

Anaerobically Digesting Hazardous Waste *Pichia pastoris* Associated with Butyric Acid Cleaner Production

Wenjun Cheng, Xuehan Chen, Lipeng Gong, Jinyu Wei, Jian Ding, and Zhongping Shi*

Cite This: *ACS Omega* 2022, 7, 2918–2928

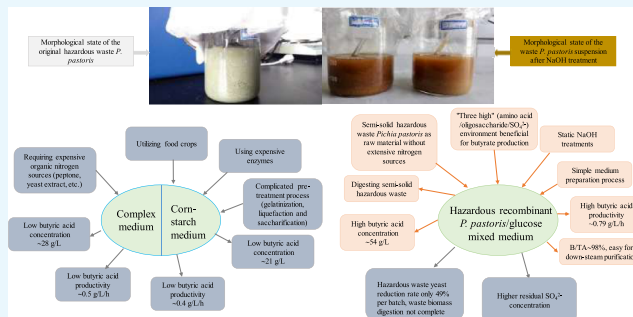
Read Online

ACCESS |

Metrics & More

Article Recommendations

ABSTRACT: Recombinant *Pichia pastoris* semisolid hazardous waste treatment is difficult and traditional solid waste treatment is not applicable. However, *P. pastoris* wastes have features of high density and enriched proteins/polysaccharides, which could supply nitrogen/carbon sources for butyric acid production. The waste *P. pastoris* was first treated using NaOH to form a waste yeast suspension, and then the suspension was mixed with glucose to obtain a starting medium containing 5.6 g DCW/L (dry cell weight) yeast to initiate butyrate fermentation. The suspension was intermediately supplemented to bring the total waste yeast concentration to 26.3 g DCW/L while continuously feeding the concentrated glucose solution. With the proposed strategy, butyrate concentration reached high levels of 51.0–54.0 g/L using *Clostridium tyrobutyricum* as the strain. Amino acids/oligosaccharides/ SO_4^{2-} in the suspension, raw material costs, complicated pretreatment process, and butyric acid cleaner production could be effectively utilized, reduced, eliminated, and realized. However, the apparent waste *P. pastoris* reduction rate was only 49% per batch, thus a “tanks in-series type’s repeated waste treating system” model was developed to theoretically explore the possibility of increasing the waste yeast reduction rate R . The simulation results indicated that when setting the treatment unit numbers at 4, waste solid concentration could decrease from 26.3 to 3.37 g DCW/L and the hazardous waste yeast reduction rate R would increase from 49 to 97%.



1. INTRODUCTION

Pichia pastoris is a common expression system for heterologous proteins. The production of proteins via methanol induction is implemented under very high cell density,¹ and the value-added target products are mainly secreted in the fermentation broth. The dry cell weight (DCW) could reach ~140 g DCW/L (400 g WCW/L, WCW: wet cell weight) after centrifugation when fermentation is finished. The waste *P. pastoris* cells could be recognized as a typical hazardous material, as they could not be used as distillers dried grains with solubles because of their edible safety, bad odor, and toxicity (contains methanol). The storage of *P. pastoris* wastes is also a huge problem. The traditional methods, such as burning, drying-powder-making, landfilling, and composting, are not applicable. The former two would require huge amounts of heating energy accompanied with air pollution, while the latter two may cause severe soil contamination. However, *P. pastoris* wastes have the features of high-density biomass and enriched proteins/polysaccharides (46% protein and 36% polysaccharide),² which could supply nitrogen/carbon sources for platform chemical productions such as organic acids (butyric acid). In butyric acid or butanol fermentations, abundant carbon/nitrogen sources are required, which could anaerobically digest the hazardous waste *P.*

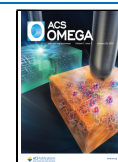
pastoris to reduce its amount while efficiently producing butyric acid or butanol simultaneously.

Butyric acid is a platform chemical with very wide applications in pharmaceutical and fine chemicals industries. In butyrate fermentation, the most commonly used substrates include corn, cassava, molasses, and so forth.³ It is recognized that the complex medium containing organic nitrogen sources (peptone, yeast extract, etc.), inorganic salts, and glucose is the most efficient fermentation of raw materials for butyrate biosynthesis. However, the high cost of the medium limits its industrial application. Butyrate fermentation is a typical semigrowth associated process. Higher cell growth rate and concentration could promote butyrate synthesis, but they require the energy supports that originated from carbohydrates, organic nitrogen sources, and other nutrients.

Received: October 18, 2021

Accepted: December 31, 2021

Published: January 11, 2022



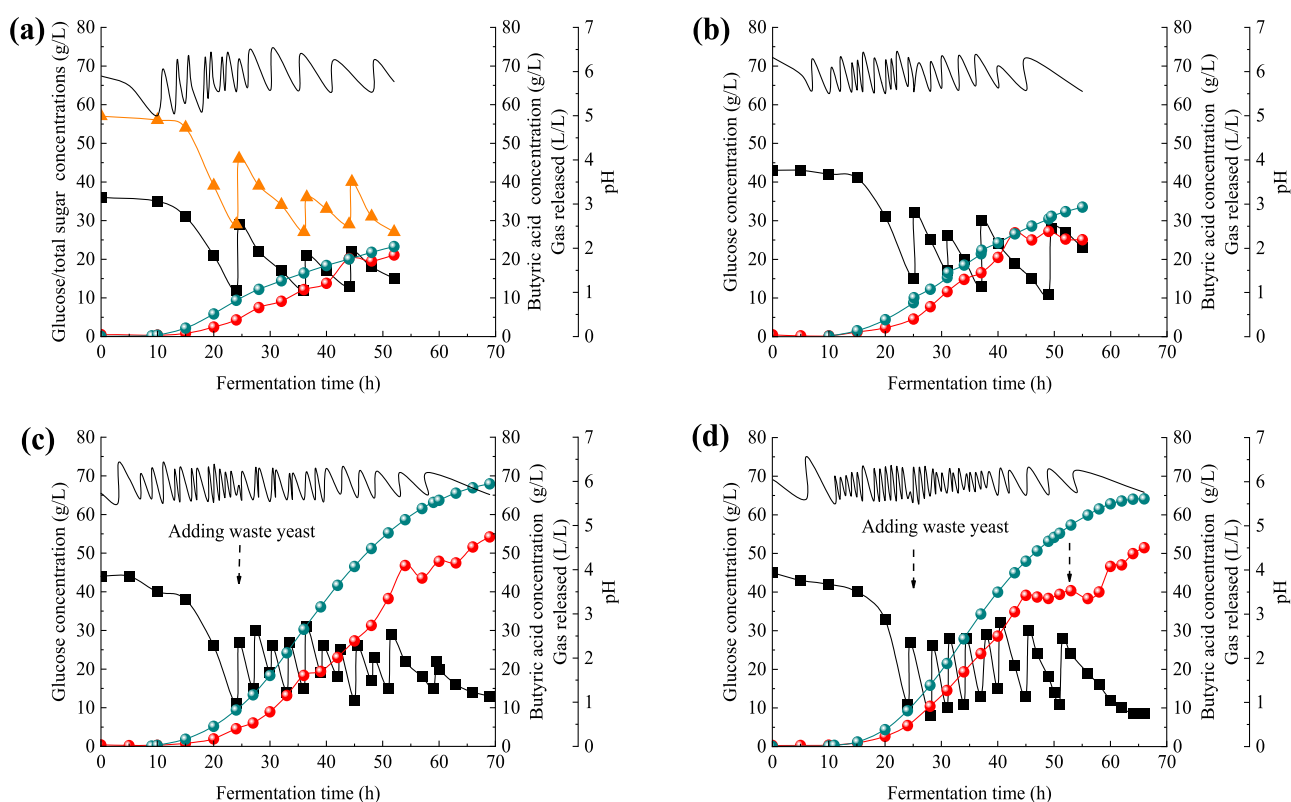


Figure 1. Butyric acid fermentation performance under different operating conditions in a 7 L fermentor. (a) Using 80 g/L corn starch medium, control; (b) waste yeast suspension (5.6 g DCW/L)/glucose-based medium; (c) waste yeast suspension (5.6 g DCW/L)/glucose-based medium with one waste yeast suspension (21.5 g DCW/L) addition during fermentation; (d) waste yeast suspension (5.6 g DCW/L)/glucose-based medium with two waste yeast suspension (10.8 g DCW/L) addition during fermentation; and runs #A–#D: consecutively supplementing the concentrated glucose solution during fermentations. Red circle solid: butyrate; box solid: glucose; yellow triangle up solid: total sugar; green circle solid: gas released; and emdash: pH.

Utilizing agricultural wastes such as straw, wood chips, and other cellulosic wastes in place of the traditional raw materials for butyric acid fermentation has gradually become the trend of research in this area.^{4–6} However, agricultural wastes have some shortcomings in their practical applications for platform chemical productions, such as difficulty in collection, high costs in transportation and storage, and so forth. In addition, the major components of agricultural wastes are carbohydrates such as cellulose, hemicellulose, lignin, and so forth, with very fewer nitrogen sources. The agricultural waste pretreatment process could hydrolyze the carbohydrates into fermentable monosaccharides, but it is complicated with the formation of many inhibitory substances, which indirectly deteriorates the subsequent fermentation performance.³ Furthermore, extra-expensive organic nitrogen sources have to be supplemented to ensure normal fermentation in the case of agricultural wastes, which increases the overall fermentation economics.

In previous studies, using 50 g/L NaOH to treat semisolid waste *P. pastoris* could form a solid–liquid suspension at room temperature in 2–3 days. The treated waste yeast suspensions (17.5 or 28.0 g DCW/L) were added into the fermentation broth when butyric acid and butanol fermentation entered the production phases at ~20–25 h.^{4,7} The recirculative utilization of waste yeast greatly enhanced butyric acid concentration compared with that of using expensive complex medium (from 28 to 45 g/L)^{3,7} and increased total sugar utilization yield in butanol fermentation (from 50%, using 150 g/L corn starch medium to 90–100%, using 80 g/L corn starch/waste yeast mixture).⁸ However, in both cases, 80 g/L corn starch medium

must be used as the “starter or inducer” for the fermentation, otherwise the fermentation could not be initiated.

A couple of problems remained unsolved or unclarified: (1) using 80 g/L corn starch medium as the “starter/inducer” increased the raw material cost and the complexity of the pretreatment process (α -amylase and glucoamylase utilization, gelatinization operation required, etc.); (2) the utilization (for waste yeast reduction/recirculation) ratio of the released nutrients (amino acids/oligosaccharides/ SO_4^{2-}) of the waste yeast suspension could not be quantified as waste yeast was mixed with corn starch powders in the previous study;⁷ and (3) the full utilization of waste yeast and complete elimination of traditional organic nitrogen sources use were not realized yet.

In this study, focusing on solving and clarifying the abovementioned problems, a novel fermentation strategy of “anaerobically digesting hazardous waste *P. pastoris* associated with efficient butyric acid production” was proposed, aiming at further improving butyric acid fermentation performance and economics; maximizing waste yeast reduction/recirculative utilization rates; and realizing efficiency and cleaner butyric acid production and promoting environmental effects.

2. RESULTS AND DISCUSSION

2.1. Pretreatment of the Semisolid Waste Yeast. Many other pretreatment measures are promising in dealing with sewage sludge, such as the 2 M NH_4Cl + NaOH method.^{9,10} Both proteins and carbohydrates could also be released.

Table 1. Butyric Acid Fermentation Performance Using Different Media and Operating Modes in a 7 L Fermentor^a

medium and fermentation operation mode	butyric acid (g/L)	acetic acid (g/L)	butyric acid productivity (g/L/h)	butyric acid yield (g/g)	B/TA (g/g)	total gas released (L/L)	total glucose consumption (g/L)	waste yeast adding dosage (g DCW/L)	residual mass dry weight (g DCW/L)
80 g/L corn control, #A	21.05 ± 0.2	2.92 ± 0.1	0.40 ± 0.01	0.32	0.88	23.26	67.7	0	27.50
WY/Glu starting medium, #B	27.20 ± 0.3	1.99 ± 0.3	0.45 ± 0.01	0.31	0.94	33.46	86.3	5.6	7.81
WY/Glu starting medium + WY*, #C	54.22 ± 0.1	1.27 ± 0.2	0.79 ± 0.02	0.35	0.98	67.95	154.0	26.3	16.40
WY/Glu starting medium + WY**, #D	51.46 ± 0.3	1.40 ± 0.3	0.78 ± 0.01	0.29	0.97	64.11	157.7	26.3	16.71

^a#A–#D: run number, consecutively supplementing concentrated glucose solution during all runs; WY: waste yeast suspension, 5.6 g DCW/L; Glu: glucose; WY*: adding 21.5 g DCW/L waste yeast suspension once during fermentation; WY**: adding 10.8 g DCW/L waste yeast suspension twice during fermentation; and residual mass dry weight: dry weight of solid residuals (including unliquefied waste yeast and *Clostridium tyrobutyricum* cells).

Table 2. Nutrients/Beneficial Substances Contents in the Corn Starch Medium and Waste Yeast Suspension

fermentation raw materials	glucose (g/L)	disaccharides (g/L)	trisaccharides (g/L)	trisaccharides (g/L)	total amino acids (g/L)	SO ₄ ²⁻ (g/L)
80 g/L corn starch	32.0 ^a	10.5 ^a	1.9 ^a	12.7 ^a	0.3 ^a	0.0 ^a
waste yeast suspension	0.0/45.0 ^a	9.1/0.36 ^a	20.9/0.84 ^a	7.2/0.29 ^a	25.0/1.0 ^a	61.0/2.4 ^a

^aVarious substance concentrations in corn starch medium and 5.6 g DCW/L waste yeast suspension/glucose-based starting medium.

However, as the biomass density of the waste yeast is much higher than that of sewage sludge, using the NH₄Cl + NaOH method could not crash the cell wall enough to release proteins and polysaccharides, so the strongest NaOH agent must be utilized.

2.2. Optimization of Waste Yeast Addition Amounts in 100 mL Anaerobic Bottles. The optimal waste yeast addition amounts were preliminarily determined in 100 mL anaerobic bottles. In bottle-scaled fermentations, butyrate concentration using complex medium (control) could only reach a level of 5.2 g/L as the pH could not be controlled. When using *P. pastoris*/glucose mixed media, the final butyrate concentration ended at 3.2 g/L with a low waste yeast initial amount (1.4 g DCW/L). After increasing the waste yeast initial amount to 5.6 g DCW/L, the final butyrate concentration reached 4.4 g/L. However, as a large amount of glucose remained unused (~32.0 g/L) at the fermentation end, in another experiment, 10 mL of waste yeast suspension was added at 20 h. The final butyrate concentration increased furthermore to 6.4 g/L. Consecutively increasing the waste yeast initial amount to higher levels of 11.2–28.0 g DCW/L, the final butyrate concentrations declined to very low levels of 0.7–1.4 g/L. In this case, the SO₄²⁻ concentration reached very high levels of 4.8–12.0 g/L, which might severely inhibit cell growth and butyrate synthesis. Based on the results, fermentations were conducted in a 7 L fermentor with pH control, with the initial *P. pastoris*/glucose mixed medium containing 5.6 g DCW/L waste yeast.

2.3. Butyric Acid Fermentation Performance in a 7 L Fermentor. **2.3.1. Butyric Acid Fermentation Using Corn Starch Medium.** Corn starch medium with 80 g/L was used for butyric acid fermentation (control, Figure 1 and Table 1, run #A). Fermentation ended when gas release ceased at 52 h, and the final butyrate concentration was 21.0 g/L. Total gas released, yield of butyrate over glucose, butyrate productivity, and butyric acid/total organic acid (B/TA) were 23.0 L/L, 0.32 g/g, 0.40 g/L/h, and 88%, respectively.

2.3.2. Butyric Acid Fermentation Using *P. pastoris*/Glucose Mixed Medium. Using the predetermined initial *P. pastoris*/glucose mixed medium (5.6 g DCW/L waste yeast, SO₄²⁻ ~2.4 g/L) to start butyric acid fermentation, the fermentation ended at 55 h, and the final butyrate concentration reached 25.0 g/L. Total gas released, butyrate yield, productivity, and B/TA were 27.0 L/L, 0.31 g/g, 0.45 g/L/h, and 94%, respectively.

2.3.3. Butyric Acid Fermentation Using *P. pastoris*/Glucose Mixed Medium with Intermediate Suspension Supplements. Fermentation was initiated using the same medium described in Section 2.3.2. At ~24 h, 400 mL of the waste yeast suspension was added at one time, allowing total waste yeast concentration to the level of 26.3 g DCW/L (run #C, Figure 1 and Table 1; maximum SO₄²⁻ concentration of ~12.0 g/L). The concentrated glucose solution was consecutively fed 11 times. The gas release rate increased significantly after adding the suspension. The fermentation ended at 69 h and the final butyrate concentration reached 54.0 g/L, which was much higher than that of control (run #C, Figure 1 and Table 1). Total gas released, butyrate yield, productivity, and B/TA significantly increased and reached 68.0 L/L, 0.37 g/g, 0.79 g/L/h, and 98%, respectively. However, the large amounts of suspension supplements at one time might vary fermentation environments remarkably and suddenly, particularly that of the SO₄²⁻ concentration (from 2.4 to 11.4 g/L), which would potentially deteriorate the entire fermentation performance.

In butyric acid fermentation run #D (Figure 1 and Table 1), 200 mL of waste yeast suspensions were added twice at 24 and 50 h, respectively, to avoid drastic environmental variations. The total waste yeast concentration also reached 26.3 g DCW/L when the concentrated glucose solution was fed eight times. The fermentation ended at 66 h and the final butyrate concentration reached 51.0 g/L (run #D, Figure 1 and Table 1). Total gas released, butyrate yield, productivity, and B/TA also reached high levels of 64.0 L/L, 0.33 g/g, 0.78 g/L/h, and 97%, respectively.

Table 3. Content Variations of Nutrients/Beneficial Substances during Butyric Acid Fermentation in a 7 L Anaerobic Fermentor

fermentation batch		aspartic acid group (g/L)	glutamic acid group (g/L)	total amino acids (g/L)	disaccharides (g/L)	trisaccharides (g/L)	trisaccharides (g/L)	total oligosaccharides (g/L)	SO ₄ ²⁻ (g/L)
run #A	initial instant	0.12 ± 0.01	0.09 ± 0.01	0.42 ± 0.01	5.17 ± 0.08	1.35 ± 0.05	5.88 ± 0.10	12.40 ± 0.12	0.00 ± 0.00
	supplement amount	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	end	0.02 ± 0.01	0.01 ± 0.00	0.16 ± 0.01	4.67 ± 0.09	0.00 ± 0.00	1.47 ± 0.06	6.14 ± 0.09	0.00 ± 0.00
run #B	initial instant	0.18 ± 0.02	0.13 ± 0.01	0.63 ± 0.02	0.25 ± 0.04	0.00 ± 0.00	0.45 ± 0.02	0.70 ± 0.01	2.73 ± 0.06
	supplement amount	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	end	0.01 ± 0.00	0.02 ± 0.00	0.28 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.15 ± 0.01	0.15 ± 0.02	1.53 ± 0.06
run #C	initial instant	0.16 ± 0.01	0.11 ± 0.01	0.55 ± 0.02	0.22 ± 0.02	0.00 ± 0.00	0.38 ± 0.02	0.60 ± 0.06	2.86 ± 0.09
	supplement amount	0.77 ± 0.02	0.72 ± 0.03	4.14 ± 0.09	2.14 ± 0.04	3.00 ± 0.05	1.12 ± 0.03	6.26 ± 0.08	9.67 ± 0.14
	end	0.11 ± 0.01	0.18 ± 0.01	1.41 ± 0.08	1.05 ± 0.03	1.03 ± 0.04	0.76 ± 0.02	2.84 ± 0.04	7.08 ± 0.11
run #D	initial instant	0.18 ± 0.01	0.12 ± 0.01	0.63 ± 0.04	0.31 ± 0.01	0.00 ± 0.00	0.53 ± 0.01	0.84 ± 0.02	2.48 ± 0.09
	supplement amount 1	0.38 ± 0.02	0.34 ± 0.01	2.19 ± 0.03	0.71 ± 0.03	1.95 ± 0.01	0.58 ± 0.01	3.24 ± 0.07	5.96 ± 0.08
	supplement amount 2	0.27 ± 0.01	0.22 ± 0.02	1.62 ± 0.05	0.77 ± 0.05	1.70 ± 0.01	0.33 ± 0.03	2.80 ± 0.06	5.36 ± 0.13
	end	0.14 ± 0.01	0.22 ± 0.01	1.54 ± 0.03	0.89 ± 0.04	0.15 ± 0.03	1.32 ± 0.04	2.36 ± 0.06	8.89 ± 0.15

2.4. Apparent Waste Yeast Reduction Rate in Each Run. Waste *P. pastoris* contains 46% proteins and 36% polysaccharides. The insoluble proteins and polysaccharides were degraded into soluble amino acids and oligosaccharides, respectively, with the NaOH pretreatment process. The oligosaccharides were disaccharides, trisaccharides, and the oligosaccharides over trisaccharides (>trisaccharides).⁸ By high-performance liquid chromatography (HPLCs) analysis, total amino acid and oligosaccharide concentrations in the suspension (140 g DCW/L specification) were 25 and 37 g/L (Table 2), respectively, and the degradation rates of proteins and polysaccharides were 39 and 73%, respectively. The apparent waste yeast reduction rate *R* refers to the ratio of the waste yeast reduction amount during digestion over total waste yeast supplemental dosages (eq 15 in Section 4.6). During digestion or fermentation, a portion of amino acids and oligosaccharides could be converted into targeted products (butyric acid), so-called recirculative resource rates. At the same time, another portion of amino acids and oligosaccharides degraded into CO₂, H₂, other small molecular organic acids, and so forth, the so-called waste biomass reduction rate. *R* included both the waste biomass recirculative resource rate and reduction rate.

When using the *P. pastoris*/glucose mixed medium for butyric acid fermentations (Table 1, runs #B–#D), the residual solid dry weights at each fermentation batch end were measured (*W_F*, eq 15, Table 1). When calculating the waste yeast reduction rate *R*, the data of *C. tyrobutyricum* cell dry weight *W* must be known. Thus, butyric acid fermentation using the complex medium (clear liquid medium, no solid particles except the cells) was conducted. The *C. tyrobutyricum* cell dry weight reached 3.0 g DCW/L.

Assuming that the dry weight of *C. tyrobutyricum* cells obtained by using waste *P. pastoris*/glucose mixed medium was equal to that of using the complex medium, then according to Table 1 and eq 15, the apparent waste yeast reduction rate *R* could be determined as ~49% (= (26.3 – 16.4 + 3.0)/26.3) per each run (eq 15, run #C and #D, Table 1). However, a higher apparent reduction rate is expected.

2.4.1. Effects of Amino Acid Utilization on Enhancing Butyric Acid Synthesis and Waste Biomass Recirculative Resource Rates. 17 amino acids were detected, and the total amino acid concentration was ~25.0 g/L in the suspension (Table 2). Butyric acid fermentation is a typical semigrowth associated process. Plenty of both carbon and nitrogen sources are required to increase the growth rate and concentration of *C. tyrobutyricum*, which would contribute to butyric acid synthesis indirectly. As shown in Table 3, the initial total amino acid concentration in corn starch medium (run #A, Figure 1) was about 0.40 g/L, while those in the initial *P. pastoris*/glucose mixed medium (runs #B–#D) were over 0.55 g/L stably. In runs #C and #D, by intermediately supplementing the suspension, total amino acid concentration was further increased to ~4.0 g/L (Table 3). The high amino acid concentration environments promoted cell growth rate and concentration, which enhanced butyric acid synthesis during the middle/late fermentation phases in an indirect manner.

At the fermentation end, the total amino acid concentration in runs #C and #D dropped to 1.41–1.54 g/L, the amino acid utilization rate was as high as 70% [= (0.55 + 4.14 – 1.41)/(0.55 + 4.14), run #C] and 65% (run #D), respectively. Studies have pointed out that the glutamic acid family (glutamic acid and proline) and aspartic acid family (aspartic acid, methionine, threonine, lysine, and isoleucine) amino acids are beneficial for *Clostridium* spp. growth/survivals and butyric acid/butanol synthesis.^{11,12} The results shown in Table 3 indicated that the utilization rates of the glutamic acid family and aspartic acid family amino acids in runs #C and #D were 78–88 and 68–83%, respectively.

It was speculated that the amino acids consumed were fully utilized in cell synthesis as organic acids, CO₂, and so forth do not contain N elements (ignoring tiny ammonia nitrogen compounds possibly formed). Therefore, the high amino acid utilization rate (65–70%) is closely correlated with the recirculative resource rate because *C. tyrobutyricum* cells could be indirectly considered as one of the targeted products (the catalyst to convert glucose into butyrate). In runs #C and #D, at least 39% proteins [= 25.0/(140 × 0.46)] in the

suspension were hydrolyzed into amino acids. Here, 0.46 is the waste yeast protein content. In run #C, the recirculative resource rate of waste yeast proteins was determined as 27% (=70% × 39%) due to the lower protein hydrolysis rate (39%).

2.4.2. Effects of High SO_4^{2-} Concentration in *P. pastoris*/Glucose Mixed Medium on Butyric Acid Fermentation Performance Improvement. Both *C. tyrobutyricum* and *Clostridium acetobutylicum* have an electron transport shuttle system where additional NADH forms are associated with H_2 release.^{13–15} Butyrate synthesis by *C. tyrobutyricum* is NADH dependent, while acetic acid (the major by-metabolite) synthesis is NADH independent. Under a higher intracellular NADH concentration environment, the by-metabolite formations could be repressed, which would increase the B/TA ratio in turn.

In the waste yeast pretreatment process by NaOH, H_2SO_4 must be used to adjust the pH of the suspension. When mixing the suspension with glucose or feeding the suspension during fermentation, the high SO_4^{2-} concentration environments were naturally created. According to the results shown in Sections 2.2, 2.3.2, and 2.3.3, *C. tyrobutyricum*'s tolerance ability against high SO_4^{2-} concentration environments was limited. Butyrate fermentation by *C. tyrobutyricum* must be initiated under a lower SO_4^{2-} concentration environment (<~2.4 g/L, 5.6 g DCW/L waste yeast). However, when *C. tyrobutyricum* continuously grew and its concentration reached a certain high level, SO_4^{2-} tolerance ability (the cells) could largely be enhanced to a higher level of ~12 g/L (runs #C and #D), allowing a large amount of waste yeast (26.3 g DCW/L, runs #C and #D) to be digested or treated. This is the reason that the new butyric acid fermentation operation strategy was proposed.

The literature has reported that electron receptors (SO_4^{2-}) could also alter electron/proton (e^-/H^+) distributions in the intracellular electron transport shuttle system, directing more electron/proton pairs (e^-/H^+) into the NADH synthesis route to strengthen reductive metabolite synthesis. The addition of electron receptor (either intently or passively) could enhance butanol or butyric acid production.^{7,8} With the proposed butyric acid fermentation operation strategy, butyric acid concentration and B/TA (54 g/L, 98%, run #C, Table 1) increased 157 and 11%, respectively, as compared with those of using corn starch medium (21 g/L, 88%, run #A-control, Table 1). SO_4^{2-} was consumed in fermentation runs #C and #D, although the utilization rate was relatively slow, SO_4^{2-} concentrations declined to 7.08 and 8.89 g/L at the fermentation ends from their maximum value of ~12 g/L, respectively. It should be noted that the higher residual SO_4^{2-} concentrations would increase the working loads of the downstream waste water treatment process, and this is one of the major shortcomings of the proposed fermentation operation strategy.

2.4.3. Effective Utilization/Reduction of Oligosaccharides in Butyric Acid Fermentation. Unlike the previously reported corn starch/waste yeast medium-based fermentation systems,^{7,8} the oligosaccharides solely originated from the waste yeast in the proposed butyric acid fermentation operation system. This provided the possibility of determining waste yeast oligosaccharide utilization/reduction rates in butyric acid fermentation.

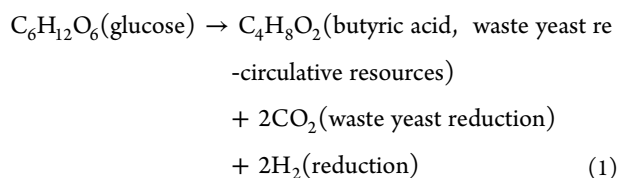
By HPLC measurements and analysis, the polysaccharides in the waste yeast subjected to NaOH pretreatment could only be hydrolyzed into oligosaccharides without monosaccharide

formation (Table 2). The oligosaccharides included disaccharides, trisaccharides, and the oligosaccharides above trisaccharides (>trisaccharides), with a total oligosaccharide concentration of ~37 g/L in the suspension.

The concentration changes of oligosaccharides in each fermentation run are summarized in Table 3. The polysaccharide degradation rate was ~73% [=37/(140 × 0.36)]. Here, 0.36 is the waste yeast polysaccharide content. In run #C, the oligosaccharide utilization rate (OUR) was 59% [= (0.60 + 6.26 - 2.84)/(0.60 + 6.26), refer to Table 3]. The actual oligosaccharide consumption amount was 4.02 g/L. In run #D, the OUR was 66%. The polysaccharide utilization/reduction rates per batch were determined as 43–48% (=73% × OUR, runs #C and #D, Table 3). It should be addressed that, the ratios of consumed polysaccharides for recirculative resources (butyrate synthesis and cell growth) or real waste reduction (gas release, other small molecular organic substances formations, etc.) still could not be identified.

2.5. Determination of the Maximum Waste Yeast Loading Amount and Reduction Rate in Each Run. To identify the maximum possible waste yeast loading (addition) amount and reduction rate in each fermentation run, multiple (three times) waste yeast suspension additions were conducted on the operation basis of runs #C and #D. 200 mL of the suspension was supplemented at 24, 36, and 54 h, respectively. The total waste yeast addition amount reached 34.4 g DCW/L (31% higher than that in runs #C and #D). However, the final solid amount (including residual waste yeast and *C. tyrobutyricum* cells), butyric acid concentration, and fermentation time were 18.2 g DCW/L, 51 g/L, and 77 h, which were 11% higher, 6% lower, and 12% longer than those of run #C, respectively. On the other hand, the apparent waste yeast amount reduction rate did reach the highest level of 56% [= (34.4 - 18.2 + 3.0)/34.4]. Measurement data indicated that amino acids were consumed no longer, and the concentrations of oligosaccharides and SO_4^{2-} decreased very slowly, after the third suspension addition. Based on the abovementioned results, it could be concluded that the maximum waste yeast loading amount (upper waste yeast digestion ability/limit of *C. tyrobutyricum*) and reduction rate in each fermentation run approximately ranged between 26.0–30.0 g DCW/L and 50%, respectively. Furthermore, raising the waste yeast loading amount up to ~35.0 g DCW/L would decrease the butyric acid concentration, increase the residual solid amount, and prolong fermentation time, deteriorating the overall butyric acid fermentation performance.

2.6. Theoretical Interpretation on Waste Yeast Reduction/Recirculative Resources in Butyric Acid Fermentation Based on C/O/H Element Balance Analysis. The data shown in Table 1 were used to analyze the C/O/H element balance in butyric acid fermentations. The metabolites and gas released were considered the outcome, while glucose/carbohydrate (polysaccharides) in waste yeast consumed represented the income. Due to the fact that only limited *C. tyrobutyricum* cells (3.0 g DCW/L) and miscellaneous acids (acetic and lactic acids) were formed during fermentations, and the cell element composition (C/N/O/H/S) was unknown, they were not considered in calculating in C/O/H element balances/yield calculations. The calculations were based on the following biochemical reaction equation (eq 1, molar base) and formula (eqs 2–4) proposed by Tashiro.¹⁶



$$Y_C = \frac{C_{\text{BA}} \times 4 + 2 \times C_{\text{CO}_2} \times 1}{C_{\text{GLC}} \times 6} \quad (2)$$

$$Y_H = \frac{C_{\text{BA}} \times 8 + 2 \times C_{\text{H}_2} \times 2}{C_{\text{GLC}} \times 12} \quad (3)$$

$$Y_O = \frac{C_{\text{BA}} \times 2 + 2 \times C_{\text{CO}_2} \times 2}{C_{\text{GLC}} \times 6} \quad (4)$$

Here, C_{BA} and C_{GLC} represented the final butyric acid concentration and glucose consumption amount (mol/L), respectively. C_{CO_2} and C_{H_2} were CO_2 and H_2 released (mol/L, determined by the gas data and ideal gas equation), assuming that the volume ratios of H_2 and CO_2 over total gas released were 30 and 70% (runs #C and #D, Table 4) under high SO_4^{2-}

Table 4. C/H/O Element Balances in Butyrate Fermentation Using Different Media^a

fermentation run	Y_C (P/%)	Y_H (P/%)	Y_O (P/%)
run #A	91	75	118
run #C	120	79	169
run #D	111	73	155

^a Y_C , Y_O , and Y_H represented the ratios/yields of outcome over income of C, O and H elements.

concentrations, while those volume ratios were 40 and 60% (run #A, control, Table 4) under the non- SO_4^{2-} existence condition, respectively.¹³ Y_C , Y_O , and Y_H represented the ratios/yields of outcome over income of C, O, and H elements, respectively. The results are shown in Table 4.

In runs #C and #D, both Y_C and Y_O largely increased, but Y_H only varied a little, compared with those of control. Y_C in run #A (control, Table 4) was 0.91, while Y_C in runs #C and #D reached 1.11–1.20, which already exceeded the ideal yield of 1.00. On the other hand, Y_O in runs #C and #D reached very

high levels of 1.55–1.69, which was much higher than the ideal yield of 1.00.

As the polysaccharide in the waste yeast was an extra carbohydrate (besides glucose), the enhanced Y_C indirectly reflected the increase in either waste yeast recirculative resource rate or reduction rate, or simultaneously increases. On the other hand, it was speculated that the largely raised Y_O was mainly correlated with extra CO_2 formations (waste yeast reduction), which coincided with the fact that gas release rates were greatly increased and the CO_2 over the total gas ratio rose to 70% under the high SO_4^{2-} concentration environment after intermediate waste yeast suspension supplements (runs #C and #D). In summary, the increases in both Y_C and Y_O indicated that the oligosaccharides in the waste yeast suspension could be at least partially utilized or consumed, which theoretically interpreted the fact that waste yeast amount reduction/recirculative resources did occur during fermentations.

2.7. Advantages and Shortcomings of the Butyric Acid Fermentation Strategy by Using *P. pastoris*/Glucose Mixed Medium. Figure 2 summarized the advantages and drawbacks of using the proposed waste yeast/glucose mixed medium-based butyric acid fermentation strategy over those of the traditional strategy using corn starch medium (control).

The major advantages included are as follows: (1) by replacing traditional/expensive organic nitrogen sources by waste *P. pastoris*, raw material costs were largely saved; (2) compared with control, high butyric acid concentration (>50.0 g/L), productivity (0.79 g/L/h), and B/TA ratio (~98%) could be achieved. The high B/TA ratio was conducive to the downstream product purification process; (3) the medium preparation process was very simple and straightforward; and (4) the waste biomass was digested/consumed during fermentation, so that butyric acid synthesis could be realized in a cleaner production manner. On the contrary, two major shortcomings remained unsolved: (1) the apparent waste yeast reduction rate was only 49% per batch, waste biomass digestion or consumption was not complete; and (2) SO_4^{2-} concentration in the fermentation broth was high (7.0–9.0 g/L), which increased the working loads or difficulties in the subsequent sewage treatment process.

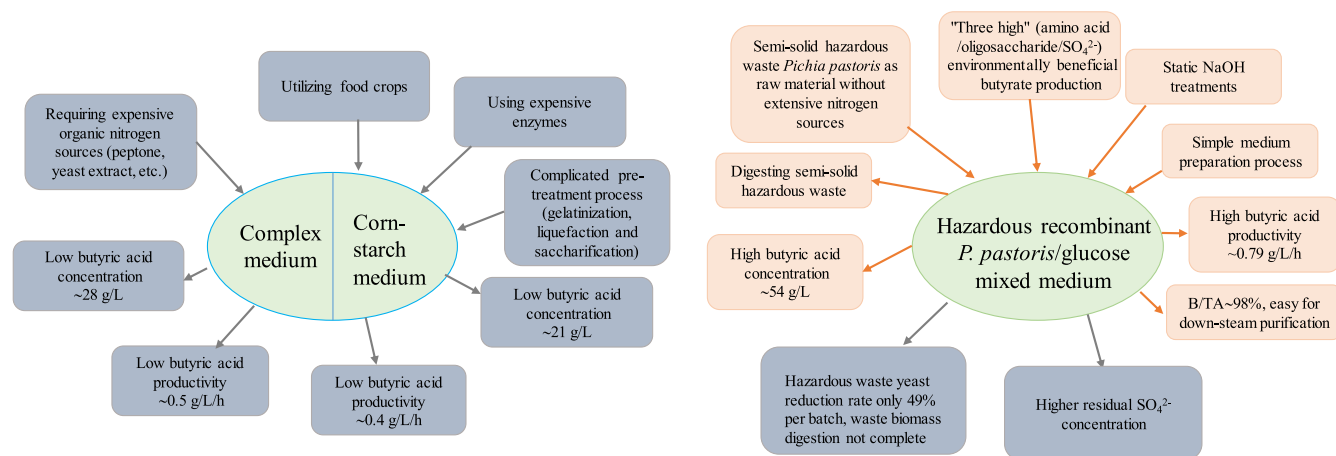


Figure 2. Butyric acid fermentation performance comparison using traditional raw material-based medium and the proposed waste yeast suspension/glucose-based medium. Yellow backgrounds: the merits and grey backgrounds: the shortcomings.

2.8. Modeling and Simulation of “Tanks In-Series Type’s Repeated Waste Yeast Treating System”. As pointed out in Section 2.7, the proposed butyric acid fermentation strategy has the problems of incomplete waste biomass digestion per batch and high residual SO_4^{2-} concentration. In runs #C and #D, the residual solid could only decrease from 26.3 to 16.4–16.7 g DCW/L (including *C. tyrobutyricum* cells, Table 1) in one batch. The waste yeast reduction rate R was only 49%. Abundant valuable amino acids (1.4–1.5 g/L) and oligosaccharides (2.4–2.8 g/L) still remained in the broth at the fermentation end. The effluent SO_4^{2-} concentrations were as high as 7.1–8.9 g/L (Table 3), which severely increased the burden of the subsequent sewage treatment process. As a result, the “repeated treating waste solids in-series tanks” system was proposed to solve those problems.

As shown in Figure 3, the system consisted of a series of fermentation tanks, while the exit waste solids from the

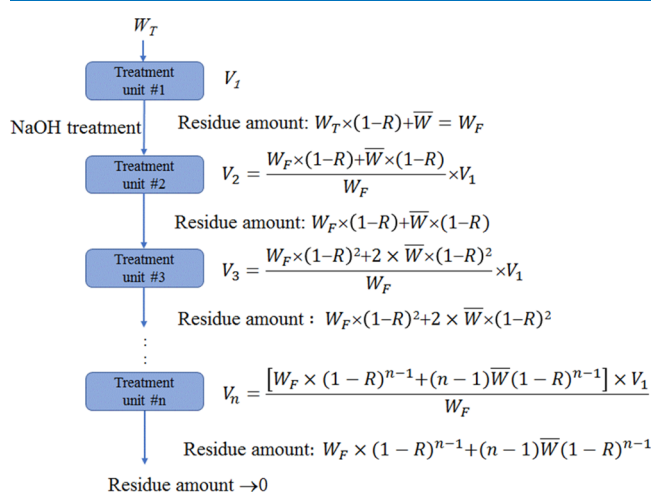


Figure 3. Tanks in-series type’s repeated waste yeast treating system.

upstream unit were used as the input of the subsequent unit, while butyric acid was harvested in each unit. In the subsequent tanks (second tank and the afterward units), the input waste solids originated from the upstream tank were treated using the same procedure proposed and fermentation was consecutively implemented with the same operation mode described (but without the intermediate extra suspension supplement). The proposed multistage-repeated fermentation system is presented in Figure 3, and is described by the following consecutive mass balance model.

- 1 The residual solid concentration at each treatment unit exit (g DCW/L)

$$W(k) = W_F(1 - R)^{k-1} + (k - 1)\bar{W}(1 - R)^{k-1} \quad (5)$$

- 2 The broth volumes in each unit and total treatment units (L) were determined by the residual solid mass balance (g)

$$W_F \times V(k) = [W_F(1 - R)^{k-1} + (k - 1)\bar{W}(1 - R)^{k-1}] \times V_1 \quad (6)$$

$$V(k) = \frac{[W_F(1 - R)^{k-1} + (k - 1)\bar{W}(1 - R)^{k-1}] \times V_1}{W_F} \quad (7)$$

$$V = V_1 \times \sum_{k=1}^n \left[\frac{W_F(1 - R)^{k-1} + (k - 1)\bar{W}(1 - R)^{k-1}}{W_F} \right] \quad (8)$$

- 3 The total residual solid treatment amount in the treatment system of tanks in-series (g DCW)

$$T_W = T_{W \lim [W_F(1-R)^{k-1} + (k-1)\bar{W}(1-R)^{k-1} \rightarrow 0]} \\ = W_T \times V_1 - W(k) \times V(k) \quad (9)$$

- 4 The residual amino acid concentration in each unit (g/L)

$$C_A(k) = W(k) \times (1 - H_A) \times C_p \times (1 - A) \quad (10)$$

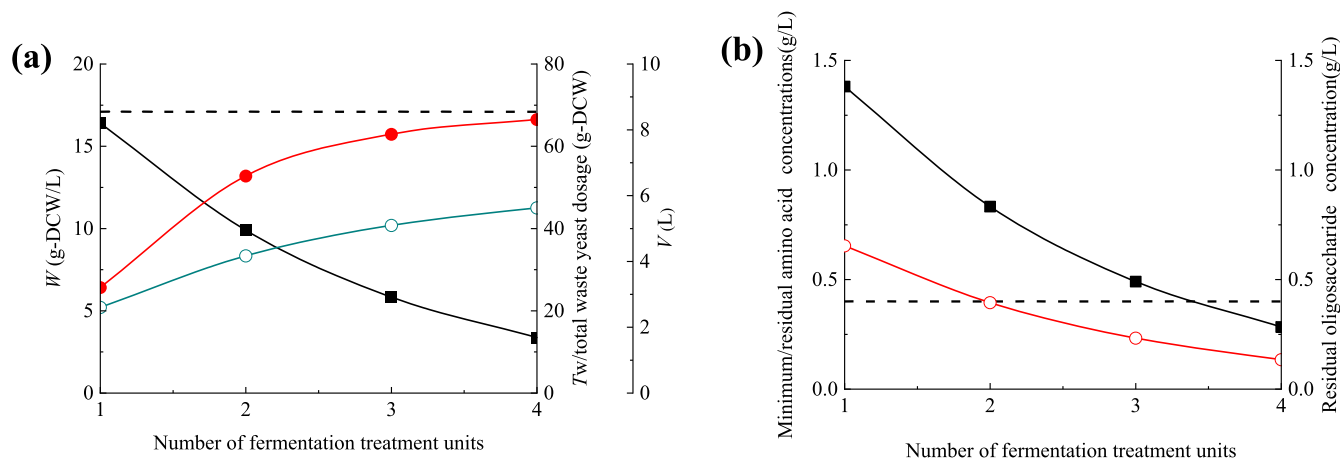


Figure 4. Simulation results of the “tanks in-series type’s repeated waste yeast treating system” model. (a) Total waste yeast digesting amount in all fermentation treatment units (T_W), the total volume of all fermentation treatment units (V), and the exit waste yeast concentration in each fermentation treatment unit [$W(k)$]. Red circle solid: total waste yeast digesting amount, green solid open: total residual solid volume, box solid: waste yeast concentration, and broken line: total waste yeast addition amount. (b) Amino acid and oligosaccharide residual concentrations in each fermentation treatment unit exit. Box solid: residual amino acids concentrations, red solid open: residual oligosaccharide concentrations, and broken line: minimum amino acid concentration required for running butyric acid fermentation by repeatedly using waste yeast.

5 The residual oligosaccharide concentration in each unit (g/L)

$$C_{\text{OA}}(k) = W(k) \times (1 - H_{\text{OA}}) \times C_{\text{OA}} \times (1 - \text{OA}) \quad (11)$$

The simulation was conducted using the fermentation data (run #C, Table 1) and the abovementioned model. Here, W_T , W_F , $W(k)$, T_W , and \bar{W} represented the waste yeast loading dosage in the first unit (26.3 g DCW/L), the residual solid concentration in the first unit (16.4 g DCW/L), the residual solid concentrations in k -th unit, the total solid treatment amount of the system (g DCW), and *C. tyrobutyricum* cell concentration in the first unit (3.0 g DCW/L), respectively. $V(k)$, V_1 , and V represented broth volumes in the k -th unit (L), the first unit (2.6 L), and the entire tanks in-series system (L), respectively. R represented the apparent waste yeast reduction rate (49%, same in each unit). $C_A(k)$ and $C_{\text{OA}}(k)$ represented amino acid and oligosaccharide outlet concentrations (g/L) in the k -th unit, respectively. H_A , H_{OA} , C_P , C_{OA} , A , and OA were constant parameters obtained by the relevant experiment (run #C) and literature data, representing the protein degradation rate (39%), polysaccharide degradation rate (73%), protein and polysaccharide contents in waste yeast (46 and 36%), and the utilization rates of amino acids (70%) and oligosaccharides (59%), respectively. The simulation results are shown in Figure 4.

Equations 5–9 are based on the following assumptions and restrictions: (1) the protein and polysaccharide contents of *C. tyrobutyricum* cells were equivalent to those of waste *P. pastoris*; and (2) the inlet amino acid concentration in each unit must be larger than 0.4 g/L, otherwise the insufficient nitrogen source would lead to incomplete fermentation in this unit and deterioration in the apparent waste solid reduction rate. Although insufficient oligosaccharides would not affect the butyric acid concentration, but the butyrate conversion yield would decline.

Figure 4A shows that the variations of the total waste solid treated amount (T_W , g) in the tanks in-series system, total broth volume (V) in the system, and the outlet waste yeast concentration in each unit [$W(k)$]. Along with the increase in the treatment unit (n), the outlet solid residue concentration in each unit continuously decreased. When $n = 4$, V increased to 5.63 L (from 2.6 L to 5.63 L), W fell to 3.37 g DCW/L, and the total solids treatment amount (T_W) reached 66.5 g-DCW (eqs 2–9), which was very close to the total waste yeast loading dosage of 68.4 g DCW (26.3 g DCW/L \times 2.6 L). Figure 4B shows amino acid and oligosaccharide concentrations in each unit exit. When $n = 4$, the outlet amino acid concentration was only 0.28 g/L (inlet concentration of 0.49 g/L); the continuously increasing treatment unit ($n = 5$) should be stopped as the restriction #2 would be violated. As a result, the unit numbers of the tanks in-series repeated treatment system were set at 4 ($n = 4$). With the system, the residual oligosaccharide concentration at the exit was reduced to a much lower level of 0.13 g/L.

In the third tank, the outlet solid concentration W had fallen to \sim 5.0 g DCW/L, but the residual solid still required NaOH/ H_2SO_4 treatments. Assuming that the required H_2SO_4 dosage (g/L) is proportional to W , and the similar fermentation procedure was adopted in the fourth unit. Then in the fourth tank, the initial SO_4^{2-} concentration would have dropped down to a low level of 2.2 g/L ($=61 \times 5/140$, refer to Sections 4.2 and 4.3; the initial *C. tyrobutyricum*'s tolerance ability

against SO_4^{2-} is \sim 2.0–4.0 g/L). The outlet SO_4^{2-} concentration in the fourth tank would even decrease down to 2.0 g/L. As a result, the problems of both incomplete solid waste digestion and high SO_4^{2-} concentration residue could be potentially solved.

2.9. Preliminary Economic Evaluation of the Proposed Process. Revenues: hazardous waste *P. pastoris* treatment benefits (I, positive value) + butyric acid (II).

(I): \$615/t-WCW waste yeast treatment income (WCW: wet cell weight). The set price is based on the treatment charge of an environmental protection company in Yancheng city, Jiangsu, China. The company specializes in landfilling semisolid hazardous materials using a special underground pool to ensure no infiltration and leakage of the hazardous components into the surrounding soils.

(II): \$1800/t, 54 g/L butyric acid was produced by digesting about 37 g WCW/L waste yeast. Please note that the apparent waste *P. pastoris* reduction rate was about 50% per each butyric fermentation batch.

Raw material and operation costs: glucose (I) + NaOH/ H_2SO_4 (II) + heating (III, distillation, for purification) + utility (IV) + labor (V) + pre-treatment (VI).

(I): C_{GLU} , \$450/t; the only raw material for the fermentation. 3 t glucose is roughly consumed in producing 1 t butyric acid according to the data of this study and other literature;

(II): NaOH/ H_2SO_4 , ignored as only little amounts are used and their prices are lower;

(III): Assuming R_1 accounts for heating cost against the raw material (glucose) cost;

(IV): Utilities, ignored. Static fermentation, no agitation or electric power required;

(V): Labor cost accounts for 9–11% ($R_2 = 10\%$) total cost,¹⁷ while the raw material cost generally occupies 70% of the total cost. Thus, the labor charge was determined on the base of glucose price/0.7;

(VI): Slight mixing only, cost ignored.

The rough cash flow balance (\$/m³): with the WCW-waste yeast (wet cell weight) as the calculation basis

$$P_T = P_I + P_{II} = \$615 \times W + \$1800 \times C_{\text{BA}} \quad (12)$$

$$\begin{aligned} C_T &= C_I + C_{II} + C_{III} + C_{IV} + C_{VI} = \$ \\ &450 \times C_{\text{CLU}} \times (1 + R_1 + R_2/0.7) = \$ \\ &450 \times C_{\text{CLU}}(1.14 + R_1) \end{aligned} \quad (13)$$

Gross profit (\$/m³)

$$\begin{aligned} \text{cross profit} &= P_T - C_T \\ &= \$615 \times W + \$1800 \times C_{\text{BA}} - \$ \\ &450 \times C_{\text{CLU}}(1.14 + R_1) \end{aligned} \quad (14)$$

Here, P_T is the total avenue and C_T is the raw material/operation cost. C_{BA} and C_{GLU} are the formation/consumption amounts of butyric acids and glucose (g/L or ton/m³), respectively. Figure 5 depicts R_1 as the X-axis and $P_T - C_T$ as the Y-axis to show the preliminary economic evaluation when digesting waste yeast while efficiently producing butyric acid. The economic analysis using CGM medium and corn starch-based medium was also shown as the comparison.

Salvachúa et al.¹⁸ produced industrial class butyric acid using corn stover hydrolysate as the raw material. The overall process

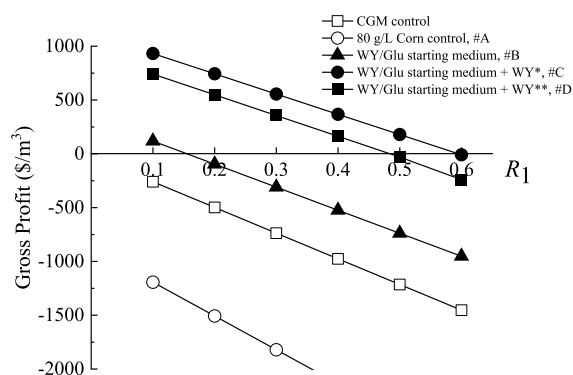


Figure 5. Preliminary economic evaluation of butyric acid production associated with waste *P. pastoris* digestion and amount reduction. WY: waste yeast suspension, 5.6 g DCW/L (16 g WCW/L); Glu: glucose; WY*: adding 21.5 g DCW/L (61 g WCW/L) waste yeast suspension once during fermentation; and WY**: adding 10.8 g DCW/L (31 g WCW/L) waste yeast suspension twice during fermentation.

was complex and combined liquid–liquid extraction, flash distillation, and distillation units. In their case, the heating (distillation) cost is about \$0.02/kg BA (butyric acid), and the total production cost is \$0.12/kg BA. Based on the abovementioned assumptions and the reference data, the R_1 ratio against the raw material (glucose) cost is about 24%. As a result, the gross profit of the proposed system producing butyric acid alongside waste yeast digestion ranges around \$500–\$700/ m^3 , a big positive margin.

3. CONCLUSIONS

A novel strategy of “anaerobic digesting waste *P. pastoris* associated with butyric acid cleaner production” was proposed. With the strategy, the final butyrate concentration reached 51.0–54.0 g/L, which was ~160% higher than that of control; reduction/recirculative resource of amino acids/oligosaccharides in waste yeast and butyrate cleaner production were realized. A “tanks in-series type’s repeated waste treating system” model was developed to theoretically explore the possibility of increasing the yeast reduction rate R . The simulation results indicated that when setting the treatment unit numbers at 4, waste solid concentrations could decrease from 26.3 to 3.37 g DCW/L and R could increase from 49 to 97%.

4. EXPERIMENTAL SECTION

4.1. Strain. *C. tyrobutyricum* ATCC 25755 was purchased from Microbial Strains Collection Centre, China.

4.2. Media. The seed culture and complex media contained (in g/L) glucose (30/60), peptone 5.0, yeast extract 5.0, NaCl 6.0, $(NH_4)_2SO_4$ 3.0, $CaCO_3$ 2.0, K_2HPO_4 1.5, $MgSO_4 \cdot 7H_2O$ 0.6, L-cysteine-HCl· H_2O 0.3, and $FeSO_4 \cdot 7H_2O$ 0.03 (pH 6.0). 80 g/L corn flour was used as the control medium (pH 6.5). The corn flour was hydrolyzed using the method previously described¹⁹ and initial glucose concentrations ranged 35–50 g/L.

Feeding media: glucose solution, 500 g/L and pretreated waste yeast suspension (140 g DCW/L, pH 6.5).

All the media (except the suspension) were sterilized at 121 °C for 20 min.

4.3. Waste *P. pastoris* Pretreatment. *P. pastoris* expressing human lysozyme (*hLYZ*) under high cell density with methanol induction was used. The cells obtained (after

centrifugation) were semisolid biomass with a dry weight ratio of ~35%. The pretreatment procedure was the same as that described in the literature, by placing the waste yeast in NaOH solution under room temperature and static conditions for 2–3 days to form a waste yeast suspension.⁸ The NaOH dosage and waste yeast concentration were fixed at 50 g/L and 140 g DCW/L, and the SO_4^{2-} concentration in the suspension was ~61.0 g/L (for pH adjustment using H_2SO_4).⁸

4.4. Preparation of *P. pastoris*/Glucose Mixed Medium. The waste yeast suspension was directly mixed with glucose solution at appropriate concentrations.

4.5. Fermentation Conditions. *C. tyrobutyricum* seed culture and butyrate fermentation were first carried out in 100 mL anaerobic bottles (working volume 50 mL) at 37 °C by adding 2.8–28.0 g DCW/L waste yeast suspension initially (SO_4^{2-} concentration of 1.2–12.0 g/L). The waste yeast suspensions were supplemented at ~20 h to raise the total waste yeast concentration to 26.1–51.3 g DCW/L (total SO_4^{2-} increase of ~11.3–22.0 g/L). All runs were terminated until no gas was released any longer.

Butyrate fermentations were then implemented in a 7 L anaerobic fermentor (Baoping Ltd., China) loaded with 2.0 L of *P. pastoris*/glucose mixed medium, 10% v/v seed cell cultures were inoculated into the medium to bring the initial broth volume to 2.2 L (waste yeast, 5.6 g DCW/L; glucose, 45 g/L; SO_4^{2-} , ~2.4 g/L). The corn starch-based fermentations with the same medium loading amount and inoculation size were also implemented as the control (corn starch concentration of 80 g/L). The operation procedures in both cases were exactly the same as those described in the literature.^{7,8} The pH was controlled at 5.5–6.5 using 50% commercial ammonia (36% purity). If the residual glucose concentration declined to ~10–15 g/L, a concentrated glucose solution was fed to elevate the glucose concentration to ~25 g/L. According to the requirements, at ~20 h, the yeast suspensions were added once (0.4 L) or twice (0.2 L each), raising the broth volume to ~2.6 L. All fermentations were operated in static mode, relying on self-generated gas for glucose/cell mixing and bioreactions. Slow agitation (~50 rpm) was imposed at the instants of glucose/yeast suspension supplements, sampling, and pH adjustment.

4.6. Analytical Methods. Organic acids (butyric acid and acetic acid) were measured using gas chromatography (Shanghai Precision Scientific Instrument Company, GC126). Glucose and lactic acid concentrations were detected using a biosensor (SBA-40C, Shandong Science Academy, China). The concentrations of oligosaccharides (disaccharides/trisaccharides/>trisaccharides) and amino acids were measured using HPLC (Waters Co., USA, 1525EF; Agilent Technologies Co., USA, 1100).^{8,13} SO_4^{2-} concentrations were determined using the barium chromate photometric method.²⁰ Total sugar concentrations (corn starch medium-based fermentation) were measured using the previously described methods.¹⁹ Residual waste yeast and harvested *C. tyrobutyricum* cells were placed in a baking oven at 90 °C until the weights did not vary any longer. Their dry weights were thus determined. All of the measurements were conducted in triplicate, and the average values were used.

Calculation of waste *P. pastoris* reduction rate in each fermentation run

$$R = \frac{W_T - (W_p + \bar{W}) + \bar{W}}{W_T} = \frac{W_T - W_F + \bar{W}}{W_T} \quad (15)$$

The waste *P. pastoris* reduction rate per each run (R) could be calculated using eq 15. Here, W_T (g DCW/L) was the total waste yeast addition dosages; W_p (g DCW/L) was the residual waste yeast dry weight at the fermentation end; and \bar{W} represented the *C. tyrobutyricum* cell dry weight (g DCW/L) assuming that the dry weight when using *P. pastoris*/glucose mixed medium could reach the equivalent levels of the fermentation using the complex medium (clear liquid medium). W_F (g DCW/L) was the dry weight sum of the residual waste yeast and *C. tyrobutyricum* cells at the fermentation end. W_F and \bar{W} were used to estimate the unmeasurable parameter W_p ($W_F = W_p + \bar{W}$).

AUTHOR INFORMATION

Corresponding Author

Zhongping Shi – The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China; orcid.org/0000-0001-5897-1510; Phone: (86) 510-85918292; Email: zps@jiangnan.edu.cn; Fax: (86) 510-85326276

Authors

Wenjun Cheng – The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China; orcid.org/0000-0002-1996-7452

Xuehan Chen – The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China

Lipeng Gong – The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China

Jinyu Wei – The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China

Jian Ding – The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China

Complete contact information is available at:

<https://pubs.acs.org/10.1021/acsomega.1c05840>

Author Contributions

W.C. fulfilled the experimental design, W.C., X.C., L.G., and J.W. performed experiments, and Z.S. and J.D. performed the experimental supervision, supplied the resources, and assisted the manuscript writings.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank the financial support from the National Key Research and Development Program of China (grant no. 2021YFC2101100).

REFERENCES

- (1) Macauley-Patrick, S.; Fazenda, M. L.; Mcneil, B.; Harvey, L. M. Heterologous protein production using the *Pichia pastoris* expression system. *Yeast* **2005**, *22*, 249–270.
- (2) Chen, C. N. Isolation, Purification and bioactivities of polysaccharide from *Pichia pastoris*. MSC Thesis, Dalian University of Technology, 2011.
- (3) Luo, H.; Yang, R.; Zhao, Y.; Wang, Z.; Liu, Z.; Huang, M.; Zeng, Q. Recent advances and strategies in process and strain engineering for the production of butyric acid by microbial fermentation. *Bioresour. Technol.* **2018**, *253*, 343–354.
- (4) Maiti, S.; Gallastegui, G.; Suresh, G.; Sarma, S. J.; Brar, S. K.; Drogui, P.; LeBihan, Y.; Buelna, G.; Verma, M.; Soccol, C. R. Hydrolytic pre-treatment methods for enhanced biobutanol production from agro-industrial wastes. *Bioresour. Technol.* **2018**, *249*, 673–683.
- (5) Câmara-Salim, I.; González-García, S.; Feijoo, G.; Moreira, M. T. Screening the environmental sustainability of microbial production of butyric acid produced from lignocellulosic waste streams. *Ind. Crops Prod.* **2021**, *162*, 113280.
- (6) Zhao, L.; Sun, G.; Jiang, J.; Chen, L.; Huang, J. Role of microbubbles coupling fibrous-bed bioreactor in butyric acid production by *Clostridium tyrobutyricum* using Brewer's spent grain as feedstock. *Biochem. Eng. J.* **2021**, *172*, 108051.
- (7) Cheng, W.; Cheng, C.; Gong, L.; Ding, J.; Shi, Z. Re-circulative utilization of waste *Pichia pastoris* as efficient nitrogen source for enhancing butyric acid production. *Biochem. Eng. J.* **2020**, *161*, 107661.
- (8) Ding, J.; Xu, M.; Xie, F.; Chen, C.; Shi, Z. Efficient butanol production using corn-starch and waste *Pichia pastoris* semi-solid mixture as the substrate. *Biochem. Eng. J.* **2019**, *143*, 41–47.
- (9) Wang, D.; Liu, B.; Liu, X.; Xu, Q.; Yang, Q.; Liu, Y.; Zeng, G.; Li, X.; Ni, B.-J. How does free ammonia-based sludge pretreatment improve methane production from anaerobic digestion of waste activated sludge. *Chemosphere* **2018**, *206*, 491–501.
- (10) He, D.; Xiao, J.; Wang, D.; Liu, X.; Fu, Q.; Li, Y.; Du, M.; Yang, Q.; Liu, Y.; Wang, Q.; Ni, B.-J.; Song, K.; Cai, Z.; Ye, J.; Yu, H. Digestion liquid based alkaline pretreatment of waste activated sludge promotes methane production from anaerobic digestion. *Water Res.* **2021**, *199*, 117198.
- (11) Mead, G. C. The amino acid-fermenting clostridia. *J. Gen. Microbiol.* **1971**, *67*, 47–56.
- (12) Heluane, H.; Evans, M. R.; Dagher, S. F.; Bruno-Bárcena, J. M. Meta-analysis and functional validation of nutritional requirements of solventogenic *Clostridia* growing under butanol stress conditions and cointilization of D-glucose and D-xylose. *Appl. Environ. Microbiol.* **2011**, *77*, 4473–4485.
- (13) Ding, J.; Luo, H.; Xie, F.; Wang, H.; Xu, M.; Shi, Z. Electron receptor addition enhances butanol synthesis in ABE fermentation by *Clostridium acetobutylicum*. *Bioresour. Technol.* **2018**, *247*, 1201–1205.
- (14) Deng, M. R.; Guo, J.; Zhu, H. H. Progress on biotechnological production of butyric acid. *Chin. Biotechnol.* **2009**, *29*, 117–122.
- (15) Jiang, L.; Fu, H.; Yang, H. K.; Xu, W.; Wang, J.; Yang, S.-T. Butyric acid: applications and recent advances in its bioproduction. *Biotechnol. Adv.* **2018**, *36*, 2101–2117.
- (16) Tashiro, Y.; Shinto, H.; Hayashi, M.; Baba, S.-i.; Kobayashi, G.; Sonomoto, K. Novel high-efficient butanol production from butyrate by non-growing *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) with methyl viologen. *J. Biosci. Bioeng.* **2007**, *104*, 238–240.
- (17) Xiao, Z.; Cheng, C.; Bao, T.; Liu, L.; Wang, B.; Tao, W.; Pei, X.; Yang, S.-T.; Wang, M. Production of butyric acid from acid hydrolysate of corn husk in fermentation by *Clostridium tyrobutyricum*: kinetics and process economic analysis. *Biotechnol. Biofuels* **2018**, *11*, 164.
- (18) Salvachúa, D.; Saboe, P. O.; Nelson, R. S.; Singer, C.; McNamara, I.; del Cerro, C.; Chou, Y.-C.; Mohagheghi, A.; Peterson, D. J.; Haugen, S.; Cleveland, N. S.; Monroe, H. R.; Guarnieri, M. T.; Tan, E. C. D.; Beckham, G. T.; Karp, E. M.; Linger, J. G. Process intensification for the biological production of the fuel precursor butyric acid from biomass. *Cell Rep. Phys. Sci.* **2021**, *2*, 100587.
- (19) Zhang, L.; Yang, Y.; Shi, Z. Performance optimization of property-improved biodiesel manufacturing process coupled with butanol extractive fermentation. *Chin. J. Biotechnol.* **2008**, *24*, 1943–1948.

(20) Masion, E.; Amine, J.; Marczak, R. Influence of amino acid supplements on the metabolism of *Clostridium acetobutylicum*. *FEMS Microbiol. Lett.* **1987**, *43*, 269–274.