). Further increase of crude glycerol concentration to 130 g/L resulted in a significantly longer $\triangle T > 10$ h, and the cell growth failed to recover even after 20 adaptation cycles. This might be due to the growth inhibition caused by the impurities and the osmotic pressure from 130 g/L crude glycerol. The cells were then kept in the automatic ALE system for another 10 repeated cultivations with 120 g/L crude glycerol for growth stabilization. The end-point adapted strain obtained from the automatic ALE system was named as *C. pasteurianum* G8 and used for characterizing the crude glycerol tolerance in the subsequent fed-batch fermentations.

As shown in Figure 4 and Table 1, fed-batch fermentations of crude glycerol by *C. pasteurianum* G8 were performed with two feeding strategies (continuous feeding with the feed rate of 40 g/L/h vs. pulse feeding with 80 g per feed). Meanwhile, the initial glycerol concentration was set at 80 g/L, which was the same as that in the pure glycerol fermentation. As expected, *C. pasteurianum* G8 showed excellent improved tolerance against the high initial concentration of crude glycerol. No lag phase was observed after inoculation and the μ_{max} ($0.52 \pm 0.02\ h^{-1}$ in pulse fed-batch and $0.59 \pm 0.02\ h^{-1}$ in continuous fed-batch) reached the same level as that in the pure glycerol fermentation. It should be noted that the same initial concentration of crude glycerol caused a long lag phase of over 10 h when the parental strain C8 was used (data not shown). Furthermore, the maximum glycerol consumption (22.19 g/L/h with pulse and 21.44 g/L/h with continuous feeding) and PDO production rate (8.74 g/L/h with pulse and 10.18 g/L/h with continuous feeding) also recovered to the same level as those achieved in pure glycerol fermentation owing to the increased utilization of crude glycerol and the fast feeding strategy. A significant metabolic shift from butyrate to acetate production was also observed after the automatic ALE. For example, the produced acetate was 59% higher (17 92 g/L vs. 11.21 g/L) and the butyrate was 15% lower (11.62 g/L vs. 13.66 g/L) by G8 than those by C8 in the pulsed-fed batch fermentation of crude glycerol. The final PDO concentration and overall productivity reached 71.47 g/L and 3.97 g/L/h with the pulse feeding (Figure 4a), and with continuous feeding (Figure 4b), 74.23 g/L and 5.30 g/L/h, respectively. These results clearly indicate that the automatic long-term ALE can effectively promote the production level of PDO in the crude glycerol fermentation by *C. pasteurianum*. Despite the similar PDO titer achieved with both feeding strategies, the yield of PDO from crude glycerol in the pulsed fed-batch fermentation was about 6% lower than that in the continuous fed-batch fermentation (0.49 vs. 0.52 g_{PDO}/g_{Gly}). This is probably due to the stress response of the strain caused by multiple instant exposure to a large amount of

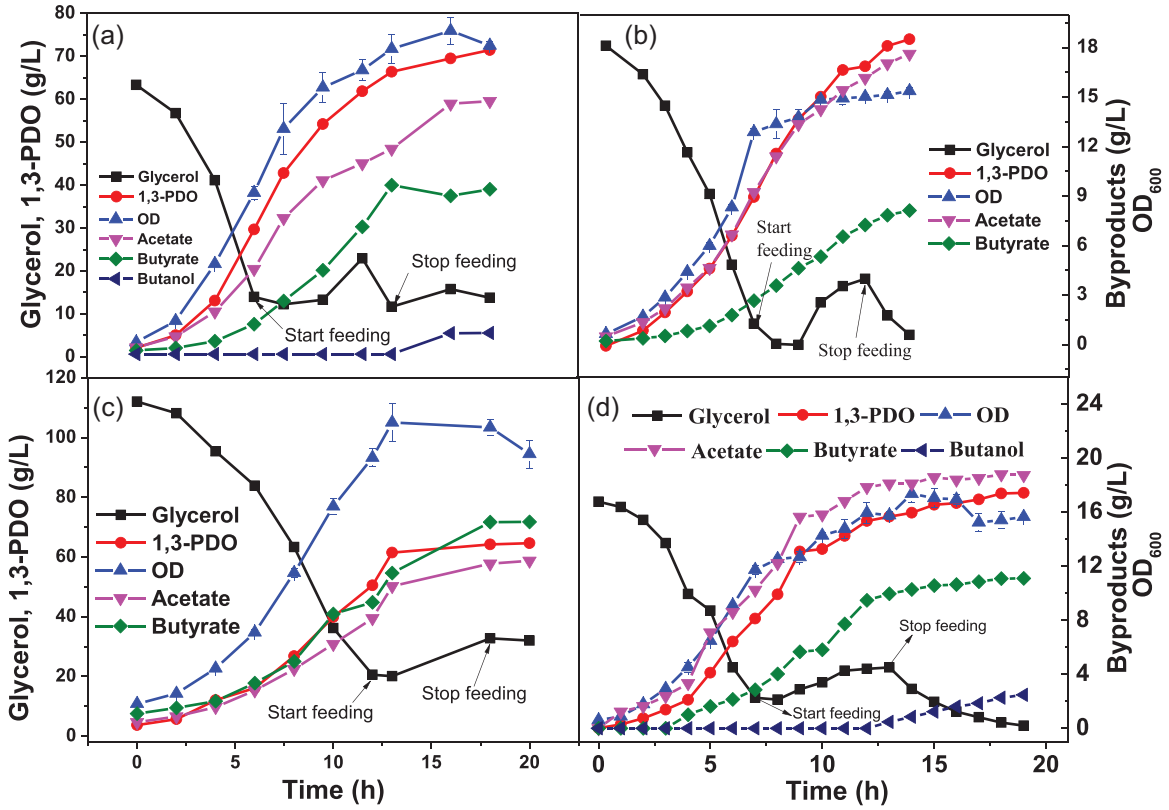

FIGURE 4 Fed-batch fermentations of adapted *C. pasteurianum* using crude glycerol as substrate. (a) 80 g/L crude glycerol + pulse feeding in 1 L bioreactor; (b) 80 g/L crude glycerol + continuous feeding in 1 L bioreactor; (c) 120 g/L crude glycerol + continuous feeding in 1 L bioreactor; (d) 80 g/L crude glycerol + continuous feeding in 1 m^3 bioreactor

crude glycerol impurities during the pulse feeding period, leading to a reduced acetate/butyrate ratio at the end of the fermentation (1.53 with pulse and 2.17 with continuous feeding). Similarly, further increasing the initial crude glycerol concentration to 120 g/L (Figure 4c) also resulted in marked decrease of acetate/butyrate ratio to 0.81, even with continuous feeding strategy. As a result, the titer, productivity and yield of PDO were all negatively affected (Table 1). Nevertheless, *C. pasteurianum* G8 exhibited good growth even with 120 g/L initial crude glycerol (μ_{max}, 0.27 ± 0.01 h^{-1}) and produced much less butanol (<1.5 g/L) in all the fed-batch fermentations, indicating much enhanced and stable tolerance to crude glycerol than that of the parental strain.

Since the fed-batch fermentation with continuous feeding described above resulted in the best PDO production, the same conditions were used for a scale-up fermentation in 1 m^3 bioreactor. As shown in Figure 4d and Table 1, the 1 m^3 scale nonsterile crude glycerol fed-batch fermentation without using yeast extract and N_2 aeration after inoculation was successful carried out. The highly crude glycerol-tolerant strain *C. pasteurianum* G8 was capable of producing 81.21 g/L PDO within 19 h, along with an overall productivity of 4.27 g/L/h and a yield of 0.49 g_{PDO}/g_{Gly}. It should be noted that more glycerol supply with a longer feeding time resulted in a slightly higher PDO titer in the 1 m^3 scale fermentation, but it also triggered higher butanol production (2.47 g/L) due to the stress induced by both high PDO concentration and crude glycerol impurities. Butanol production can be avoided and a significantly higher PDO yield of 0.53 g_{PDO}/g_{Gly} can be reached by simply stopping the fermentation at an early processing time of 14 h (Figure 4d and Table 1). These results represent one of the most efficient glycerol fermentation processes reported so far in terms of PDO titer, yield, productivity, and process economy (Table 2). To further increase the PDO titer, automatic long-term adaptation to a high PDO concentration could be considered in future work.

3.3 | Downstream processing for the coproduction of PDO and organic acid esters

3.3.1 | Process design and preparation for the esterification of organic acids in crude glycerol fermentation broth

Fermentation in 1 m^3 bioreactor clearly shown that, along with 80 g/L PDO, almost 30 g/L organic acids (acetate and butyrate) were coproduced by *C. pasteurianum* G8, which makes up more than 20% of the carbon atoms of glycerol consumed. Therefore, the process economy can be significantly improved if the organic acids are recovered as coproducts along with PDO from the fermentation

TABLE 2 Comparison of high-level 1,3-propanediol production from crude glycerol by nonpathogenic natural producers in fed-batch fermentations

Strain	PDO (g/L)	Yield PDO/gly (g/g)	Q_{PDO} (g/L/h)	Yeast extract	Medium sterilization	N_2 aeration	References
Clostridium butyricum DL07	94.23	0.52	3.04	40 g/L in the feed solution	Yes	0.15 vvm	Wang et al. (2020)
C. butyricum AKR 102a	76.20	0.51	2.30	40 g/L in the feed solution	Yes	0.6 L/h	Wilkens et al. (2012)
C. butyricum VPI 1718	67.90	0.55	0.78	1% in the feed solution	No	0.1 vvm	Chatzifragkou et al. (2011)
C. butyricum	66.23	0.51	1.38	5 g/L in both medium and feed solution	No	0.1 vvm	A. H. Zhang et al. (2019)
C. butyricum NCIMB 8082	73.07	0.55	2.48	1 g/L in the medium	Yes	No	Martins et al. (2020)
Lactobacillus reuteri CH53	68	0.82	1.27	5 g/L in the medium	Not mentioned	No	Ju et al. (2020)
Lactobacillus diolivorans DSM14421	92	0.78	0.56	4 g/L in the medium	Yes	2 sL/min	Lindlbauer et al. (2017)
Clostridium pasteurianum K1	55.00	0.52	2.30	No	No	No	Kaeding et al. (2015)
C. pasteurianum DSMZ 525	53.70	-	0.81	1 g/L in the medium	Yes	No	Groeger et al. (2016)
C. pasteurianum G8 (1 L)	74.23	0.52	5.3	No	No	No	This study
C. pasteurianum G8 (1 m^3)	81.21	0.49	4.27	No	No	No	This study

broth. In this scenario, the total product titer, overall productivity, and yield (PDO + organic acids) from the 1 m^3 crude glycerol fermentation reached 110 g/L, 5.78 g/L/h and 0.67 g/g, respectively. However, a direct separation of free organic acids from PDO usually requires the use of a distillation column with a high reflux ratio, which increases the investment and reduces the production efficiency (Kaeding et al., 2015; Rousseaux et al., 2013). More seriously, PDO and the organic acids can form esters during the distillation process, and the high boiling point of the PDO ester impurities significantly reduces the recovery yield of organic acids and the final purity of PDO (C. Zhang et al., 2021). To overcome this problem, a DSP that can convert the organic acids to the low-boiling alcohol esters before the acid separation is of special interest. In particular, a conversion of acetic and butyric acids to their corresponding methyl esters is beneficial to the acid recovery via evaporation owing to the significantly reduced boiling point for the esters (acetic acid 117.9°C vs. MA 56.8°C; butyric acid 164.35°C vs. MB 102.8°C, at atmosphere pressure). The occupation of the carboxylic group in the methyl esters also prevents the formation of PDO-acid esters during the thermal separation process. This can significantly increase the efficiency and reduce the costs of PDO recovery. Furthermore, both MA and MB have a higher market price than those of the free acids (acetic acid 0.6 USD/kg vs. MA 1.1 USD/kg; butyric acid 1.01 USD/kg vs. MB 4.04 USD/kg, according to market survey made on August 16, 2021), which represents a great gain or "compensation" for the production cost of PDO.

Based on the advantages mentioned above, the development of a novel DSP for the coproduction of PDO, MA, and MB was investigated using the fermentation broth from the 1 m^3 bioreactor. It is well known that the presence of water will greatly inhibit the esterification process (Dange et al., 2015). To achieve high conversions of acetic and butyric acids, the removal of water from the fermentation broth is necessary. In the study by Orjuela et al., a similar process was developed to recover fermentation-derived succinic acid by first converting the succinic acid to diethyl succinate through esterification with ethanol (Orjuela et al., 2013). By partially concentrating the fermentation broth, sodium succinate was precipitated and completely separated from water. The dried salt was then mixed with ethanol and sulfuric acid for the esterification reaction. In our case, however, acetate and butyrate salts cannot be crystallized and precipitated from a concentrated broth due to their solubility in PDO and glycerol. Moreover, acetic and butyric acids are more easily evaporated along with water compared with succinic acid (boiling point at atmosphere: 236.15°C), resulting in a high acid loss in the water removal process (Kaeding et al., 2015; C. Zhang et al., 2021). To ensure a high recovery yield of the acids and a complete water removal at the same time, a 50 wt% NaOH solution was used to adjust the pH of the ultra-filtrated fermentation broth to pH9. The formation of sodium salts of acetate and butyrate is expected to prevent the evaporation of acids. In addition, the glycerol content in the fermentation broth was increased to 4% by adding crude glycerol to prevent precipitation of the inorganic salts during the water evaporation process. As shown in Figure 5 (Content 3) and Table 3, owing to high contents of PDO and glycerol, no obvious salt crystallization and precipitation were observed after a nearly complete evaporation of water (rest water content <1%). Meanwhile,

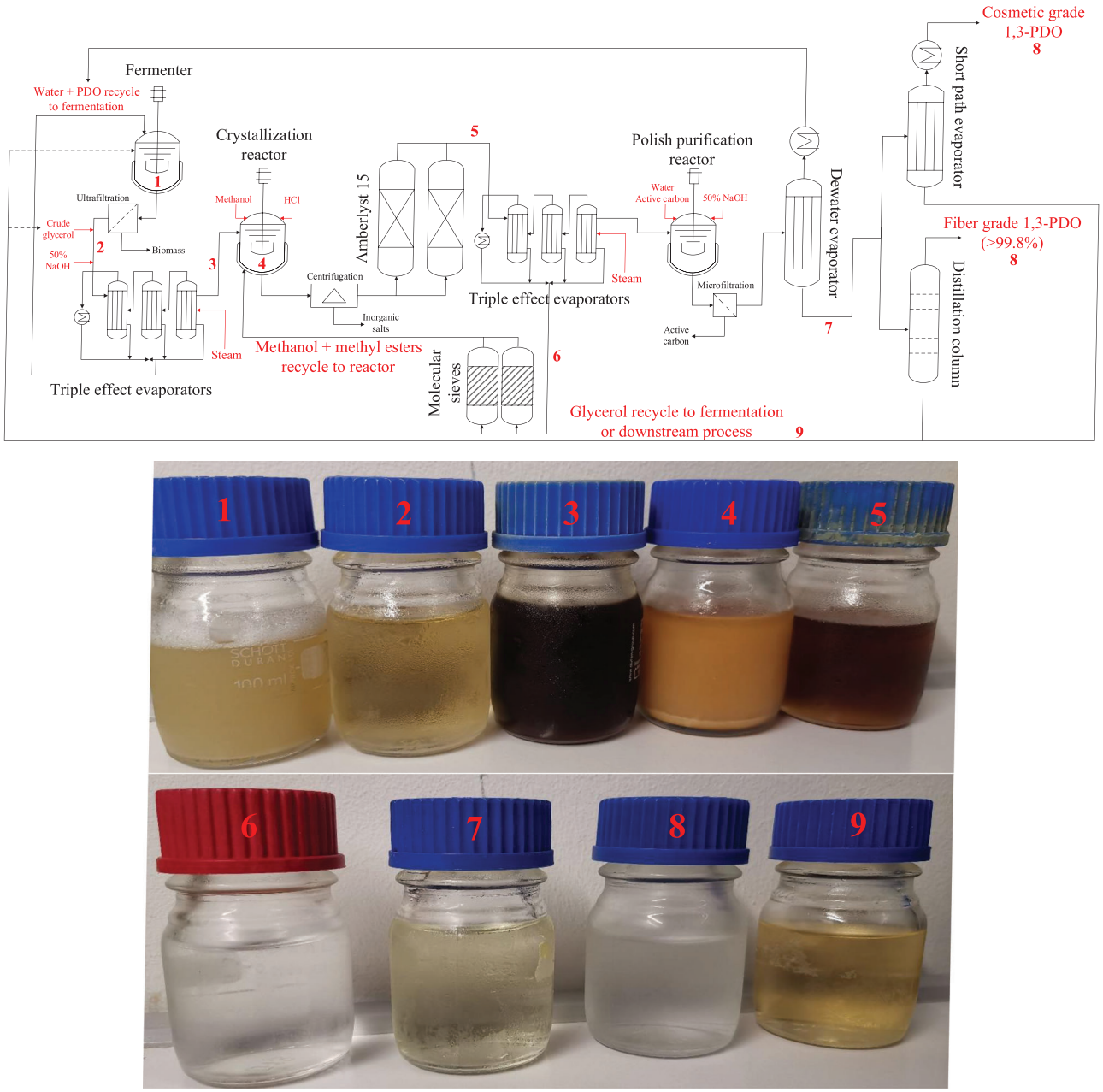

FIGURE 5 Flow diagram of industrial process for the coproduction of PDO and organic acid esters. 1. Crude glycerol fermentation broth; 2. permeate from ultrafiltration of fermentation broth; 3. dewatered fermentation broth; 4. mixture of dewatered broth and acidified methanol; 5. mixture after esterification; 6. recycled methanol solution containing MA and MB; 7. residue after polishing purification; 8. cosmetic grade or fiber grade PDO; 9. residual glycerol from PDO distillation. PDO, 1,3-propanediol

the recovery yield of PDO, acetic acid and butyric acid in the residue were 96%, 92% and 96%, respectively. Surprisingly, only 3 g/L PDO but no acids were detected in the distillated water (data not shown), indicating a complete retention of sodium acetate and sodium butyrate in the residue. The loss of acids might be only due to the dead volume and sampling from the rotary evaporator. The distillated water with a small amount of PDO can be directly reused in the fermentation process (Figure 5), leading to a theoretical yield of 100% for PDO, acetic and butyric acid in the water removal process.

To further convert the acetic acid and butyric acid in the dewatered broth to their corresponding methyl esters, fresh anhydrous methanol was added, and 37% concentrated HCl solution was used to acidify the mixture for liberating the free organic acids from the sodium salts. As a result, the medium salts and the NaCl formed by the acidification of the sodium acid salts were precipitated due to the reduced solubility in the methanol solution and subsequently removed by centrifugation (Content 4 in Figure 5). The remaining mixture containing methanol, PDO, glycerol, and free acetic and butyric acids was finally ready for the esterification reaction. Gao et al. previously evaluated the salt crystallization effect by adding ethanol to the concentrated fermentation broth of PDO (Gao et al., 2007). It was reported that the volume ratio of ethanol to the

TABLE 3 Key components and mass flow in the purification process for the coproduction of PDO and methyl esters from crude glycerol fermentation broth

Stream	Mass (g)	Methanol (%)	PDO (%)	Glycerol (%)	Acetic acid (%)	Butyric acid (%)	Methyl acetate (%)	Methyl butyrate (%)
1: Fermentation broth	4010	<0.1	7.80	0.5	1.80	0.91	<0.1	<0.1
2: Ultra-filtrated broth + crude glycerol	4162	<0.1	7.14	4	1.65	0.84	<0.1	<0.1
3: Dewatered broth	552	<0.1	51.90	28.24	11.50	6.10	<0.1	<0.1
4: Desalinated broth	1844	69	13.60	7.96	3.67	1.60	<0.1	<0.1
5: Esterified broth	1800	65.27	13.54	7.98	<0.1	<0.1	4.52	1.72
6: Recycled methanol	1344	86.80	<0.1	<0.1	<0.1	<0.1	5.87	2.27
7: Residue after polish purification	450	<0.1	53.08	30.96	0.13	<0.1	<0.1	<0.1
8: Rectified PDO	240	<0.1	96	3	<0.1	<0.1	<0.1	<0.1
9: Residual glycerol	210	<0.1	10	71	0.31	0.28	<0.1	<0.1

Abbreviation: PDO, 1,3-propanediol.

condensed broth should be over 2: 1, and the pH of the mixture should be reduced to 2 for a complete crystallization of the salts. Similarly, the mass ratio of methanol to the dewatered broth in this study was set at 2.5: 1, and the pH was reduced to 2 to ensure a complete removal of inorganic salts. In this way, the molar ratios of methanol, PDO, and glycerol to the total organic acids (acetic acid + butyric acid) were 27: 1, 2.3: 1, and 1.1: 1, respectively (Table 3). Considering the fact that the organic acids can be also esterified with the poly-hydroxyl PDO and glycerol, a high molar ratio of methanol to the organic acids was used to greatly increase the esterification selectivity to the targeted methyl esters.

3.3.2 | Optimization of the esterification process using synthetic broth

Before performing the esterification of organic acids in the real fermentation broth, a synthetic broth containing similar compositions of the desalinated fermentation broth (Table 3) was prepared and used for optimization of the esterification conditions. To achieve better separation and recovery of catalyst from the reaction mixture, a heterogeneous catalyst Amberlyst-15 resin, which is reported to have an excellent catalytic activity in many esterification reactions (Dange et al., 2014), was used for the formation of methyl esters. As shown in Figure 6a,b, the conversion of both acetic and butyric acid increased markedly as the reaction temperature was increased from 25 to 70°C. An equilibrium maximum conversion of 99.54% ± 0.14% in 90 min reaction time for acetic acid (X_{eA}), and 98.27% ± 0.03% in 150 min for butyric acid (X_{eB}), were reached at the boiling temperature of 70°C, respectively. In addition, it can be also seen that the X_{eB} decreased to 62.02% ± 0.80% when the temperature was reduced to 25°C, while a relatively high X_{eA} of 90.88% ± 4.4% was maintained at the same reaction temperature, indicating that the activation energy needed for the formation of MB is higher than that for MA. The reaction temperature was fixed at 70°C with a reaction time of 2.5 h for the rest of the esterification experiments to ensure a high conversion of both acetic and butyric acid and thus a high recovery yield of the organic acids from the fermentation broth.

Catalyst loading was also observed to have positive influence on the conversion of acetic and butyric acids (Figure 6c). Regardless of whether the catalyst loading was 10% or 20%, the conversion of both acids were over 98%. A reduction to 5% loading led to a similar acetic acid conversion, whereas the butyric acid conversion slightly decreased to 96%. By contrast, in control samples without catalyst, less than 10% acid conversion was reached, indicating an excellent catalytic activity of Amberlyst-15 for simultaneous synthesis of MA and MB. We also examined whether the high catalyst loading of 20% can accelerate the reaction rate for reaching the same maximum acid conversion yield, but no significant reduction of the time was observed (data not shown). These findings illustrate that a 10% loading of Amberlyst-15 is enough for the efficient conversion of acetic and butyric acids in the synthetic broth.

In contrast to catalyst loading, the initial water content presented in the synthetic broth negatively affected the acid conversion (Figure 6d). The X_A and X_B under the optimal reaction conditions decreased to 94.08% ± 0.69% and 87.21% ± 1.11% with 10% water, which was 5.5% and 11.3% lower than those achieved in reactions without water, respectively. Thus the initial water content in the fermentation broth should be carefully controlled before starting the esterification reaction. In our designed DSP, water from the fermentation medium can be completely removed by vacuum distillation. However, some water will be inevitably introduced to the fermentation broth during the acidification step using HCl solution. To remove the trace amount of water and improve the equilibrium conversion in the real fermentation broth, addition of molecular sieves or silica gel that has good water absorption can be considered in the DSP.

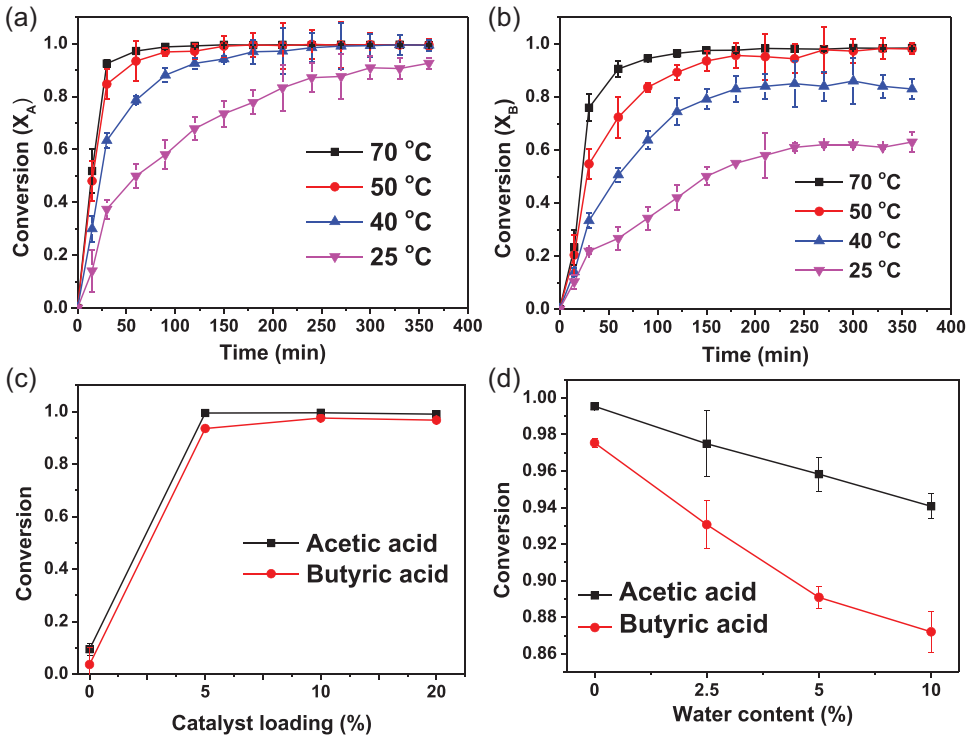

FIGURE 6 Effects of temperature (a and b), catalyst loading (c) and water content (d) on the esterification conversion of acetic acid and butyric acid with methanol

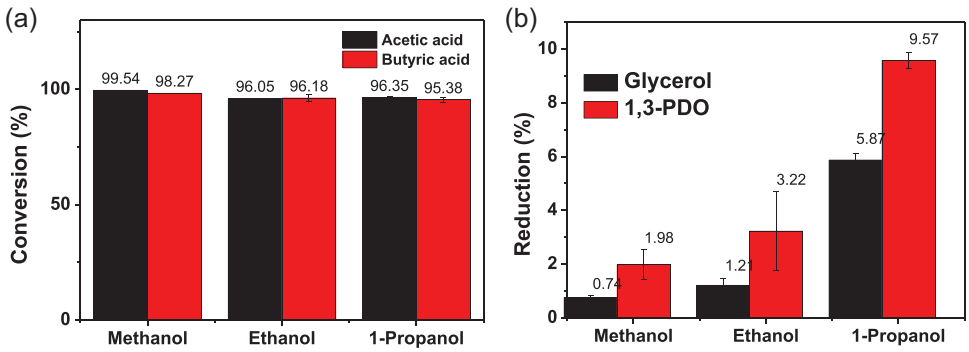

FIGURE 7 Effect of different alcohols on the esterification conversion of organic acids (a) and polyols (b)

In principle, alcohols other than methanol, such as ethanol and 1-propanol can also be esterified with the free organic acids in the fermentation broth, forming corresponding ethyl ester or propyl esters of acetic and butyric acid. These esters are also high value-added products widely used in manufacturing perfumeries and flavoring food (Dange et al., 2015; Nemati et al., 2021; Xue et al., 2020). In fact, we found that ethanol and 1-propanol were also effective for salt crystallization from the fermentation broth. Therefore, the reactivity of ethanol and 1-propanol with acetic and butyric acids in the synthetic broth was also investigated. As shown in Figure 7a, both X_A and X_B slightly decreased to around 96% when the alcohol was changed to same molar of ethanol or 1-propanol. The total consumption of acids by the esterification reaction maintained at a similar level regardless of the carbon chain length of the used alcohol. However, there are large differences in the esterification selectivity of the organic acids to the different alcohols including PDO and glycerol under the same reaction conditions (Figure 7b). The content of PDO and glycerol decreased markedly after the esterification reaction as the number of carbons in the linear alkyl chain of alcohol increased, indicating more acids were esterified with the polyols when ethanol or 1-propanol was used. In particular, the reduction of PDO in all three different alcohol-based synthetic broths was almost two times higher than the corresponding reduction of glycerol. This may be attributed to the increased steric hindrance and thus reduced reactivity between the acids and the ortho-hydroxylated glycerol compared with the meta-hydroxylated PDO (Cui et al., 2017). Although esterification between the acids and the polyols is inevitable, both acetic and butyric acids exhibited

the highest esterification selectivity to methanol in the synthetic mixture. The reacted polyols (PDO + glycerol) in the methanol-based reaction was 1.6 and 5.4 times lower than those in the ethanol and 1-propanol based reactions, respectively (Figure 7b). More than 95% of the reacted acetic and butyric acids were successfully converted to the corresponding methyl esters. As discussed above, less PDO-derived ester impurities facilitate the separation of acids and the rectification of highly pure PDO. Therefore, considering the ester recovery and the PDO purification efficiency, methanol is a good choice for the esterification conversion of organic acids in the fermentation broth.

3.3.3 | Esterification conversion of organic acids and rectification of highly pure PDO from fermentation broth

The esterification between the organic acids and methanol in the desalinated fermentation broth was performed under the optimal conditions selected above: reaction temperature of 70°C, 10% catalyst loading and 2.5 h reaction time. As shown in Tables 3, 4.52% MA and 1.72% MB were detected in the esterified fermentation broth. This indicates that 97.3% acetic acid and 92.9% butyric acid were successfully converted to their corresponding methyl esters. The slightly lower conversion of acids compared with that in the synthetic broth could be due to the presence of a small amount of water (1.52%) in the desalinated broth before the esterification reaction. Since the conversion of both acetic and butyric acids can be maintained over 93% with an initial water content lower than 2.5% (Figure 6d), considering the process efficiency, water absorption treatment by molecular sieves or silica gel was not applied before the esterification. Followed by a simple vacuum distillation operated at 60°C and 200 mbar, methanol, MA, and MB were completely evaporated from the fermentation broth, resulting in a colorless methanol solution containing 5.87% MA and 2.27% MB (Content 5 in Figure 5). The total yield of the recovered acids in their methyl ester form was therefore more than 88%. These results demonstrate that the organic acids can be separated from PDO and extracted from the fermentation broth simply and efficiently by integration of the esterification conversion process. Furthermore, the content of PDO and glycerol in the fermentation broth was basically unchanged before and after the esterification and methanol recycling process (Table 3), indicating that very few PDO or glycerol ester impurities were formed. On the contrary, more than 8% PDO was reported to be esterified and lost during the direct separation of PDO and free organic acids by vacuum distillation in our previous study (C. Zhang et al., 2021). Although the lost PDO could be recovered by a subsequent alkaline hydrolysis treatment, the elimination of a large amount of PDO ester impurities required more consumption of NaOH (50 g NaOH/kg PDO) and resulted in a sodium organic acids content of more than 4% in the residual from the final PDO distillation step. This makes the residual glycerol impossible to be reused in the fermentation process due to the growth inhibition of organic acids to *C. pasteurianum* (Sabra et al., 2016). Therefore, because of the significantly reduced formation of PDO and glycerol ester impurities, the methyl esterification strategy also improved the process economy and efficiency for the final rectification of highly pure PDO. After neutralization of the excessive HCl by adding 50% NaOH solution, and active carbon treatment to polish odor and color, the residual was subjected to a rotary evaporator for the vacuum distillation of PDO. As a result, a colorless and odorless distillate product containing 96% PDO and 3% glycerol (Content 8 in Figure 5) was successfully obtained, with a total PDO recovery yield of 77% for the whole DSP. Organic acids were hardly detectable in the final product (Table 3), indicating that the purified PDO has a high quality and could be directly used as an effective moisturizer in cosmetic manufacturing (Becker & Wittmann, 2015). The residual glycerol from the PDO distillation process still contained 10% PDO and a small amount of organic acids (0.31% acetic acid and 0.28% butyric acid). It is highly possible that these acids were released from the hydrolysis of the trace amount of PDO or glycerol esters in the neutralization step. Since the content of organic acids was very low, the residual glycerol from the new DSP is more promising to be directly reused in fermentation. In fact, if the water from the concentration step (4% PDO lost), and the residual glycerol from the final distillation step (5.5% PDO lost) can be both recycled in the fermentation, the total PDO recovery yield from the DSP would increase to 86.5%, which is economically more competitive.

Based on the successful lab-scale experimental results, a process flow diagram for the industrial-scale coproduction of PDO and organic acid esters from the crude glycerol fermentation broth is presented in Figure 5. Compared with the laboratory process, the most significant changes in the industrial process are listed as follow: 1. Two sets of triple effect evaporators are used for the removal of water and recycling of methanol to minimize the energy consumption; 2. The desalinated fermentation broth in methanol solution is fed to a fix-bed reactor packed with Amberlyst-15 for the continuous esterification conversion of organic acids, which is expected to reduce the reaction time and facilitate the reuse of catalyst (Salvi et al., 2018). In fact, Amberlyst-15 shows excellent reusability for catalyzing the formation of both methyl esters (Figure S2). The conversion rates were similar by reusing the catalyst three times, indicating that Amberlyst-15 may also have a long-term and stable catalytic activity in the fix-bed reactor. Considering possible need of regeneration or replacement of the catalyst, the feed can be switched to a second fix-bed reactor in series, and the regeneration of catalyst in the first fix-bed reactor can be carried out simultaneously; 3. The recycled methanol is fed to a molecular sieves column for water absorption, and subsequently reused for the crystallization and esterification process. It should be noted that the content of methyl esters in the recycled methanol was less than 8% (Table 3). Accumulation of methyl esters by multiple reuse of the methanol solution in the esterification reaction with organic acids could be beneficial for the

separation of methyl esters from methanol. In fact, the difficulty of separating the methanol-esters mixture lies in the separation of the MA-methanol azeotrope, which cannot be separated using the ordinary distillation technique. Instead, special distillation strategies, such as pressure swing distillation which realizes azeotropic separation by changing the azeotropic composition with different operating pressures (Wang et al., 2019), will be further evaluated in our future work. Since the water content in the recycled methanol will be increased to over 4% due to the esterification reaction (data not shown), to ensure high acid conversion and avoid excessive use of energy, the absorption of water from the recycled methanol involving molecular sieves is therefore proposed; 4. The PDO containing stream from the polishing purification can be fed to different PDO rectification process according to product applications. For application in cosmetics, where glycerol and PDO are both used as moisturizers in the formula, the stream can be fed to a short path evaporator for the continuous and efficient distillation of PDO. To produce fiber grade PDO for PTT synthesis, the stream can be fed to a distillation column for a complete separation of PDO and glycerol. Apart from the above-mentioned designs for the industrial-scale production, it is also worth mentioning that other alcohols such as ethanol and 1-propanol can also be used in our integrated process for producing a variety of high value-added organic acid esters for different applications. Generally, the novel industrial process presented here is able to exploit a variety of metabolic products from an efficient crude glycerol fermentation for the coproduction of PDO and various organic acid esters. The process itself is flexible and environmental friendly without producing any waste water, giving it great potential for improving the economy and efficiency of industrial PDO bio-production. Detailed cost evaluation for our proposed process including the separation of methanol, MA, and MB by pressure swing distillation will be considered based on process simulation and verification at pilot plant scale in the future work.

4 | CONCLUSION

To further increase the utilization capacity of crude glycerol and thus make the PDO bio-production more economically feasible, a low-butanol-producing strain *C. pasteurianum* C8 was subjected to a novel automatic ALE system developed for long-term adaptation of the strain to crude glycerol. Using a real-time monitoring of cell growth and precise control of sufficiently low biomass, automatic ALE of the strain with more than 100 adaptation cycles was successfully performed within two months. Compared with the parental strain, the adapted strain *C. pasteurianum* G8 can tolerate up to 120 g/L crude glycerol with a satisfactory growth rate. The PDO titer, volumetric productivity, and yield in the crude glycerol fed-batch fermentation reached 74.23 g/L, 5.30 g/L/h and 0.52 g_{PDO}/g_{Gly}, respectively, corresponding to the same performance as using pure glycerol. Furthermore, the broth from a successful scale-up fermentation (m^3 scale) using the evolved strain and crude glycerol was used to investigate a novel DSP for coproduction of PDO and organic acid esters. After biomass removal by ultrafiltration and water removal by vacuum distillation, the dewatered broth was mixed with an acidified alcohol for salts crystallization and removal, followed by esterification conversion of free organic acids to their corresponding esters. High conversion yields (97.3% for acetic acid and 92.9% for butyric acid) were achieved using methanol as a reactant under optimized conditions. The total recovery yield of organic acids in methyl ester form was more than 88%. Cosmetic grade PDO without acid contamination was easily recovered from the esterified broth via short path evaporation with a total process yield more than 77% which could be further improved by recycling the residuals. More pure PDO for fiber applications can be obtained by rectification. Overall, the highly efficient and integrated process elaborated for the first time in this study for coproduction of PDO and a variety of value-added organic acid esters from crude glycerol without waste water represents significant advances in developing more economic and ecological bio-manufacturing processes for industrially appealing green chemicals.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Chijian Zhang: Conceptualization; methodology; validation; formal analysis; investigation; data curation; writing—original draft; review & editing; visualization. **Shubhang Sharma**: Conceptualization; Methodology; validation; investigation. **Chengwei Ma**: Conceptualization; methodology; resources; software; investigation; data curation. **An-Ping Zeng**: Conceptualization; supervision; writing—review & editing; funding acquisition.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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