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Batch, design optimization, and DNA sequencing study for continuous 1,3-propanediol production from waste glycerol by a soil-based inoculum

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Abstract 1,3-propanediol (1,3-PD) was produced with a robust fermentation process using waste glycerol feedstock from biodiesel production and a soil-based bacterial inoculum. An iterative inoculation method was developed to achieve independence from soil and selectively breed bacterial populations capable of glycerol metabolism to 1,3-PD. The inoculum showed high resistance to impurities in the feedstock. 1,3-PD selectivity and yield in batch fermentations was optimized by appropriate nutrient compositions and pH control. The batch yield of 1,3-PD was maximized to ~0.7 mol/mol for industrial glycerol which was higher than that for pure glycerin. 16S rDNA sequencing results show a systematic selective enrichment of 1,3-PD producing bacteria with iterative inoculation and subsequent process control. A statistical design of experiments was carried out on industrial glycerol batches to optimize conditions, which were used to run two continuous flow stirred-tank reactor (CSTR) experiments over a period of >500 h each. A detailed analysis of steady states at three dilution rates is presented. Enhanced specific 1,3-PD productivity was observed with faster dilution rates due to lower levels of solvent degeneration. 1,3-PD productivity, specific productivity, and yield of 1.1 g/l hr, 1.5 g/g hr, and 0.6 mol/mol of glycerol were obtained at a dilution rate of 0.1 h^{-1}which is bettered only by pure strains in pure glycerin feeds.

Keywords 1,3-propanediol · Mixed bacterial inoculum · CSTR · Batch fermentation · Design of experiment · 16S sequencing

Introduction

A paradigm shift in fuels and chemicals toward renewables has led to a growing market for biorenewable chemicals such as 1,3-propanediol (1,3-PD) with a plethora of applications (Ragauskas et al. 2006; Nikolau et al. 2014). The production of biodiesel, an attractive renewable fuel, (Noshadi et al. 2012; Jaliliannosrati et al. 2013), albeit, leads to excess glycerol byproduct (Haas 2006). Fermentation of crude glycerol to 1,3-PD provides a sustainable and value-added recourse to waste utilization (Cheng et al. 2007).

Naturally occurring glycerol to 1,3-PD convertors of genera *Klebsiella*, *Clostridia*, *Citrobacter*, *Enterobacter*, and *Lactobacilli* were studied by Urban and Bakshi (2009), Yang et al. (2007), Papanikolaou et al. (2004), and Biebl (2001). Bio 1,3-PD from sugars is being commercialized by DuPont, Genencor, and Tate & Lyle using a genetically modified *Escherichia coli* with a 51 wt% yield (Saxena et al. 2009). However, with the current glut and decreasing value, glycerol will likely gain preference as a sustainable feedstock platform for biorenewables (Johnson and Taconi 2007). Recent works utilize mixed bacterial consortia for fermenting industrial glycerol. These directly use heat-shocked and pretreated soil or sludge and report 1,3-PD yields from 0.41 to 0.65 mol/mol of glycerol (Selembo et al. 2009; Rossi et al. 2012; Metsoviti et al. 2012; Liu et al. 2013). While Liu et al. (2013) do not use pH control in batch fermentation, Dietz and Zheng (2014) do so for fed batch fermentation using activated sludge. A caveat is offered by the latter on the high yield by assuming the presence of additional unknown carbon sources in the sludge contributing to metabolite production.

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The present work established an iterative reinoculation procedure for preparing the starting culture. The selectivity and productivity were optimized by media and process variations. 16S rDNA sequencing was used to study bacterial population statistics in batches. A statistical design of experiment (DOE) was used to optimize batch production of 1,3-PD. The DOE results were used to run continuous flow stirred-tank reactor (CSTR) fermentations for over 500 h.

The iterative reinoculation eliminated soil particles from the culture to minimize variations due to changes in soil quality over storage and allowed easy optical density measurement. For future intended industrial application, soil elimination may reduce maintenance and ease pipeline flow. The effect of two nutrient compositions on selectivity is presented in this paper. The performance of pure glycerin (PG) and industrial glycerol (IG) feeds was compared. pH control resulted in a 0.7 mol/mol conversion with IG which is one of the highest reported in literature so far with mixed inocula. 16S rDNA sequencing results, a powerful tool for eliciting phylogenetic relationships, are reported on the starting inoculum and several batches. The DNA sequencing was carried out to obtain preliminary information about the composition of the inoculum and to briefly look at the effect of certain experimental parameters on the relative populations of species present. Sequencing on several fermentation batches are reported to underscore the fermentation results. This work also reports results of a statistical DOE with IG feed and concludes with analysis of two CSTR runs. The DOE, carried out with three variables, optimized conditions for 1,3-PD production in an attempt to integrate and reconcile the stochasticity inherent in two nonstandard inputs—the inoculum and the industrial glycerol—with their spread of species and impurities, respectively. Response surface methodology (RSM) was utilized to model and optimize conditions for maximum 1,3-PD output, which was used to run two CSTR experiments for 500 h each. Steady states were analyzed for productivities and molar yields. This appears to be the first systematic study of a mixed bacterial inoculum for fermenting industrial waste glycerol to 1,3-PD.

Mixed inocula offer advantages with cheaper inocula generation and maintenance, robustness against phage infections, and the ability to metabolize a wider range of carbon sources. They provide a cheaper and safer alternative to genetically engineered strains (Driessen 1981; Harrison 1978). Additionally, mixed cultures offer more flexibility for optimization of multiple product yields such as 1,3-PD and biohydrogen (Liu et al. 2013).

While Papanikolaou et al. (2004) and Chatzifragkou et al. (2011) continuously ferment IG to 1,3-PD using pure *Clostridium butyricum* strains, this work used a mixed bacterial inoculum with more than 100 species. The molar 1,3-PD yield ~0.66 at a dilution rate of 0.04 h^{-1} equaled Chatzifragkou's results. This is the first time, in a well-controlled CSTR fermentation with mixed inocula, that yields of 1,3-PD have been obtained comparable to those with pure cultures.

Materials and methods

Stock culture preparation

Bacterial cultures were grown from a University of Connecticut organic farm soil. An amount of 5 g of the soil were soaked in 10 ml of freshly prepared and autoclaved Difco™ Reinforced Clostridial Medium (RCM) from Becton Dickinson, MD, USA, (38 g/l) and glycerol from Sigma-Aldrich, USA, (10 g/l) and purged with high purity nitrogen for 10 min. The method to check for the absence of dissolved oxygen is as per Li et al. (2011). The test tubes containing the soil in the growth medium were anaerobically heat shocked at 80 °C for 10 min, cooled for 2 min, and incubated at 37 °C and 100 RPM (Li et al. 2011) for 7 days. The resultant supernatant medium was used for reinoculation under similar conditions. This procedure was iterated four times resulting in a thick inoculum as a seed culture for further bacterial inoculations and fermentation.

Feed media composition: batch, DOE, and CSTR

Feed compositions F1 and F2 with 3 g/l of pure glycerol (PG) were used to illustrate the effect of nutrient composition on selectivity. Compositions F2 and F3, with 20 g/l of PG and IG, respectively, were used to compare their respective performances, with and without pH control. The IG was obtained as a byproduct from converting waste cooking oil to biodiesel (Pomykala et al. 2013). Glycerol, yeast extract, potassium phosphates, and ammonium sulfate solutions were autoclaved separately at 121 C. Other nutrients and micronutrient solutions were sterile filtered by a 0.22-μm syringe filter and stabilized under refrigeration at acidic pH.

F1, composition per liter—pure glycerol 3 g, $(NH4)_2CO_3$ 2 g, KH_2PO_4 1 g, $MgSO_4{\cdot}7H_2O$ 0.1 g, NaCl 0.01 g, $MnSO_4{\cdot}7H_2O$ 0.015 g, $CaCl_2{\cdot}2H_2O$ 0.01 g, $Na_2MoO_4{\cdot}2H_2O$ 0.01 g, $FeCl_2{\cdot}4H_2O$ 2.75 mg. Feed was buffered with 0.05 M 2-N morpholino ethanesulfonic acid monohydrate. Initial pH ~5.5–5.7 (Liu et al. 2013)

F2, composition per liter—pure glycerol (PG) 3 g or 20 g, yeast extract 1 g, $(NH4)_2SO_4$ 2 g, KH_2PO_4 0.5 g, K_2HPO_4 1 g, $MgSO_4{\cdot}7H_2O$ 0.2 g, $CaCl_2{\cdot}2H_2O$ 15 mg, $FeSO_4$ 5 mg. Micronutrients: $Na_2MoO_4{\cdot}2H_2O$ 0.0072 mg, $FeCl_2{\cdot}4H_2O$ 0.3 mg, $CoCl_2{\cdot}4H_2O$ 0.38 mg, $MnCl_2{\cdot}4H_2O$ 0.2 mg, $ZnCl_2$ 0.014 mg, H_3BO_3 0.012 mg, $NiCl_2{\cdot}6H_2O$ 0.0048 mg, $CuCl_2{\cdot}2H_2O$ 0.0034 mg

F3, composition per liter—industrial glycerol (IG) 20 g, yeast extract 1 g, (NH4)2SO4 2 g, micronutrient composition was the same as F2.

All commercially available salts and chemicals were obtained from Sigma-Aldrich, USA, and used without any further purification.

For the DOE, the concentrations of IG and yeast extract were varied over selected ranges. The CSTR experiments used the optimized concentrations of IG and yeast extract from the DOE. The $(NH4)_2SO_4$, and micronutrient composition was kept identical to F3 for both DOE and CSTR.

Batches without pH control

Anaerobic, 150-ml batch fermentations were carried out in serum bottles. The pH was adjusted to 6.8–6.9 prior to inoculum injection. The media were purged with nitrogen, and the serum bottles incubated at 37C and 100 rpm. The absence of dissolved oxygen was checked as per Li et al. (2011). The inoculum transferred to the batches was either ~3 or 7 % of the batch volume. All batches were repeated in triplicate, and the results averaged.

Batches with pH control

Fermentations were carried out in a BioFlo 3000 bioreactor from New Brunswick Scientific. The pH was maintained by 2 N NaOH and 1 N HCl, and the batch volume was 300± 10 ml. The temperature and agitator were kept at 37 °C and 100 rpm, respectively, and nitrogen was purged through the headspace. The initial nitrogen flow rate of 250 ml/min, for 1.5 h, was decreased to 120 ml/min for the rest of the experiment (Li et al. 2011). Inoculum transferred was 7 % of batch volume, and each batch was repeated in triplicate.

16s rDNA sequencing for identification and characterization of consortia

The genomic DNA was isolated, amplified, sequenced, and analyzed. Sequences of the V4 hypervariable region of the DNA were analyzed to identify bacterial classification and relative abundances. An average of 68,490 gene sequences were analyzed per sample. The experimental details are delineated in Nelson et al. (2014). Sequencing was done on the stock culture (initial inoculum) and final samples of several batch fermentations. Average of triplicate results are presented for six samples: feed F1 (3 g/l PG)—no pH control, F2 (3 g/l PG)—no pH control, F2 (20 g/l PG)—pH 5.5, F2 (20 g/l PG)—pH 6.5, F3(20 g/l IG)—pH 5.5, F3(20 g/l IG)—pH 6.5. The results of the 16s rDNA sequencing were submitted to the NCBI SRA database with an accession number of SRP047483.

Design of experiment batches

A statistical DOE was performed on anaerobic batches with three process variables—pH, IG concentration, and yeast extract concentration. RSM was utilized to model and optimize the final 1,3-PD concentration as a response variable. The mineral nutrient compositions were identical to composition F3. The pH, IG, and yeast extract concentrations were varied from 4.8 to 7.2, 15 to 35 g/l, and 0.5 to 4 g/l, respectively. Experimental details are as per "Batches with pH control."

CSTR fermentations

Two CSTR runs were carried out, based on conditions computed from the RSM optimization, in a BioFlo 3000 bioreactor. The anaerobic fermentation was run in the batch mode until an exponential growth was initiated, at which point fresh medium was fed into the fermentor. The CSTR medium was a replica of the RSM optimized composition. The pH was maintained at the optimized value. The other experimental details are mentioned in "Batches with pH control." The fermentation volume was kept constant while being run at various dilution rates. The dilution (D) rate is defined as the reciprocal of feed and cellular residence time in the absence of cell retention (Li et al. 2011).

Analytical methods

The IG feedstock was analyzed by thermogravimetric analysis (TGA) on a TA Instruments TG Analyzer, using 50 ml/min nitrogen flow and 10 °C/min temperature ramp to 800 °C. Cell density was measured at 540 nm using a BioMate™ spectrophotometer (Thermo Spectronic, USA). Aliquot samples of known volume were centrifuged, and the biomass weight measured after washing with PBS media and drying to constant weight. The estimated biomass in g/l was numerically correlated to corresponding optical density. The glycerol and metabolite concentrations were analyzed on 0.22-μm syringe-filtered samples by gas chromatography (GC) using a DB-FFAP capillary column with an MS detector and a 1-μl injection volume. GC injector and detector temperatures were kept at 240 and 270 °C, respectively. For the analysis of the 16S rDNA sequencing data, QIIME versions 1.6 and 1.7 were used (Nelson et al. 2014)—a software package for statistically processing and analyzing sequencing data of microbial communities (Caporaso et al. 2010).

Results

Analysis and treatment of industrial glycerol

The industrial glycerol from the biodiesel run contained KOH transesterification catalyst, miscellaneous impurities, and

remnant methanol. Prior to methanol distillation, KOH was partly precipitated as KH_2PO_4 and K_2HPO_4 using H_3PO_4. The resultant IG had ~75 mass% glycerin, ~20 mass% methanol, and ~5 mass% inorganic residue after the TGA reached 800 °C which was likely remnant phosphates.

Batch fermentation

Effect of feed nutrient composition on 1,3-PD selectivity

The effect of media composition is illustrated by comparing feeds F1 and F2 (Fig. 1a, bars 1 and 2) in batches without pH control using a PG concentration of 3 g/l. The inoculum was 3 % of batch volume. The major metabolite in F1 was acetate (0.718 mol/mol gly) and with F2, the major metabolite was 1,3-PD (0.197 mol/mol gly). F2 yielded a better molar conversion to 1,3-PD. 1,3-PD: acid molar ratios were 0.19 with F1 and 1.01 with F2, which made F2 much more selective toward 1,3-PD. Although the 16S rDNA sequencing results, in a later section, show little difference between bacterial populations, feed F2 encouraged metabolism in favor of 1,3-PD.

Comparison of pure and industrial glycerol without pH control

The performances of PG and IG were compared with feeds F2 and F3, respectively. The product yields are shown in Fig. 1a (bars 3 and 6). Total molar liquid metabolite yields of 27 and 22 % were obtained with PG and IG, respectively. 1,3-PD yields of 0.247 and 0.204 mol/mol of glycerol were achieved with PG and IG feeds, respectively. During the experiment, the pH dropped from ~6.9 to ~4.5 with a concomitant decrease in bacterial growth resulting in limited glycerol utilization. The acid production was higher with PG while the solvent production was similar with both feeds (Fig. 1a). The 1,3-PD: total acids molar ratios were at 8.2 and 12.0 for PG and IG respectively. Comparing bars 2 and 3, the selectivity in favor

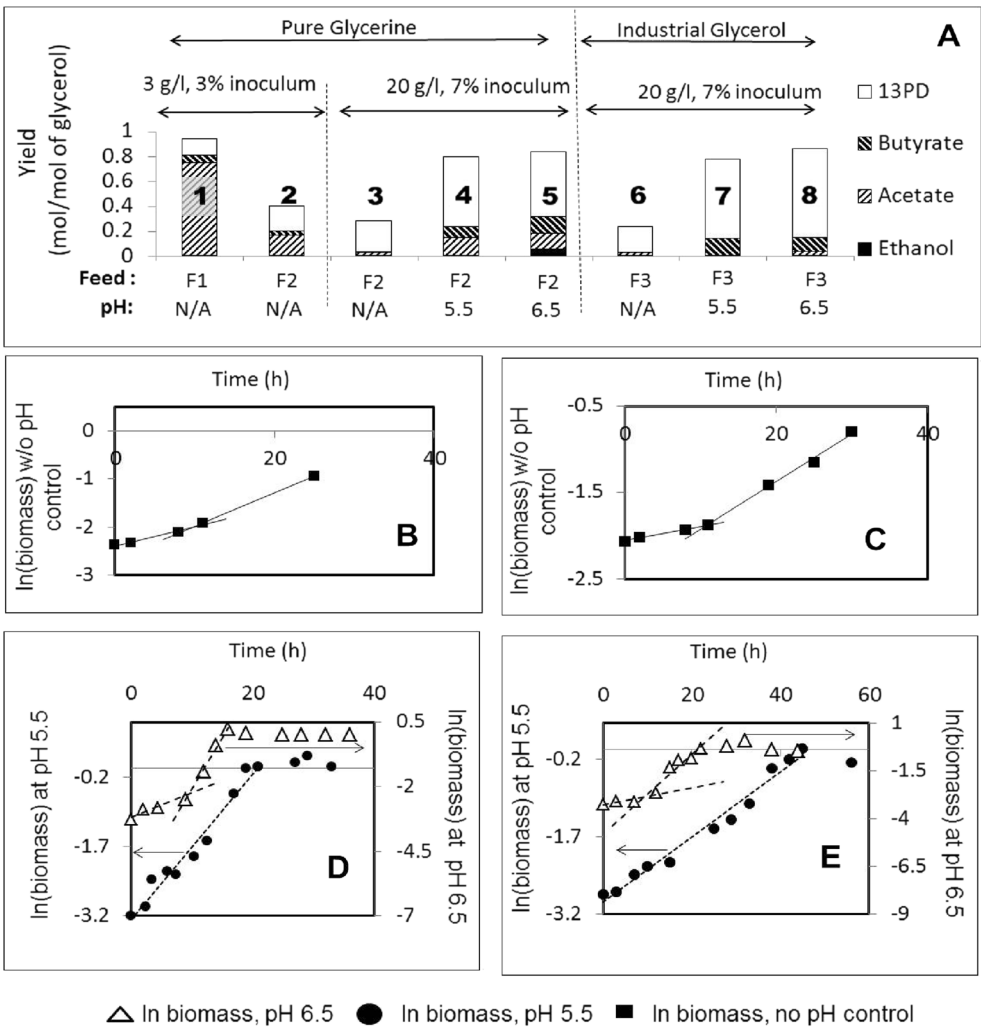

Fig. 1 Summary of batch fermentations. **a** Metabolite molar yield comparing batches at various conditions. **b**–**e** Semi-log plots ln (biomass in g dry wt/l) vs time (h) for batches with 20 g/l glycerol and 7 % inoculum. **b** Feed F2, no pH control. **c** Feed F3, no pH control. **d** Feed F2, with pH control. **e** Feed F3, with pH control

of 1,3-PD was enhanced when the glycerol feed concentration was increased from 3 to 20 g/l and the inoculum volume was increased from 3 to 7 % of the batch volume. Semi-log biomass profiles are shown in Fig. 1b, c. The liquid metabolite production was delayed with IG feed. The semi-log biomass-time plots for IG batch had a biphasic behavior (Fig. 1c) but those with PG (Fig. 1b) were not as clearly delineated. The IG feedstock with ~5 mass% phosphates and other impurities may have suppressed metabolic processes with the resultant initial lag. The metabolite profiles indicated the probable existence of *C. butyricum* as the most dominant species.

The effect of pH control on product yield

Batches with PG and IG feeds (F2 and F3, 20 g/l glycerol) were carried out at pH 5.5 and 6.5. Figure 1a (bars 4, 5 and 7, 8) summarizes the yields. At pH 5.5 (bars 4 and 7), the total metabolite molar yield of 77 % was nearly identical for the two feeds. At pH 6.5, the total metabolite molar yields were ~83 and ~85.7 %, with PG and IG, respectively, which are indistinguishable. For both feeds, the acid production increased marginally with pH, and the acetate production was slightly higher with PG than with IG. With PG, pH had little effect on the 1,3-PD molar yield. At pH 5.5, the molar 1,3-PD yields for PG and IG, respectively, were 56 and 63 %. The same changed to 52 and 71 % at pH 6.5. The molar 1,3-PD yield was higher with IG and increased with pH. This increased yield was at the expense of selectivity as the 1,3PD: acids ratio dropped to ~2.5 and ~4.9 for PG and IG, respectively. The limit of glycerol utilization here had much to do with toxic intermediate buildup.

At pH 5.5, exponential growth is seen with both feeds with no identifiable lag phase (Fig. 1d, e). The growth was faster in PG. At pH 6.5, both feeds exhibited a poor fit to an exponential pattern unless a lag phase was included (Fig. 1d, e). Fits to biphasic growth patterns at pH 6.5 (Fig. 1d, e) indicate a ~10-h lag phase for both PG and IG feeds.

Batch results at pH 6.5 with PG and IG feeds of 30 g/l showed a drop in 1,3-PD yield by 8 % for PG and ~19 % for IG compared to batches with 20 g/l glycerol concentration. The initial lag time increased from ~10 to ~15 h, but maximum biomass levels were unchanged. With even a higher IG concentration of 42 g/l, initial growth lag times increased from ~15 to ~24 h, and a stunted biomass growth, inefficient glycerol utilization, and even lower 1,3-PD yields were observed (0.27 mol/mol). With 58 g/l IG, the biomass did not reach an exponential growth stage.

16s rDNA sequencing

The DNA sequencing yielded information on the relative populations of species and genera comprising the inoculum. A total of 160 bacterial species were identified in the initial inoculum, which decreased to 133 and 146, respectively, with fermentation in feeds F1 and F2 (with 3 g/l glycerol, 3 % inoculum) without pH control. pH control with a PG feed caused further reduction, especially at pH 6.5. The reduction in the number of species or the effect of pH were less evident with IG feeds. The results are shown in Fig. 2a. Figure 2b shows Shannon index and Simpson's index of diversity. The Shannon index (SI) increases with richness and evenness of the community. Simpson's index of diversity (SID), a measure of dominance, also increases with diversity (Tuomisto 2012). The reduction in SI and SID in F1 and F2 from the starting inoculum reflected reduced diversity, rise of the dominance of specific genera, and the obliteration of some classes. The indices further dropped with pH control underscoring population unevenness and dominance by the *Clostridia* genus. In the initial inoculum, *Clostridia* and *Enterobacter* were the most populous genera comprising 45 and 30 % of the population followed by unidentifiable genera from Clostridiaceae and Enterobacteriaceae families at 15 and 9 %, respectively. A diversity of 35 identified minor members and a host of unidentified classes were grouped together, and these comprised the rest of the population in the initial inoculum. The results are presented in Fig. 2c. The average populations of *Clostridia* and *Enterobacter* genera were preserved in F1 and F2 batches without pH control.

Design of experiments and analysis of variance

Response surface methodology (RSM) was employed to optimize IG fermentation with 17 batch fermentations and final 1,3-PD concentration as the response variable. Three experimental factors, pH (A), feed IG concentration (B), and yeast extract concentration (C) as independent variables, were put in a Box-Behnken design and used at three levels between minima and maxima (Montgomery 1996). The ranges of the pH, IG concentration, and yeast extract concentration were 4.8–7.2, 15–35 g/l, and 0.5–4 g/l, respectively. The design center experiment was repeated five times to estimate the error. The regression and test factor coding is detailed in Noshadi et al. (2012). Table 1 provides the 1,3-PD concentration at the end of each batch. Biomass and metabolite profiles were noted for each experiment as a quality control check, but those details are not necessary for this discussion. The lag time for initial bacterial growth increased with glycerol feed concentration. The final metabolite concentrations were estimated by GC from samples taken once the inoculum had entered a stationary phase. The experimental response was related to independent variables by an optimization model (Eq. 1).

$$\eta = \beta_0 + \sum_{j=1}^{k} \beta_j x_j + \sum_{j=1}^{k} \beta_{jj} x_j^2 + \sum_i \sum_{<j=2}^{k} \beta_{ij} x_i x_j + e_i \quad (1)$$

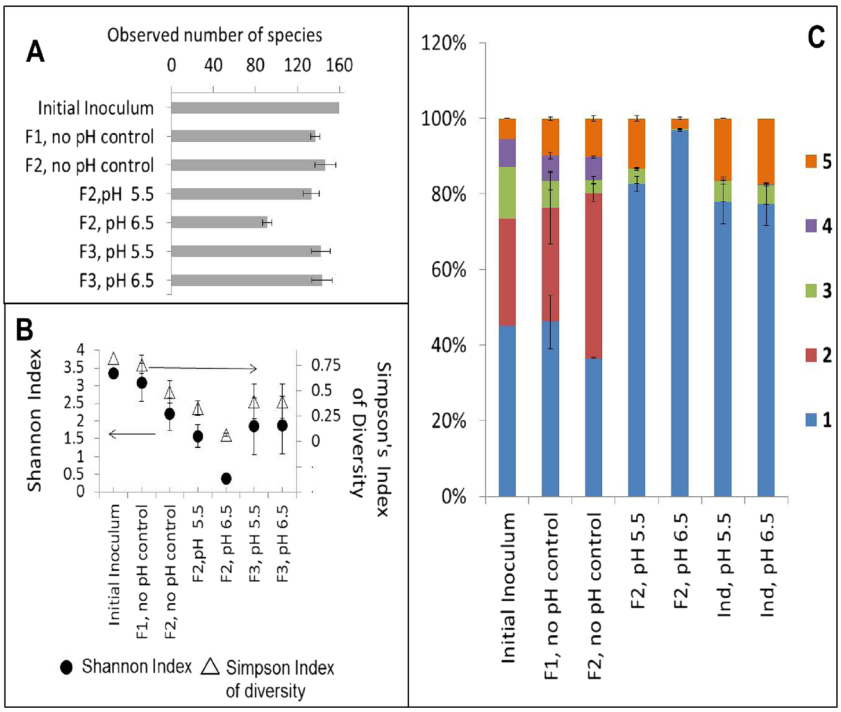

Fig. 2 Summary of 16S r-DNA sequencing results. **a** Average observed number of species. **b** Shannon index and Simpson's index of diversity. **c** Population distribution: *1*—phylum_*Firmicutes*, class_*Clostridia*, order_*Clostridiales*, family_*Clostridiaceae*, genus_*Clostridium*; *2*—p_*Proteobacteria*, c_*Gammaproteobacteria*, o_*Enterobacteriales*, f_*Enterobacteriaceae*, g_*Enterobacter*; *3*—p_*Firmicutes*, c_*Clostridia*, o_*Clostridiales*, f_*Clostridiaceae*, Other; *4*—p_*Proteobacteria*, c_*Gammaproteobacteria*, o_*Enterobacteriales*, f_*Enterobacteriaceae*, Other; *5*—miscellaneous strains

η is the final 1,3-PD concentration, x_i and x_j are the independent factors of pH, glycerol concentration, and yeast concentration, β_0 is the constant coefficient, β_j, β_{jj}, and β_{ij} are the coefficients for linear, quadratic, and interaction effects, and e_i is the error. Coefficients of determination R^2 and R^2_{adj} were used to indicate the quality of fit. Statistical significance was checked with F value, p value, and estimates detailed in Myers and Montgomery (2000) and Noshadi et al. (2012). Experimental response and independent variables were correlated by Eq. (2). Least squares regression was used to obtain a best fit model.

$$\eta = +8.35426 + 2.70258*A - 1.30514*B + 0.15521*A*B + 0.013780*B^2 + 0.61289*C^2 \quad (2)$$

Table 1 Experimental design results

Experiment no.	pH	Glycerol feed concentration (g/l)	Yeast extract concentration (g/l)	1,3-PD concentration (g/l)
1	7.2	15	2.25	7.98
2	6	25	2.25	9.14
3	6	35	4	17.50
4	4.8	25	0.5	7.22
5	4.8	25	4	7.50
6	4.8	15	2.25	4.20
7	6	25	2.25	9.71
8	6	25	2.25	9.29
9	6	15	0.5	8.82
10	4.8	35	2.25	8.16
11	6	35	0.5	16.50
12	6	15	4	6.30
13	7.2	35	2.25	19.40
14	7.2	25	4	13.92
15	6	25	2.25	8.16
16	6	25	2.25	8.82
17	7.2	25	0.5	13.10

Table 2 shows the ANOVA evaluations of this model. Appropriateness of fit was measured by F value, R^2, p value, and lack of fit (Myers and Montgomery 2000). Large F values and low p values indicate significance of linear terms for pH (A) and IG concentration (B) and underscores strong correlations of the quadratic terms for the glycerol concentration (B) and yeast concentration (C) with the response. The coefficient of term AB indicates significant coupling effect of pH with IG concentration on 1,3-PD concentration. Low p values <0.0001 indicate that the chance of the model F value arising due to experimental noise is negligible. The lack of fit F value of 3.29 indicated its insignificance relative to pure error. The regression equation and coefficient of determination were evaluated to test the fit of model. Randomly scattered studentized residuals vs responses (Fig. 3a) indicate that variation in original observations is not related to response value. The

Table 2 ANOVA for the regression model and respective model terms

Source	Sum of squares	*df*	Mean square	*F* value	*p* value Probability>F	
Model	281.507	9	31.27856	47.01922	<0.0001	significant
A	93.34042	1	93.34042	140.3132	<0.0001	
B	146.7137	1	146.7137	220.5461	<0.0001	
C	0.022184	1	0.022184	0.033348	0.8603	
AB	13.876	1	13.876	20.85897	0.0026	
AC	0.072387	1	0.072387	0.108816	0.7511	
BC	3.097019	1	3.097019	4.655568	0.0678	
A^2	0.913004	1	0.913004	1.372466	0.2797	
B^2	7.995	1	7.995	12.01841	0.0105	
C^2	14.83379	1	14.83379	22.29876	0.0022	
Residual	4.656604	7	0.665229			
Lack of fit	3.315028	3	1.105009	3.294661	0.1398	not significant
Pure error	1.341576	4	0.335394			
Correlation total	286.1636	16				

experimental vs computed 1,3-PD concentration, from Eq. 2, is plotted in Fig. 3b. A high adjusted determination coefficient underscores significance of the model (Myers and Montgomery 2000; Noshadi et al. 2012). Numerical optimization was used to find the experimental factors expected to give the highest 1,3-PD concentration. Optimum conditions of 34.9 g/l glycerol, pH 7.2, and yeast extract 3.4 g/l were determined.

Continuous production of 1,3-propanediol

Continuous fermentation (CSTR) of IG to 1,3-PD was conducted with RSM optimized conditions at progressively increasing dilution rates, and steady states were identified. The fermentation was switched to CSTR once an exponential bacterial growth initiated. This corresponded with acidogenesis as the PID control system responded by switching on the base supply to maintain

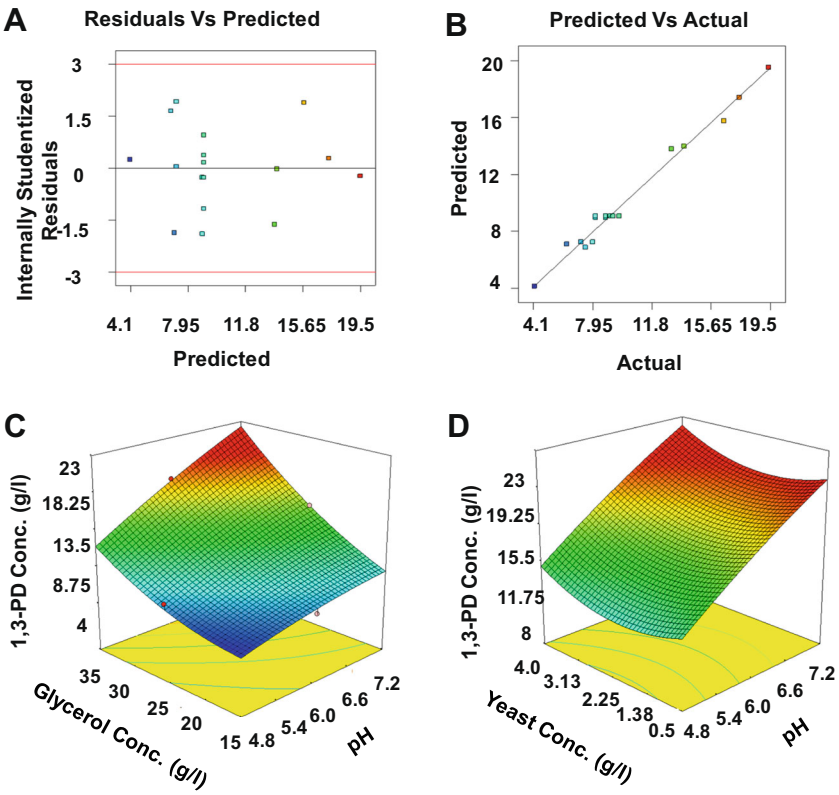

Fig. 3 Summary of design of experiments. **a** Studentized residuals and predicted response plot. **b** Predicted response vs actual response plot. **c** Surface graph of the final 1,3 propanediol concentration at different glycerol concentrations and pH. **d** Surface graph of the final 1,3 propanediol concentration at different yeast extract concentrations and pH

the pH. Two individual runs, each lasting >500 h, were carried out. The biomass, feed and remnant glycerol, and metabolite concentration profiles are presented in Fig. 4a, b, c, for the first run. Three steady states at dilution (D) rates of 0.04, 0.11, and 0.19 h^{-1} were achieved for the first CSTR run. The details of the second CSTR run and the steady states of the first run at D=0.11 h^{-1} and D=0.19 h^{-1} are presented in "Supplementary figures." Figure 4d, e, f, g show CSTR profiles on expanded time scales at D=0.04 h^{-1} for the first CSTR run. After 480 h, when the system returned to D=0.04 h^{-1}, the metabolite profiles did not recover to earlier levels, even though the biomass recovered. This indicated solvent degeneration, typical in clostridial fermentations (Li et al. 2011).

The steady state volumetric and specific glycerol consumption rates, molar yields, and productivities were computed. The 1,3-PD productivity (Fig. 5a) went through a maximum of 1.2 g/l hr and yield of 0.6 mol/mol of glycerol at D=0.11 h^{-1}. This trend was replicated by all metabolites. The volumetric glycerol consumption rate (Fig. 5b) increases with dilution rate. 1,3-PD molar yield decreased from 0.65 to 0.50 mol/mol gly with increase in dilution from 0.04 to 0.19 h^{-1} (Fig. 5c). A similar trend in molar yields of other metabolites was observed. The solvent to acid ratio increased sharply at D=0.19 h^{-1} for both runs, plausibly due to a protracted or late acidogenesis of a species in the consortium. Despite increased specific glycerol consumption, molar yield decreased with dilution rate (Fig. 5c) as the time allowed for residence and fermentative conversion decreased.

The specific metabolite productivity of 1,3-PD, specific glycerol consumption rates (Fig. 6a, b) increased with dilution rate with the former reaching a maximum of 1.5 g/g of biomass hr. The specific ethanol, acetate, and butyrate productivities went through a maximum at D=0.11 h^{-1} (Fig. 6a). The 1,3-PD productivities and specific productivities were comparable to CSTR results of *C. butyricum* VPI 3266 on

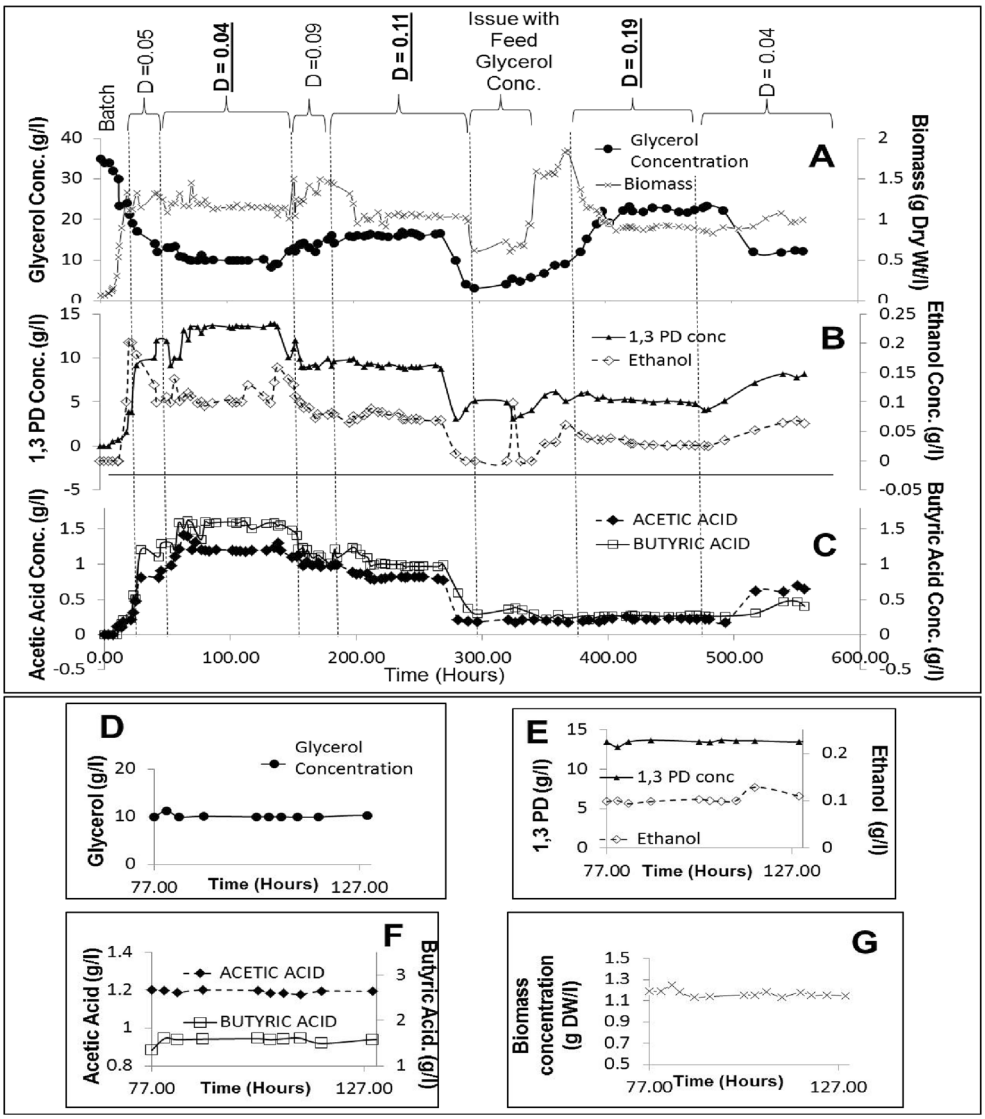

Fig. 4 Continuous fermentation profiles, run no. 1. **a** Biomass and glycerol concentrations. **b** Solvent concentrations. **c** Acid concentrations, dilution rates are indicated between *vertical dotted lines* and bold dilution rates are those for which well-developed steady states were attained. **d**–**g** Steady state profiles on expanded time scale between 77 and 127 h, D=0.04 h^{-1}. **d** Glycerol concentration. **e** Solvent concentration. **f** Acid concentration. **g** Biomass concentration

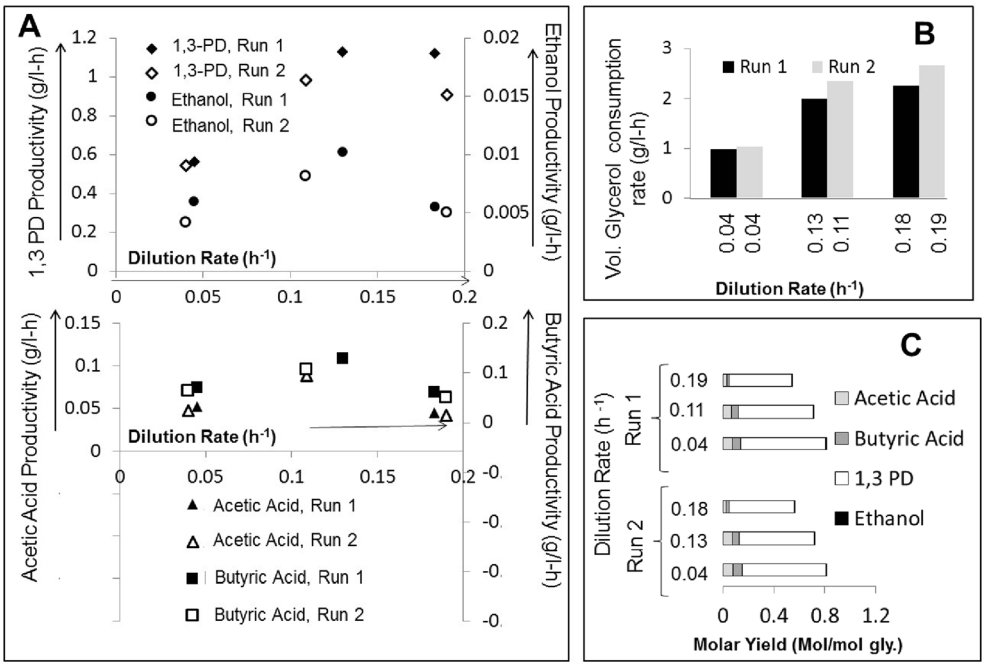

Fig. 5 **a** Metabolite productivities (g/l-h) vs dilution rate (h^{-1}). **b** Volumetric glycerol consumption rate (g/l-h) vs dilution rate (h^{-1}). **c** Molar yields of liquid metabolites (mol/mol of glycerol)

raw and pure glycerol at D=0.11 h^{-1} (Gonzalez-Pajuelo et al. 2004). Productivities and yields higher than those reported here were found in studies based on pure strains using pure glycerin feed (Kaur et al. 2012). Table 3 lists a comparison between select glycerol to 1,3-PD fermentations with a variety of cultures and feeds.

The volumetric productivity and specific productivity follow dissimilar trends with increasing dilution rate, and this apparent contradiction is discussed below.

Discussion

Batch fermentations

The 1,3-PD production appeared growth rate dependent in pH controlled batch experiments. Solventogenesis slowed bacterial growth and efficiency due to 3-hydroxypropionaldehyde buildup—a toxic intermediate (Biebl 2001). Although pH is not a solventogenesis trigger, it is a key factor in clostridial

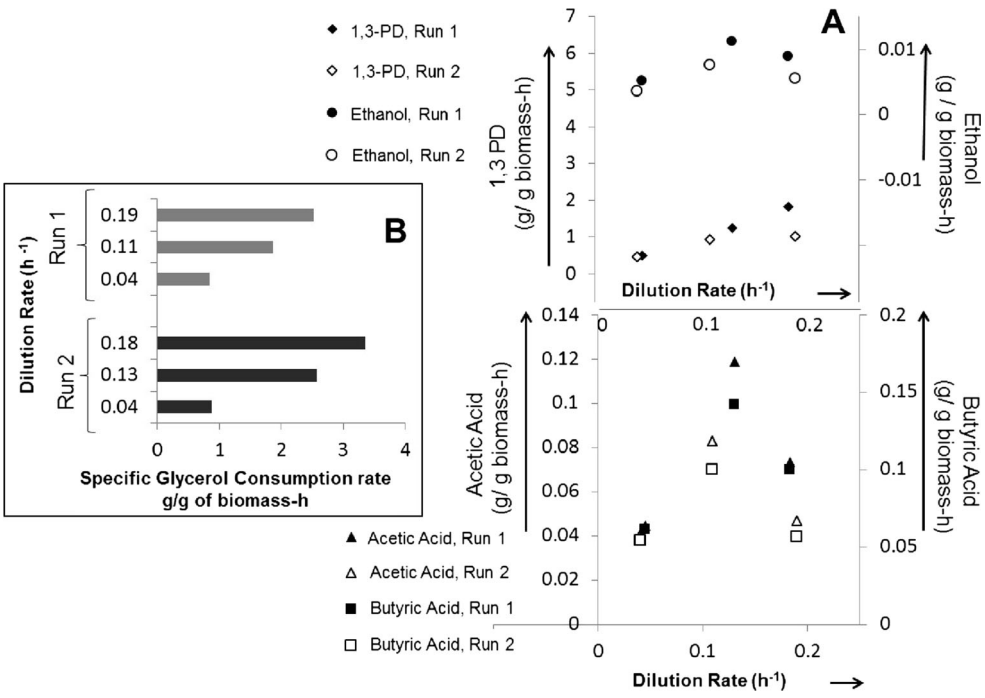

Fig. 6 **a** steady state specific metabolite productivity (g/g of biomass-h). **b** specific glycerol consumption rate (g/g of biomass-h)

Table 3 Comparison of selected studies on fermentative production of 1,3-propanediol from pure and raw glycerol

Inoculum	Process	Yield (mol/mol gly)	Reference
Feed: *pure glycerol*			
C. butyricum (VPI 3266)	Batch Fed batch	0.65 0.69	Saint-Amans et al. (1994)
K. pneumoniae M5al *K. pneumoniae* AC15 *K. pneumoniae* DSM 2016	Batch Fed batch Continuous	0.53 0.64 0.61	Cheng et al. 2007 Zheng et al. 2008 Menzel et al. (1997)
Enterobacter agglomerans	Batch	0.51–0.61	Barbarito et al. (1995)
Organic Soil: Iterative reinoculation	Batch	0.56	This Work
Feed: *raw glycerol*			
C. butyricum (VP 1718)*	Batch Fed batch Continuous	0.63 0.66 0.63 (0.02 h^{-1}), 0.64 (0.04 h^{-1}), 0.60 (0.06 h^{-1}), 0.62 (0.08 h^{-1}), 0.64 (0.1 h^{-1})	Chatzifragkou et al. (2011)
K. oxytoca FMCC-197′*	Batch Fed batch	0.56 0.24	Metsoviti et al. (2012)
Sludge bacterial culture	Batch Fed batch	0.51–0.76 0.52–0.56	Dietz and Zheng, 2014
Organic soil, heat treated	Batch	0.65	Liu et al., 2013
Tomato soil, heat treated	Batch	0.69	Selembo et al., 2009
Treated granular sludge*	Continuous (EGSB reactors)	0.52 (0.08 h^{-1})	Gallardo et al. 2014
Organic soil: inoculum prepared by iterative reinoculation procedure	Batch, DOE, RSM optimization CSTR	0.71 (batch) 0.658–0.664 (0.04–0.045 h^{-1}) 0.586–0.583 (0.11–0.13 h^{-1}) 0.506–0.517 (0.183–0.19 h^{-1})	This work

*Mass yields reported were converted to molar yields

fermentations, such as acetone-butanol-ethanol processes, where low pH is conducive to solventogenesis and detrimental to growth (Jones and Woods 1986). However, in the experiments reported here, a higher pH enhanced bacterial growth and 1,3-PD yield. Although the course of growth and metabolism is dynamically influenced by acid concentrations in clostridial fermentation (Colin et al. 2001), in these experiments, acids and 1,3-PD were produced simultaneously. 1,3-PD was the dominant product in all batches followed by butyrate, acetate, and ethanol. Remarkably, 1,3-PD molar yield at pH 6.5 was enhanced with IG feed, relative to PG, despite slower biomass growth. At pH 5.5, 1,3-PD production was delayed with IG. Clearly, both pH and nutrient conditions play a strong role in determining performance, but conditions were found that were very favorable for 1,3-PD production.

Inhibition was observed at glycerol feed concentrations of 30 g/l and higher. While the reduction of 1,3-PD yield was only 8 % with PG, it was 19 % with IG at feed concentration of 30 g/l. This may indicate glycerin inhibition in the case of PG (Biebl 1991), and glycerin and phosphate inhibition in the case of IG. The inhibition becomes greater as IG feed concentration increases until biomass growth is completely inhibited at 58 g/l. Phosphates may enhance metabolic performance of bacterial cultures, but may be toxic at higher concentrations (Stewart 1975; Qureshi et al. 2001). Each strain in the 160 species of the mixed inoculum used in this work may have individual phosphate responses. It is most probable that the phosphates in our IG feed improved fermentation performance at lower IG feed concentrations, compared to PG, but became toxic beyond a limit.

16S rDNA sequencing

The DNA sequencing results underscored the presence of 1,3-PD-producing genera in the initial inoculum. With fermentations, even without pH control, the diversity of the bacterial population decreased. While the feed composition had little effect on the relative species' populations, feed F2 made it conducive for the production of 1,3-PD for the predominant species even without pH control. For batches without pH control, a drop in pH during the first 20 h of the experiments (exponential growth) and concomitant stunted biomass growth allowed little time for disparities between the populations of different species to develop. However, the 16S rDNA sequencing of bacterial population at the end of the batches revealed a significant effect of pH on growth and obliteration

of specific genera. Control of pH allowed a marked growth of *Clostridia* genus to 80–95 % of the population while nearly obliterating the *Enterobacter* genus. With pH control, the bacterial population diversity dropped further with rising dominance of specific genera and the obliteration of some classes.

DNA sequencing results are consistent with the metabolite profiles from the fermentations. While *Clostridium* is anaerobic, *Enterobacter* is facultatively aerobic. The main byproducts with clostridial glycerol fermentation are acetate and butyrate and that with *Enterobacter* are ethanol and acetate (Barbarito et al. 1995). With *Enterobacter*, pyruvate is cleaved to acetyl-CoA or condensed to α-acetolactate with subsequent transformation to acetoin and 2,3-butanediol. Very little ethanol was produced in the fermentations while the major secondary products were acetate and butyrate, consistent with the dominance of clostridial species.

Statistical DOE—influence of experimental parameters on the final 1,3-PD concentration

The regression results in Eq. 2 indicate that pH had the strongest effect on the final 1,3-PD concentration (Figs. 3b, c). The 1,3-PD concentration was an increasing function of both pH and IG concentration but went through a minimum at ~2.5 g/l of yeast extract concentration. High final metabolite concentrations are a natural outcome of enhanced concentration of carbon source in feed, and hence, underscoring the significance may seem redundant. However, it may be stressed that batch experiments with >35 g/l glycerol failed to give appreciable bacterial growth even after >24 h of lag time, and hence, the feed IG had to be limited to a maximum of 35 g/l for the DOE. As with earlier batches, growth suppression may be linked to remnant phosphates and impurities in IG.

CSTR

It may be recalled that in pH-controlled batches, solventogenesis was apparently independent of acid concentration and 1,3-PD was concomitantly produced with the acids. Increased dilution rates in CSTR, counterintuitively, enhanced the solvent to acid ratios (Fig. 5C). This is in contrast to biphasic (e.g., ABE glucose) fermentations, where solventogenesis is subsequent to acidogenesis, high dilution rates keep the inoculum in acidogenesis, and low dilution rates allow time for solventogenesis and molecular reassimilation (Jones and Woods 1986). In ABE fermentation, butanol and butyrate are formed via butyryl CoA whose conversion to either metabolite allows for molecular reassimilation. Here, 16S sequencing results prove clostridial domination at controlled pH. In clostridial glycerol fermentation, butyric acid and 1,3-PD are on competitive, not complimentary routes. The acids and 1,3-PD form via respective intermediates—dihydroxyacetone (DHA) and 3-hydroxypropionaldehyde (3HPA) (Ragauskas et al. 2006). Simple molecular reassimilation pathway between acids and 1,3-PD is absent. The butyrate competes with 1,3-PD for regenerating $NADH_2$ equivalents. The oxidative branch converts glycerol to DHA by NAD+ dependent glycerol dehydrogenase and phosphorylates it to enter glycolysis, resulting in acids. The remaining glycerol is dehydrated to 3HPA by a dehydratase and reduced to 1,3-PD by an NAD+-dependent oxidoreductase. The reductive branch regenerates NAD+ via butyrate synthesis from acetyl-CoA. This needs two $NADH_2$-oxidizing steps per butyrate molecule and is antagonistic to 1,3-PD production (Papanikolaou, et al. 2004).

At lower D, the average age of cells is higher with longer toxic intermediate exposure, which may increase the propensity for solvent degeneration. In a CSTR, cells are washed away and replaced via cellular multiplication. At steady state, the rate of biomass production by cellular multiplication is matched by the rate at which the cells are washed out of the fermenter. The rate of biomass production is therefore given by $[m]^{\bullet}_{p} = [m]{\bullet}D$, where [m] is the steady state biomass concentration and D is the dilution rate. The computed steady state values of $[m]^{\bullet}_{p}$, in (grams dry wt) per liter per hour, were 0.046, 0.117, and 0.174 at respective D=0.04, 0.11, and 0.19 h^{-1}. Thus, mass balance reveals that bacteria multiplied faster at higher dilution rates (Fig. 4a). Younger cells, with lower toxic intermediate exposure, are more efficient 1,3-PD producers leading to increased specific 1,3-PD productivity.

Over the course of the CSTR experiment, the bacterial population and its distribution may have undergone a significant statistical evolution with the obliteration and preservation of specific species. In addition to this statistical population evolution, genetic or mutative evolutions may also be at play determining the CSTR profiles, a detailed study of which is beyond the scope of this work. Drawing from the fermentation results and the DNA sequencing experiments, both of these evolutionary changes can contribute to shifts in metabolic propensities. The CSTR study of a multispecies culture thus presents future opportunities for evaluation of a directed evolution phenomena of bacterial population statistics—a combination of systematic screening and mutative evolutions over generations of bacterial growth.

Studies on batch, 16S rDNA sequencing, statistical DOE, and CSTR production of 1,3-PD from waste glycerol with a soil-based inoculum were presented. The iteratively inoculated starting culture, feed composition, and pH control enhanced 1,3-PD selectivity, productivity, and selectively bred 1,3-PD-producing bacteria as shown by 16S rDNA sequencing. The DOE was used to optimize conditions for CSTR, in which 1,3-PD molar yield decreased but selectivity and specific productivity increased with dilution rate due to faster bacterial growth and reduced solvent degeneration. 13-PD

productivity and molar yields obtained were comparable to studies with pure strains using pure glycerin feed.

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