Increased Butanol Yields through Cosubstrate Fermentation of Jerusalem Artichoke Tubers and Crude Glycerol by *Clostridium pasteurianum* DSM 525

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**ABSTRACT:** *Clostridium pasteurianum* DSM 525 can produce butanol, 1,3-propanediol, and ethanol from glycerol. The product distribution can be tilted toward butanol when adding butyric acid. The strain predominantly produces acetic and butyric acids when grown on saccharides. Hence, butyrate formed from saccharide conversion can be used to stimulate butanol production from glycerol under cosubstrate cultivation. The optimal cosubstrate ratio was determined, and under optimal conditions, a butanol yield and a productivity of $0.27 \pm 0.01 \text{ g L}^{-1} h^{-1}$ and $0.74 \pm 0.02 \text{ g L}^{-1} h^{-1}$ were obtained. On the basis of these results, batch fermentation in a 5 L bioreactor was performed using Jerusalem artichoke hydrolysate (carbohydrate source) and crude glycerol (residue from biodiesel production) at the previously determined optimal condition. A butanol yield and a productivity of $0.28 \pm 0.007 \text{ g L}^{-1} h^{-1}$ and $0.55 \pm 0.008 \text{ g L}^{-1} h^{-1}$ were achieved after 27 h fermentation, indicating the suitability of those low-cost carbon sources as cosubstrates for butanol production via *C. pasteurianum*.

### INTRODUCTION

Butanol is a potential advanced biofuel that can be blended with gasoline and diesel.\(^1\)–\(^4\) Because of increased substrate costs and availability of less expensive petrochemically derived butanol in the 1950s, most of the acetone/butanol/ethanol (ABE) fermentation plants were closed.\(^4\),\(^5\) However, in the past decade, the interest in butanol fermentation has been revived, which led multiple studies on strain development, fermentation improvement, and in situ product removal technologies.\(^6\)–\(^9\) This has resulted in a major reduction of product toxicity to the respective microorganisms, improved substrate utilization, as well as the overall performance of the bioreactors. Nevertheless, the high cost and seasonal availability of conventional substrates (corn, molasses) are still a disadvantage for fermentative butanol processes compared to petroleum-based production.\(^6\),\(^7\) In order to realize industrial-scale butanol fermentation, it is crucially important to identify available low-cost biomass feedstock that is fermentable by *Clostridium* species.\(^8\)–\(^14\) Glycerol is a byproduct generated during biodiesel production. It is a potential substrate for bio-based production of chemicals and fuels, leading to multiple recent studies.\(^15\)–\(^19\) As a result of global increase in biodiesel production, surplus quantities of biodiesel-derived glycerol (crude glycerol) are becoming available.\(^16\) Crude glycerol is contaminated with various impurities, which makes it unsuitable for conventional outlets (cosmetics, soaps). Further purification is possible, though the high cost is rendering it less attractive after significant decrease of glycerol’s market price.\(^20\),\(^21\) Hence, effective utilization of crude glycerol is crucial to enhance the economy of biodiesel industry.\(^20\)

The most studied microorganism for butanol production from glycerol is *Clostridium pasteurianum*. It can utilize glycerol as a sole carbon source and converts it into 1,3-propanediol (1,3-PDO), butanol, and ethanol, referred to as PBE fermentation.\(^22\)–\(^25\) The process is different from the more common traditional Weizmann process converting carbohydrates to ABE.\(^26\) When using saccharides as the sole carbon source for *C. pasteurianum*, it mainly produces organic acids such as acetic and butyric acids.\(^19\),\(^27\) Butyrate is an intermediate in the respective fermentation pathway, leading to butanol, and the external addition of butyrate can significantly and efficiently enhance butanol production when glycerol is the sole carbon source.\(^28\)–\(^31\) The addition of acetate has also been reported to enhance butanol production for some *Clostridium* species.\(^31\),\(^32\) However, *C. pasteurianum* appears to not fully convert these acids when grown on saccharides, a limitation that does not exist with glycerol as the main carbon source. The addition of butyric acid to fermentative glycerol conversion by *C. pasteurianum* has been shown to shift its product distribution toward butanol.\(^33\)–\(^35\) An alternative to adding butyrate to the fermentation medium is to utilize a cosubstrate system, which can take advantage of the substrate with lower costs than butyrate.

Jerusalem artichokes (Helianthus tuberosus L.) have been shown as an alternative source of saccharides for the fermentative production of butanol in the ABE process; they...
can be cultivated on the marginal land and are resistant to typical plant diseases, hence there is no direct competition with grain crops for the arable land.\textsuperscript{36–39} Jerusalem artichoke tuber (as all members of the Asteraceae family) is a rich source of inulin, a carbohydrate storage polymer of linear chains of $\beta$ (2 $\rightarrow$ 1)-linked $\alpha$-fructofuranosyl units with $\alpha$ (1 $\rightarrow$ 2) bond-linked fructose cap.\textsuperscript{39} Inulin is the principal storage carbohydrate in the tuber (15–20%); however, monomeric sugars such as sucrose, glucose, and fructose can also be present.\textsuperscript{40} The vast number of microorganisms is incapable of directly fermenting inulin; therefore, prior hydrolysis to fructose and glucose is required (enzymatically or via an acid catalyst).\textsuperscript{36,41} By comparison, hydrolysis via an acid catalyst can reach up to 98.5% conversion in 35 min with byproduct concentrations below the inhibitory threshold, while enzymatic conversion of the same Jerusalem artichoke extract requires 24 h.\textsuperscript{36} Hydrolysis time and catalyst costs will likely render acid hydrolysis to be the favorable option. Availability and cost competitiveness of crude glycerol and Jerusalem artichoke tubers make both excellent candidates for butanol production.

Using Jerusalem artichoke hydrolysate (JAH) as the source of carbohydrates and glycerol as the main carbon source for \textit{C. pasteurianum} might lead to the conversion of carbohydrates to organic acids, which then stimulate the simultaneous butanol formation from glycerol. Therefore, this study was undertaken to assess the feasibility of employing the aforementioned cosubstrate strategy for the enhanced butanol production with the \textit{C. pasteurianum} DSM 525. In order to establish such a system, the effect of adding acetate and butyrate on butanol formation by \textit{C. pasteurianum} DSM 525 (from glycerol) was first investigated and confirmed. The product formation by the same strain using different monosubstrates was studied, followed by an optimization study of the cosubstrate ratio. On the basis of the estimated optimal conditions, JAH and crude glycerol (from the biodiesel manufacturing waste) were used as low-cost carbon sources for the cosubstrate-based butanol production in a 5 L laboratory bench bioreactor.

\section*{RESULTS AND DISCUSSION}

The main carbon source of the proposed process is glycerol, with Jerusalem artichoke-derived carbohydrates functioning as a secondary substrate. Hence, monomeric sugars were produced through acid hydrolysis of Jerusalem artichoke tubers. The Jerusalem artichoke tuber had a total solid content of $\sim$30% (oven-dried at 80 °C for 72 h).\textsuperscript{42} The carbohydrate composition (inulin and free sugars) of the material before and after acid hydrolysis is shown in Table 1. Over 91% of the initial available carbohydrates were recovered as monomeric sugars after the hydrolysis consistent with previously reported data.\textsuperscript{42} The bulk material was at random location to verify homogeneity, which was given as seen by the small standard deviation.

The goal of this study was to increase butanol formation from glycerol through the cometabolism of organic acids produced by the same organism from sugars. Therefore, initially, the effect of direct addition of acetic and butyric acids was investigated in batch cultures with a medium containing 50 g L$^{-1}$ of pure glycerol. As shown in Figure 1a, the addition of acetate improved the butanol yield from 0.28 ± 0.008 to 0.31 ± 0.015 g butanol g (glycerol+acetate)$^{-1}$, as the added acetate increased from 0 to 5 g L$^{-1}$. However, the butanol productivity started to decrease from 0.180 ± 0.005 to 0.160 ± 0.006 g L$^{-1}$ h$^{-1}$ as acetate addition was increased from 3 to 5 g L$^{-1}$ (Figure 1a). The addition of 3.0 g L$^{-1}$ acetate improved the butanol yield by 10.7% without reducing the rate. A study on the effect of acetate addition (up to 6 g L$^{-1}$) on solvent production from 60 g L$^{-1}$ of glucose by \textit{Clostridium beijerinckii} NCIMB 8052 and \textit{C. beijerinckii} BA 101 indicates that the addition of acetate could improve the butanol production, but for acetate addition greater than 4.7 g L$^{-1}$, the butanol production started to decrease.\textsuperscript{32} However, unlike the work in this study, the respective strains followed the ABE pathway using carbohydrates. It was also reported that the effect of acetate on increasing butanol production was correlated to the increase in the coenzyme-A transferase, an enzyme that plays a key role in the butanol pathway.\textsuperscript{32,43}

As shown in Figure 1b, the addition of butyrate improved the butanol production yield from 0.28 ± 0.008 to 0.37 ± 0.005 g butanol g (glycerol+butyrate)$^{-1}$, as the butyrate concentration added was increased from 0 to 5 g L$^{-1}$. The influence on the 1,3-PDO production and therefore on the butanol/1,3-PDO ratio (data not shown) is of larger relevance to this study, as also reported elsewhere.\textsuperscript{35,35} The concentration of 1,3-PDO decreased from 6.4 ± 0.17 to 4.6 ± 0.12 g L$^{-1}$ as the added butyrate concentration was increased from 0 to 5 g L$^{-1}$. The reported values for butanol yields are within the range typically achieved in the literature for pure glycerol ($0.26–0.36$ g g$^{-1}$) and crude glycerol ($0.21–0.30$ g g$^{-1}$), as reviewed elsewhere.\textsuperscript{22} The butanol production rate however started to decrease from 0.37 to 0.26 g L$^{-1}$ h$^{-1}$ as butyrate was increased from 4 to 5 g L$^{-1}$. The results show that the addition of 4.0 g L$^{-1}$ butyrate

\begin{table}[h]
\centering
\caption{Extractable Carbohydrates from Jerusalem Artichoke Tubers (Average of Triplicates ± Standard Deviation) before and after Acid Hydrolysis, Following Previously Published\textsuperscript{42} Extraction and Quantification Protocols}
\begin{tabular}{|l|c|c|}
\hline
\textbf{compound} & \textbf{Sugar Jerusalem artichoke$^{-1}$} & \\
 & \textbf{raw material} & \textbf{hydrolysate} \\
\hline
inulin & 0.52 ± 0.05 & 0.01 ± 0.01 \\
fructose & 0.16 ± 0.02 & 0.60 ± 0.09 \\
glucose & 0.10 ± 0.01 & 0.15 ± 0.06 \\
sucrose & 0.05 ± 0.01 & 0.07 ± 0.01 \\
\hline
\end{tabular}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure1.png}
\caption{Effect of acetate (a) and butyrate (b) addition on butanol yield and productivity [anaerobic, 50 mL (in 150 mL shake flasks), 30 °C, 200 rpm, 40 h, 50 g L$^{-1}$ glycerol, pH 6.8]. The values are average of triplicate measurements ± standard deviation.}
\end{figure}
improved the butanol yield to \(0.36 \pm 0.004 \, \text{g} \, \text{butanol} \, (\text{g} \, \text{glycerol} + \text{butyrate})^{-1}\) (22%) without decreasing the butanol production rate. A related study investigated the effect of butyrate addition using a fermentation medium containing 100 g L\(^{-1}\) of glycerol supplemented with 0, 2, 4, 6, 8, and 10 g L\(^{-1}\) of butyrate by \(C.\) pasteurianum CH4.\(^{28}\) The results show that the addition of butyrate could improve the butanol yield, but any further addition of butyrate greater than 6 g L\(^{-1}\) decreased the butanol production rate. Another study investigated the impact of butyric acid on butanol formation by \(C.\) pasteurianum DSM 525 under pH-controlled condition using 45 g L\(^{-1}\) glycerol supplemented with butyric acid.\(^{36}\) It was concluded that the addition of butyric acid could improve the butanol yield in moderate amounts (3 g L\(^{-1}\)) without decreasing the production rate; however, the metabolic rate decreased, and the initial lag phase increased at elevated concentrations (>4 g L\(^{-1}\)) while still increasing the butanol yield. On the basis of the current models of involved pathways, butyryl-CoA can reversibly be converted to butyric acid or directly to butanol, as reviewed elsewhere.\(^{22}\) The external addition of butyrate can therefore be assumed to result in butanol formation via butyryl-CoA. The result obtained in this work is in agreement with the results of both studies. Therefore, this study confirms that acetate and butyrate addition especially butyrate is beneficial to butanol production with \(C.\) pasteurianum DSM 525 using pure glycerol as the carbon source. A substrate that \(C.\) pasteurianum DSM 525 can convert to acetate and butyrate yielding between 3 and 4 g L\(^{-1}\) would therefore be a desirable cosubstrate when utilizing glycerol as the main carbon source.

The product formation by \(C.\) pasteurianum was therefore studied using pure glycerol, crude glycerol, fructose, glucose, fructose and glucose (same ratio as in JAH), and JAH as the sole carbon sources. The use of the pure substrates was investigated for control purposes, as batch-to-batch variations are expected for crude glycerol and JAH. As shown in Figure 2, the major products of the \(C.\) pasteurianum substrates are butanol and 1,3-PDO when grown on pure and crude glycerol, respectively. However, when the strain is cultivated on sugar (fructose, glucose, fructose and glucose, JAH), it produces mostly acetic and butyric acids. The highest butyric acid concentration was achieved using glucose as the substrate, whereas using JAH resulted in the highest acetic acid titer (Figure 2a). The highest butanol and 1,3-PDO concentrations were obtained using pure glycerol as the substrate; however, the 1,3-PDO concentration decreased significantly using crude glycerol (from 6.4 to 4.4 g L\(^{-1}\)) and no 1,3-PDO was produced when this bacterium was cultivated on sugar (Figure 2b). These results indicate that JAH (sugar source) can be utilized by \(C.\) pasteurianum to appropriately produce acids to serve as the precursor to stimulate the subsequent butanol production from glycerol. The results presented in Figure 1 indicate further that the optimal substrate ratio might exist for a maximum butanol yield.

The measured product profile obtained from pure glycerol, crude glycerol, and glucose fermentation is in agreement with values typically found for these feedstocks fermented with \(C.\) pasteurianum DSM 525.\(^{44,45}\) To the best knowledge of the authors, this is the first attempt to use fructose, a mixture of fructose and glucose, and JAH as the substrates for fermentative butanol production by \(C.\) pasteurianum DSM 525.

The effect of acetic and butyric acid addition on butanol fermentation was investigated above, showing that direct addition of acetate and butyrate into the fermentation broth enhances butanol yield and productivity (Figure 1). The results shown in Figure 2 indicate the suitability of JAH as a sugar source to be fermented by \(C.\) pasteurianum DSM 525 to produce acetic and butyric acids. Therefore, as already mentioned, JAH (sugar source) and crude glycerol can be fermented at the same time (cosubstrate strategy) to conduct acetate and butyrate formation and butanol fermentation simultaneously. To prevent inhibition due to high acid concentration arising from a high sugar concentration and to enhance butanol production yield without decreasing the butanol production rate, the initial glycerol and sugar concentration should be optimized to maximize the butanol yield and productivity.

All optimization studies were performed using pure glycerol and a synthetic medium simulating JAH at otherwise optimal butanol fermentation condition.\(^{45}\) A central composite design (CCD) was used to determine the experimental conditions. Table 2 shows the values of the independent parameters and the experimental response (averages of triplicates ± standard deviation).

The fermentation process successfully produced butanol from pure glycerol and a synthetic medium simulating JAH as
the cosubstrate under the tested conditions. The full dataset was fitted with a quadratic model for both butanol yield and productivity as described in eq 3. The estimated parameters for both responses are shown in Table 3. Both F values (57.8 and 41.4 for the butanol yield and productivity, respectively) are higher than the critical value, hence both models are considered significant. On the basis of the P values, both factors (sugar and glycerol concentration) were significant for butanol yield and productivity, while the interaction parameter was only significant in the yield model. The high coefficients of determination ($R^2$) and adjusted coefficients of determination Adj. $R^2$ confirm the goodness of fit of both models (Table 3).

The analysis yields the following model equations for yields and productivity

$$Y_{P/S} = 0.016420 + 0.001236S_1 + 0.009251S_2 + 0.0000375P_1^2 + 0.00013P_2$$

$$PR_p = -0.2567 + 0.010630S_1 + 0.03629S_2 - 0.00064S_1^2 - 0.00036S_2^2$$

where $Y_{P/S}$ is the butanol yield per total substrate (g g$^{-1}$), $PR_p$ is the butanol productivity (g L$^{-1}$ h$^{-1}$), and $S_1$ and $S_2$ are the sugar and glycerol concentrations, respectively.

Visual inspection of the residuals implies them to be normally distributed (data not shown).

The interaction of the two parameters was studied via response surface methodology. Surface plots of the effects of glycerol concentration and sugar concentration on butanol yield and productivity are shown in Figure 3a,b, respectively. The butanol yield and productivity are both a function of glycerol concentration and sugar concentration. The plots clearly indicate that an optimum exists within the observed design space for both responses.

The optimal combinations of the two parameters were determined via numerical optimization of the two model equations, yielding 53.7 g L$^{-1}$ of glycerol and 12.4 g L$^{-1}$ of sugars for optimal butanol yield and 50.0 g L$^{-1}$ of glycerol and 8.2 g L$^{-1}$ of sugar for maximum butanol productivity.

Validation experiments were carried out around the identified optima (Table 4). A T test at 95% confidence showed that the measured values did not significantly deviate from the model predictions, hence the model can be considered capable at identifying maximum butanol yield and productivity.

The optimization studies were carried out with pure substrates for reproducibility purposes. To confirm the applicability to industrial substrates, confirmatory fermentations were conducted with JAH and crude glycerol.

### Table 3. ANOVA of Fitted Model for Butanol Yield and Productivity

| response | source | remark | sum of squares | degrees of freedom | $F$ value | $P$ value | Prob > $F$ |
|----------|--------|--------|---------------|--------------------|-----------|-----------|------------|
| yield    | model  | significant | 0.011         | 5                  | 57.84     | <0.0001   |            |
| productivity | model  | significant | 0.17          | 5                  | 41.4      | <0.0001   |            |
| yield    | glycerol (A) | significant | 0.00053       | 1                  | 13.4      | 0.0080    |            |
| productivity | glycerol (A) | significant | 0.011         | 1                  | 13.07     | 0.0086    |            |
| yield    | sugar (B) | significant | 0.0018        | 1                  | 42.23     | 0.0003    |            |
| productivity | sugar (B) | significant | 0.0067        | 1                  | 8.14      | 0.0246    |            |
| yield    | AB     | significant | 0.0002        | 1                  | 5.66      | 0.0489    |            |
| productivity | AB     | not-sig. | 0.0016        | 1                  | 1.93      | 0.2073    |            |
| yield    | $A^2$  | significant | 0.0010        | 1                  | 26.46     | 0.0013    |            |
| productivity | $A^2$  | significant | 0.025         | 1                  | 30.62     | 0.0009    |            |
| yield    | $B^2$  | significant | 0.0080        | 1                  | 198.89    | <0.0001   |            |
| productivity | $B^2$  | significant | 0.013         | 1                  | 153.76    | <0.0001   |            |
| yield    | lack of fit | not-sig. | 0.0001        | 7                  | 0.87      | 1.4124    |            |
| productivity | lack of fit | not-sig. | 0.0003        | 7                  | 0.13      | 1.3426    |            |
| yield    | Adj-$R^2$ | significant | 0.96          | 0.96               | 18.85     | 15.27     |            |
| productivity | Adj-$R^2$ | significant | 0.94          | 0.94               |           |           |            |
| yield    | Adeq precision | significant | 0.0001        | 7                  | 0.87      | 1.4124    |            |
| productivity | Adeq precision | significant | 0.0003        | 7                  | 0.13      | 1.3426    |            |

![Figure 3. Surface plot of combined effect of glycerol concentration and sugar concentration on (a) butanol yield and (b) butanol productivity.](image-url)
substrates. However, the butanol productivity decreased from production yield (within error) compared to using pure fermentation with JAH and crude glycerol showed identical butanol Pasteurianum DSM 525 in cosubstrate fermentation. Fermentation with JAH and crude glycerol in (c,d) under optimal fermentation and cosubstrate condition by C. pasteurianum DSM 525 (anaerobic, 50 mL, 30 °C; 200 rpm).

Table 4. Model Validation around Optimal Conditions, Predicted Values ±95% Prediction Interval, Measured Values ± Standard Deviation

| glycerol concentration (g L⁻¹) | sugar concentration (g L⁻¹) | butanol yield (gbutanol g⁻¹ (glycerol+glucose)) | butanol productivity (gL⁻¹ h⁻¹) |
|-------------------------------|-----------------------------|-----------------------------------------------|---------------------------------|
|                               |                             | predicted | experimental | predicted | experimental |
| 53                            | 13                          | 0.273 ± 0.05 | 0.268 ± 0.04 | 0.72 ± 0.07 | 0.71 ± 0.04 |
| 54                            | 12                          | 0.274 ± 0.06 | 0.271 ± 0.04 | 0.74 ± 0.08 | 0.74 ± 0.01 |
| 50                            | 11                          | 0.262 ± 0.09 | 0.269 ± 0.05 | 0.71 ± 0.07 | 0.70 ± 0.01 |

Figure 4. Profile of substrate utilization, solvent production, and organic acid production using fructose, glucose, and pure glycerol as feedstocks in (a,b) using JAH and crude glycerol in (c,d) under optimal fermentation and cosubstrate condition by C. pasteurianum DSM 525 (anaerobic, 50 mL, 30 °C; 200 rpm).

Another study investigated the optimal glucose to pure glycerol ratio (20:60 g L⁻¹) for the strain C. pasteurianum CH4 (an isolate from anaerobic sludge). The simultaneous cosubstrate strategy obtained a final butanol concentration, an overall productivity, and a yield of 13.2 g L⁻¹, 0.19 g L⁻¹ h⁻¹, and 0.21 gbutanol g(glycerol+glucose)⁻¹, respectively, whereas using pure glycerol as the sole carbon source resulted in a butanol concentration, productivity, and yield of 11.5 g L⁻¹, 0.13 g L⁻¹ h⁻¹, and 0.16 gbutanol g(glycerol+glucose)⁻¹, respectively. Moreover, bagasse and crude glycerol as cosubstrates were also converted into butanol with a butanol concentration, an overall productivity, and a yield of 11.8 g L⁻¹, 0.14 g L⁻¹ h⁻¹, and 0.19 gbutanol g(glycerol+glucose)⁻¹, respectively, with a fermentation time of 4–5 days (96–120 h), substantially longer than the 35 h used in this study. Higher fermentation temperature and iron-limiting condition may explain lower butanol yields and productivity. It has been reported that the optimal fermentation temperature for butanol production by C. pasteurianum is 30 °C⁴⁵,⁴⁶ and that the iron-limited condition enhances 1,3-PDO production over butanol.¹⁹ Additional deviation can be potentially explained by strain characteristics of the Clostridia (CH4 vs DSM 525). However, pure glycerol and glucose as carbon sources (wt ratio 1:1) have been reported to be fermented by C. pasteurianum DSM 525, with a butanol concentration, an overall productivity, and a yield of 21.1 g L⁻¹, 0.69 g L⁻¹ h⁻¹, and 0.23 gbutanol g(glycerol+glucose)⁻¹ as carbon sources for fermentative butanol production in a cosubstrate system.
Butanol production by C. pasteurianum DSM 525 from glycerol was significantly enhanced by adding organic acids, especially butyric acid, directly to the fermentation medium. These organic acids can be directly produced by C. pasteurianum DSM 525 through the conversion of sugars. A cosubstrate system was characterized and optimized for pure glycerol in a cosubstrate strategy is also a more relevant carbon source.

To verify the validity of the cosubstrate fermentation method by C. pasteurianum DSM 525 in larger scale, fermentations were carried out in a 5 L benchtop bioreactor using JAH and crude glycerol as feedstocks, and the results are presented in Figure 5. In addition to the offline-determined substrate and product concentrations, pH, CO₂ formation, and cell density were measured online (Figure 5b). A butanol yield and a productivity of 0.28 ± 0.01 g\textsubscript{butanol} (g\textsubscript{glycerol}+g\textsubscript{sugar})\textsuperscript{−1} and 0.55 ± 0.01 g L\textsuperscript{−1} h\textsuperscript{−1} were achieved after 27 h fermentation using a cosubstrate strategy. The fermentation was considered complete after 27 h based on the online signals of CO₂ formation and cell dry weight (CDW). The online signal for CO₂ represents biological CO₂ production as well as CO₂ release from the CaCO₃ buffer with decreasing pH (Figure 5b). Therefore, the shown value does not exclusively result in biological activity. The pH of the fermentation medium was not controlled; it was initially adjusted to 6.8 (optimal initial pH, estimated elsewhere\textsuperscript{46}) and was subsequently allowed to decrease until it reached 5.01 (Figure 5b). Cell growth was continuously measured with an online turbidity probe, and a short lag phase is followed by exponential growth, leading to high final CDW (Figure 5b).

At optimized conditions, the butanol production achieved in the 5 L reactor vessel and in anaerobic shake flasks were within error of each other, indicating that the scaled-down shake flask conditions used in the optimization study were a suitable representation of reactor conditions.

This study focused on the cosubstrate fermentation of Jerusalem artichoke tubers and crude glycerol for the production of butanol. The small-scale batch fermentation is not intended to represent an industrial process, where more advanced fermentation process design and control would be applied. Overall productivity improvements could be achieved through continuous fermentation and/or in situ product removal, as evaluated elsewhere for different feedstocks.\textsuperscript{47,48}

### CONCLUSIONS

Butanol production by C. pasteurianum DSM 525 from glycerol was significantly enhanced by adding organic acids, especially butyric acid, directly to the fermentation medium. These organic acids can be directly produced by C. pasteurianum DSM 525 through the conversion of sugars. A cosubstrate system was characterized and optimized for pure feedstocks and could directly be transferred to the relevant carbon sources of crude glycerol and JAH. The system is a potential way to utilize an industrial waste stream and a dedicated energy crop for the efficient production of an advanced biofuel.

### MATERIALS AND METHODS

Complex media ingredients (peptone, yeast/beef extract) were purchased from BD-Becton, Dickinson and Company (New Jersey, USA). Sodium acetate, soluble starch, thiamine, and resazurin were obtained from Alfa Aesar (Massachusetts, USA), while CaCO₃ and dextrose were purchased from Amresco (Ohio, USA) and CaCl₂ from EMD Millipore (Massachusetts, USA). Sulfuric acid (18.0 M), MnSO₄·H₂O, (NH₄)₂SO₄, KH₂PO₄, MgSO₄·7H₂O, and K₂HPO₄ were obtained from Caledon (Ontario, Canada). L-Cysteine, pure glycerol, NaCl, and FeSO₄·7H₂O were purchased from BDH (Georgia, USA). Biotin, sodium butyrate, p-aminobenzoic acid, and 2-(N-morpholino) ethanesulfonic acid (MES) were obtained from Sigma-Aldrich (Missouri, USA).

Crude glycerol was kindly provided by Newalta Corp. (AB Canada). The received crude glycerol is a gel-like viscous material of dark brownish color. For further use, the material was homogenized through mechanical shaking, followed by preparing an aqueous solution (250 g of crude glycerol in 500 mL of deionized water) which was filtered three times (0.2 μm grade filters) to remove solids. The stock solution was diluted as required for fermentation trials and filter prior to use. Glycerol analysis was conducted with a 250-fold dilution of the stock solution (a clear liquid), indicating that the crude glycerol concentration in the stock solution was 240 ± 3 g L\textsuperscript{−1}, while the methanol concentration (residue) was considered...
too low to negatively impact the microbial activity (data not shown).

Jerusalem artichoke tubers were kindly provided by the Institute for Chemicals and Fuels from Alternative Resources (ICFAR), University of Western Ontario. The raw material was prepared and characterized as described elsewhere. In brief, tubers were washed and cut to ~2 cm³ cubes, dried (105 °C for 72 h), ground (250 μm mesh), and stored at 4 °C.

JA characterization and inulin extraction: 15 g of JA powder and 300 mL of water were stirred for 1 h (300 rpm, 25 °C), and solids were separated through centrifugation (20 min at 12,000g). The supernatant contained 0.52 g g⁻¹ of inulin, 0.16 g g⁻¹ of fructose, 0.1 g g⁻¹ of glucose, and 0.05 g g⁻¹ of sucrose (extractables), while 0.03 g g⁻¹ of cellulose and 0.02 g g⁻¹ of hemicellulose were found in the precipitate (nonextractable). Because of the low cellulose and hemicellulose content, only hemicellulose were found in the precipitate (nonextractable).

Because of the low cellulose and hemicellulose content, only hemicellulose were found in the precipitate (nonextractable).

Precultures (RCM) were obtained from the frozen stock after intermittent samples were reached in OD₆₀₀ of 0.8) was stored at -15 °C and used as inoculum once they reached ~50 of the final growth (~16 h).

Fermentation and optimization studies were conducted in 150 mL flasks containing 50 mL of modified Bioblu medium, containing glycerol (as needed), 1 g L⁻¹ of yeast extract, 0.5 g L⁻¹ of KH₂PO₄, 0.5 g L⁻¹ of K₂HPO₄, 5 g L⁻¹ of (NH₄)₂SO₄, 0.2 g L⁻¹ of MgSO₄·7H₂O, 0.02 g L⁻¹ of CaCl₂·2H₂O, 0.1 g L⁻¹ of FeSO₄·7H₂O, 2 g L⁻¹ of CaCO₃, 0.01 mg/L of biotin, 1 mg/L of thiamine, 1 mg/L of p-amino benzoic acid, 4 mL/L of trace element solution (St), as described elsewhere. Flasks (40 mL medium) were inoculated with 0.4 g L⁻¹ of actively growing culture and cultivated for 40 h (anaerobic chamber, 30 °C, 200 rpm, initial pH of 6.8). Intermittent samples were filtered (0.2 μm grade filters) and stored at ~20 °C prior to analysis.

A CCD was selected to evaluate the response pattern and to determine the optimal combination of glycerol and sugar concentration for maximizing the butanol yield and productivity. The general design space was chosen based on previous experiments (data not shown), yielding the following parameter values (uncoded) [low star point, low central point, center point, high central point, high star point]:

- Glycerol concentration in g L⁻¹: [23.6, 30, 50.0, 70, 76.4]
- Sugar concentration in g L⁻¹: [1.8, 5, 15, 25, 28.2].

The resulting conditions (including three center points) were tested in triplicates, resulting in 33 experiments (12 factorial + 12 augmented + 9 center points) that were randomized prior to testing.

The experimental data were fitted to a second-order polynomial model via linear regression analysis using Design-Expert 8.0.7.1

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Y = \beta_0 + \sum_{i=1}^{2} \beta_i x_i + \sum_{i=1}^{2} \beta_{i1} x_i^2 + \sum_{1 \leq i < j \leq 2} \beta_{ij} x_i x_j + \epsilon
\]  

Analysis of variance (ANOVA) was used to determine the significance of each model term based on an α of 0.05 using the F-test. Numerical optimization was done via Design-Expert 8.0.7.1, followed by the experimental validation point near the predicted optimum.

Lab-scale stirred-tank bioreactor fermentation: a Labfors 4 system (Infors, Quebec, Canada) was used (5 L of nominal volume, 2.7 L of modified Bioblu medium, 0.3 L of preculture, 0.2 mL of antifoam, 30 °C, 150 rpm using one Rushton impeller). The pH was monitored but not controlled (Hamilton EasyFerm, Switzerland), so were redox potential (Mettler Toledo, Switzerland) and cell density (TruCell2, Finesse Solutions, LLC, USA). The online cell density signal was correlated with CDW measurements from offline samples (CDW was determined via filtration of 5 mL samples (cellulose filter) as the weight difference of the filter before and after drying for 48 h at 80 °C). Anaerobic conduction and flow for off-gas analysis (Infors, Quebec, Canada) were achieved via nitrogen sparging (0.3 L min⁻¹). Offline analysis was conducted on samples taken and prepared as described above.

The fermentation broth was analyzed for substrates and products via Agilent 1260 infinity HPLC (Agilent USA, Santa Clara). The analytes were separated on an Agilent Hi-plex H (7.7 x 300 mm) column (Agilent USA, Santa Clara) at 35 °C and detected with a refractive index detector. The mobile phase was 0.005 M H₂SO₄ (isocratic, 0.4 mL min⁻¹).

Product yields were calculated as the highest detected butanol concentration divided by the sum of substrates consumed at the point expressed as R_butanol (g/glycerol or sugar)⁻¹ for glycerol and carbohydrate fermentations or as R_butanol (g/glycerol or sugar + added acid)⁻¹, when the medium was supplemented with organic acids. Productivities were expressed as the highest detected butanol concentration over fermentation time (g L⁻¹ h⁻¹).

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

#### Notes

The authors declare no competing financial interest.

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