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Production of butyric acid at constant pH by a solventogenic strain of *Clostridium beijerinckii*

*Marek Drahokoupil, Petra Patáková**

Department of Biotechnology, University of Chemistry and Technology Prague, Prague, Czech Republic

**Corresponding author: petra.patakova@vscht.cz*

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Abstract: A solventogenic strain of *Clostridium beijerinckii*, NRRL B-598, was cultured for the production of butyric acid as the main fermentation product. However, unlike typical acetone-butanol-ethanol (ABE) fermentations, where pH is not regulated, in this study the pH was kept constant during fermentation. From the five pH values tested, 6.0, 6.5, 7.0, 7.5 and 8.0, pH 6.5 and 7.0 resulted in the highest concentrations of butyric acid, at 9.69 ± 0.09 g L^{-1} and 11.5 ± 0.39 g L^{-1}, respectively. However, a low concentration of solvents, 1.8 ± 0.22 g L^{-1}, was only reached at pH 7.0. These results are comparable with those from typical butyric acid producers, i.e. *Clostridium butyricum* and *Clostridium tyrobutyricum* strains. At pH 7.0, we succeeded in suppressing sporulation and prolonging the population viability, which was confirmed by flow cytometry combined with double fluorescence staining.

Keywords: butyrate; *Clostridium beijerinckii* NRRL B-598; solventogenic clostridia; controlled pH; flow cytometry

Although butyric acid itself has an unpleasant odour, butyric esters such as methyl, ethyl and amyl butyrate are used as fragrances and flavourings in the beverage, food and cosmetic industries (Armstrong & Yamazaki 1986; Shu et al. 2011). Ethyl butyrate is commonly used as artificial flavouring resembling orange juice and hence it is used in nearly all orange juices (including those sold as "fresh" or "concentrated") in the market. It is also used in alcoholic beverages (e.g. martinis, daiquiris etc.) (Rodriguez-Nogales et al. 2005). Methyl butyrate is a component of pineapple essence. In addition, butyric acid has a beneficial role in both the human and animal gastrointestinal tract (Bedford & Gong 2018; Załęski et al. 2013) and might be considered a prebiotic molecule.

At the industrial scale, butyric acid is mainly produced by chemical synthesis. This involves the oxidation of butyraldehyde which is obtained from propylene that originates from petroleum by oxosynthesis (Cascone 2008). The chemical synthesis of butyric acid remains at the forefront in terms of lower production costs and the availability of starting materials.

Butyric acid is also a fermentation end product of some strictly anaerobic bacteria. This method is currently too expensive compared to chemical synthesis, but it is gaining more attention due to the growing demand from consumers for organic and natural products (Zigová & Šturdík 2000; Cascone 2008). Various strains of the genera *Clostridium*, *Butyrivibrio*, *Butyribacterium*, *Sarcina*, *Eubacterium*, *Fusobacterium*, *Megasphaera*, *Roseburia* and *Coprococcus* may be used for microbial production of butyric acid (Zigová & Šturdík 2000; Duncan et al. 2002; Zhang et al. 2009; Dwidar et al. 2012). Several species, including *C. butyricum*, *C. tyrobutyricum* and *C. thermobutyricum*, produce butyrate as a major

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product with relatively high levels of production and yield, and are therefore the most commonly studied species because of their high commercial potential for butyric acid production.

In this study, we used the solventogenic strain *Clostridium beijerinckii* NRRL B-598 as a production microorganism for butyric acid. *C. beijerinckii* belongs to the group of solventogenic clostridia that is characterized by its ability to produce solvents, i.e. acetone, butanol and ethanol, by ABE fermentation, which can be divided into two basic phases: acidogenic and solventogenic. Acidogenesis, together with vegetative cell growth generates mainly acetic and butyric acid, together with hydrogen and CO_2 as the main products. Solventogenesis begins with a decrease in pH and accumulation of acids in the medium and is usually accompanied by the onset of sporulation.

During solvent production, some of the acids formed, together with carbohydrates, are transformed into 1-butanol and acetone, while ethanol, hydrogen and CO_2 are formed from saccharides (Jones & Woods 1986; Dürre 2015; Lipovský et al. 2016; Patáková et al. 2019). The main goal of the research was to determine the pH below which the solventogenic switch and sporulation start would be blocked, resulting in the production of butyric acid as the main fermentation product.

MATERIALS AND METHODS

Bacterial strains. *C. beijerinckii* NRRL B-598 [obtained as *C. pasteurianum* NRRL B-598 from the ARS/NRRL collection but re-classified as *C. beijerinckii* NRRL B-598 in 2017, see Sedlář et al. (2017)] was maintained as a spore suspension.

Fermentation medium. TYA (tryptone yeast extract acetate) nutrient medium was used for all experiments. This medium was selected because of its common use in fermentation by solventogenic clostridial cells. This medium consisted of: glucose (Penta, Czech Republic) 50 g L^{-1}, yeast extract (Merck KGaA, Germany) 2 g L^{-1}, tryptone (Merck KGaA, Germany) 6 g L^{-1}, ammonium sulphate (Penta, Czech Republic) 3 g L^{-1}, potassium dihydrogen phosphate (Penta, Czech Republic) 0.5 g L^{-1}, magnesium sulphate heptahydrate (Penta, Czech Republic) 0.3 g L^{-1} and ferrous sulphate heptahydrate (Penta, Czech Republic) 0.01 g L^{-1}. The pH of the medium was adjusted to the desired value with 10% NaOH solution. The medium was then transferred to laboratory bioreactors and sterilized with all components at 21 °C for 20 minutes.

Batch cultivation in bioreactor. Prior to inoculation, a spore suspension of *C. beijerinckii* NRRL B-598 was heat-shocked for 2 min at 80 °C and cultured in TYA medium in the anaerobic chamber (Concept 400; Ruskinn Technology, UK) at 37 °C, for 24 h. Fermentations were performed at 37 °C in 1 L parallel Multiforce bioreactors (Infors HT, Switzerland) filled with 630 mL TYA medium at 200 rpm agitation with pH online control. Prior to fermentation, N_2 bubbling for 30 min was used for oxygen removal, the pH was adjusted to 6.0, 6.5, 7.0, 7.5 or 8.0 with 10% NaOH and all bioreactors were inoculated with 70 mL of inoculum. The inoculum was prepared by culturing the strain in an anaerobic chamber (Concept 400; Ruskinn Technology, UK) for 18 hours. Samples from bioreactor fermentation were taken every 3 h for further analyses.

Determination of metabolites, biomass and glucose. Glucose and metabolite (butyric acid, acetic acid, lactic acid, ethanol, acetone and butanol) concentrations were determined by HPLC (Agilent Series 1200 HPLC; Agilent, Spain) using refractive index detection (Agilent Series 1200 Refractive Index Detector; Agilent, Spain) in samples of culture media. An IEX H^+ polymer column (Watrex, Czech Republic) was used under the following conditions: isocratic elution, mobile phase (5 mM H_2SO_4) with stable flow rate of 1 mL min^{-1}, column temperature 60 °C, injection sample volume 20 µL. Results are presented as mean values from parallel fermentations with standard deviations.

Flow-cytometric (FC) analysis. Flow cytometry was used for rapid analysis of cell population viability and spore formation of the *Clostridium* strain. A procedure described in detail by Kolek et al. (2016) and Branská et al. (2018) was chosen. The main principle of the method is double staining of the cells with 6-carboxy-fluorescein diacetate (CFDA) and propidium iodide (PI). Further, flow cytometry analysis of the labelled population is performed together with evaluation of standard FC parameters, i.e. side and forward scatters. While double staining is used for estimation of culture viability, spores, which are not stained, are recognised in the population based on their size, shape and autofluorescence.

Microscopy. Phase contrast and fluorescence microscopy (Olympus BX51) were used in the study at ×400 and ×1000 magnifications.

Calculation of the parameters of butyric acid/butanol formation. The yield and rate of product formation (productivity) for the first 24 h of fermen-

https://doi.org/10.17221/95/2020-CJFS

Table 1. A summary of the results of batch cultivation at different pHs (mean value ± SD)

pH	Remaining glucose (g L^{-1})	Total butyric acid (g L^{-1})	Total acids (g L^{-1})	Total solvents (g L^{-1})	Productivity of butyric acid formation 24 h (g L^{-1} h^{-1})	Productivity of butanol formation 24 h (g L^{-1} h^{-1})	Yield of butyric acid 48 h (%)
6	12.03 ± 0.22	2.97 ± 0.01	8.43 ± 0.09	9.01 ± 0.15	0.23 ± 0.01	0.09 ± 0.01	8.26 ± 0.20
6.5	2.25 ± 0.12	9.69 ± 0.09	17.29 ± 0.18	7.21 ± 0.09	0.39 ± 0.01	0.05 ± 0.01	21.62 ± 0.20
7	5.05 ± 2.01	11.49 ± 0.39	20.60 ± 0.62	1.76 ± 0.22	0.40 ± 0.01	0.05 ± 0.02	25.73 ± 0.30

tation in the form of butyric acid or butanol were calculated from the results of HPLC analysis. Although the fermentations were run for 48 h, the yield and productivity were calculated for the first 24 h to compare the values at the time when most cells in the population remained active (see population viability in Figure 1). The formulas for the calculations are given below.

1. Product yield was calculated according to Equation 1:

$$Y_{P/S} = \frac{P_{t24} - P_{t0}}{S_{t0} - S_{t24}} \times 100\ (\%) \quad (1)$$

where t_0 is the time of bioreactor inoculation and t_{24} is 24 h after inoculation.

S_{t0} and S_{t24} are glucose concentrations at times t_0 and t_{24}, respectively; P_{t24} and P_{t0} are product (butyric acid or butanol) concentrations at times t_{24} and t_0, respectively.

2. Productivity was calculated according to Equation 2:

$$P = \frac{P_{t24} - P_{t0}}{t_{24}} \quad (2)$$

where t_0 is time of bioreactor inoculation and t_{24} is 24 h after inoculation; P_{t24} and P_{t0} are product (butyric acid or butanol) concentrations at times t_{24} and t_0, respectively.

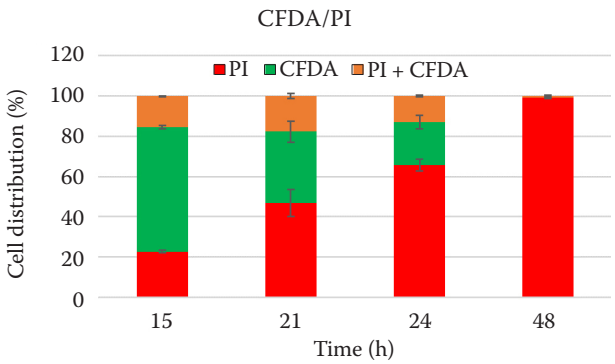

Figure 1. Proportions of PI and CFDA stained *C. beijerinckii* NRRL B-598 cells in the population during fermentation at constant pH 7.0

PI – dead cells; CFDA – viable cells; PI + CFDA – doubly stained cells with an unclear status; values are in mean ± standard deviation (SD)

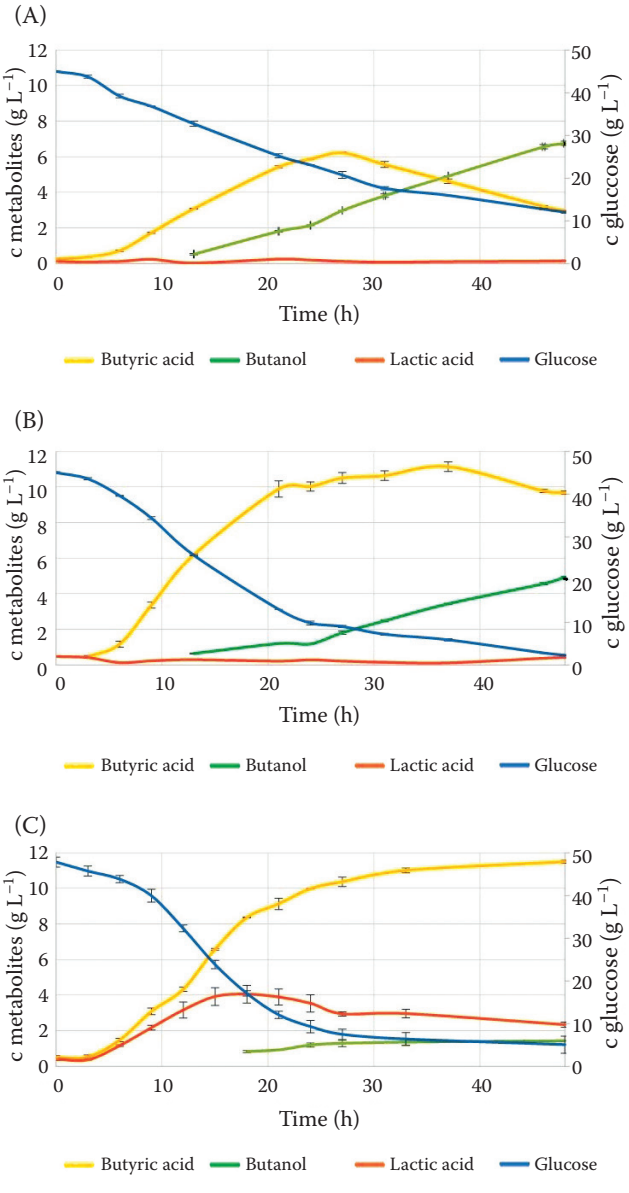

Figure 2. Concentration profiles for the consumption of glucose, production of butyric acid and yields of other cultivation products during batch fermentation in a bioreactor

(A) concentration profile for pH 6; (B) concentration profile for pH 6.5; (C) concentration profile for pH 7; values are in mean ± standard deviation (SD)

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RESULTS AND DISCUSSION

Results. The results are shown graphically in Figure 2. The values of the process parameters for all fermentations are summarized with the mean standard deviations in Table 1. The results for pH 7.5 and 8.0 are not shown because these pH values were strongly inhibitory for clostridial cells, with almost no cell growth. Individual experiments show that the lowest concentration of butyric acid was achieved at pH 6. At this pH, the highest concentration of solvents was also achieved and cell sporulation was seen, as shown in Figure 3A. At pH 6.5, a comparable peak concentration of butyric acid was reached like at pH 7.0 (fermentation time 36 h), but in the late phase of fermentation, part of the butyric acid was transformed into butanol (see Figure 2B) and spore formation was observed. At pH 7, a high concentration of butyric acid was achieved, while at the same time there were minimal solvent production and no sporulation, as shown in Figure 3C.

It can be seen from Figure 3 that spore formation occurred at pH 6 and this phenomenon declined with increasing pH. At pH 7, spores were not formed. As shown in Figure 3C, higher pH caused cellular stress, leading to filament formation. Further, flow cytometry with double fluorescence staining was chosen for monitoring the cell viability at pH 7.0, i.e. under conditions where cells did not sporulate (see Figure 4). Propidium iodide, which stains damaged cells, generally serves as an indicator of membrane integrity. CFDA is a non-fluorescent compound that is cleaved by active enzymes inside the cells into green fluorescent carboxy-fluorescein (CF). As CF is charged unlike CFDA, it is retained in the cells. Viable and dead cells were easily distinguishable as green (CFDA-stained) and red (PI-stained) cells, respectively. Orange cells, which are the result of double staining (CFDA + PI), represent compromised cells but they were considered also viable in this study (see Figure 1). For detailed explanation of the FC analysis see Kolek et al. (2016) and Branská et al. (2018).

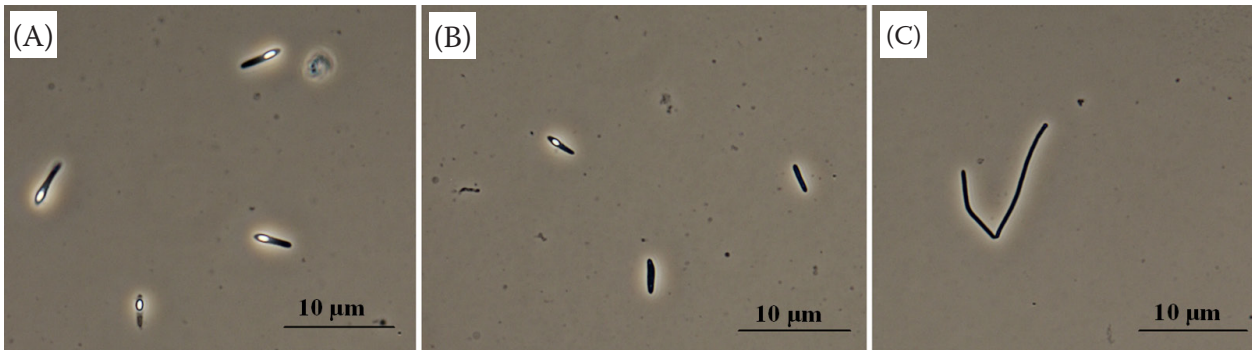

Figure 3. Microphotographs showing the typical morphology of cells after 24 h of culture for given pH values

(A) cells after 24 h of fermentation at constant pH 6; (B) cells after 24 h of fermentation at constant pH 6.5; (C) cells after 24 h of fermentation at constant pH 7

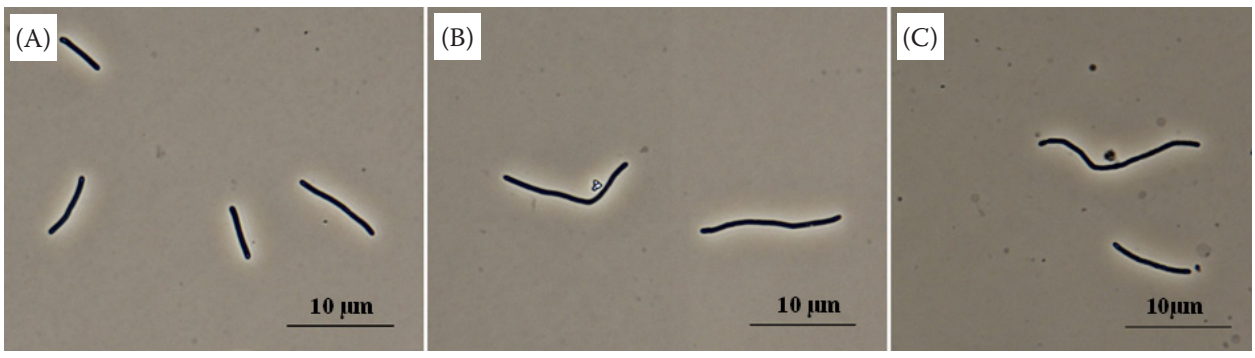

Figure 4. Microphotographs showing the morphology of cells during fermentation at pH 7

(A) cells after 12 h of fermentation; (B) cells after 24 h of fermentation; (C) cells after 48 h of fermentation

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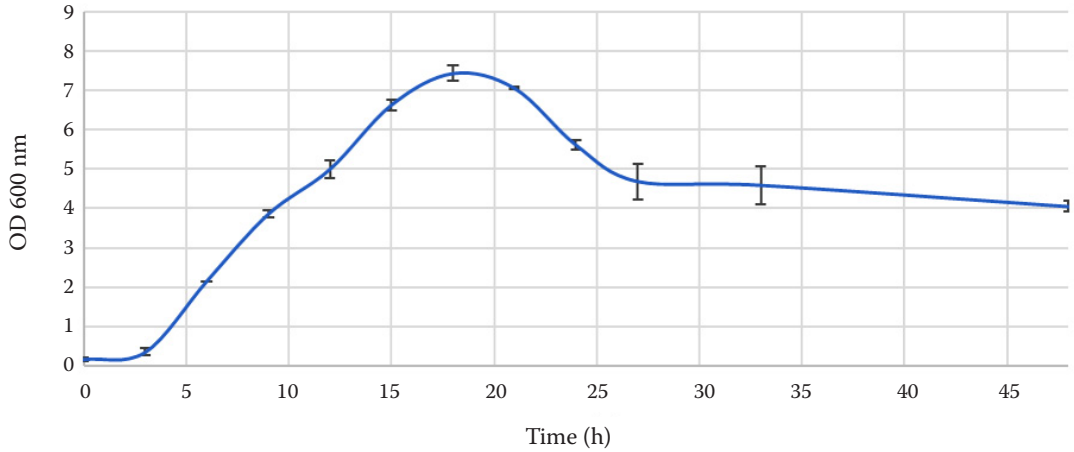

Figure 5. The time course of cell population growth during fermentation while maintaining a constant pH 7.0 (mean ± SD)
OD – optical density

The results from flow cytometry showed that at the 15th hour of fermentation, approximately 80% of cells were viable, corresponding to a growth curve in exponential phase (see Figure 5). After 24 h of fermentation, viability dropped to approximately 20%, which again corresponds to the growth curve shown in Figure 5. Consumption of glucose also decreased, and the formation of butyric acid slowed down after 24 h of fermentation.

Discussion. As reported in a number of studies (Jones & Woods 1986; Haus et al. 2011; Li et al. 2014; Wang et al. 2019), the initial pH of the fermentation broth in solvent/butanol production is an important factor that significantly affects the fermentation process, mainly the butanol yield. All studies agree that a neutral pH leads to a higher level of production of acids, while a weakly acidic pH promotes solvent formation. This trend was confirmed during our experiments. Usual final butyric acid concentration achieved with the same strain under the same culture conditions (Lipovský et al. 2016; Branská et al. 2018; Patáková et al. 2019) is about 1.8 g L^{-1} while the concentration of 11.5 g L^{-1} was reached at pH regulated to 7.0. If the same strain, *C. beijerinckii* NRRL B-598, was cultured under the same conditions but the pH was not regulated (Lipovský et al. 2016), butanol productivity of 0.15 g L^{-1} h^{-1} and butanol concentration of 7.3 g L^{-1} were reached. Under pH regulation, see Table 1, maximum butanol productivity and concentration were obtained at pH 6.0, i.e. 0.09 g L^{-1} h^{-1} and 6.8 g L^{-1}, respectively, while at constant pH 7.0, maximum butanol productivity and concentration were 0.05 g L^{-1} h^{-1} and 1.7 g L^{-1}, respectively. For *C. acetobutylicum*, Al-Shorgani et al. (2018) reported the maximum productivity of butanol at uncontrolled pH to be 0.19 g L^{-1} h^{-1} compared to the productivity of 0.06 g L^{-1} h^{-1} at controlled pH 6.0.

The effect of pH on the production of organic acids by solventogenic clostridia including *C. beijerinckii* was rarely tested, however an increased formation of lactic acid by *C. acetobutylicum* under "alkaline" pH (value 7.0 and higher) was reported by Katagiri et al. (1960). This corresponds with our results when the unusually high concentration of lactic acid (up to 4 g L^{-1}) was determined at pH 7.0. At pH 7.5 and 8.0, no growth of the strain was detected, which might be explained by the necessity to ensure a lower redox potential or to add a higher amount of inoculum because the same phenomenon was described for *C. thermoaceticum* and acetic acid formation at pH 7.0 (Schwartz & Keller 1982).

Kolek et al. (2016) stated that the number of viable cells during typical ABE fermentation without pH regulation was about 20% after 24 h of fermentation; similar results were confirmed by Branska et al. (2018). At a constant pH of 7, about 35% of viable cells were still present after 24 h of fermentation. This result suggests that by maintaining a constant pH and preventing cell sporulation, the population viability can be increased and prolongation of the time of metabolic activity of cells was found.

At pH 7, 11.5 g L^{-1} of butyric acid was produced at an initial glucose concentration of 50 g L^{-1} and a total of 1.8 g L^{-1} of solvents. This butyric acid concentration (11.5 g L^{-1}) was more than five times higher than the butyric acid concentration obtained in standard fermentation of solventogenic clostridia, which

https://doi.org/10.17221/95/2020-CJFS

is around 2 g L^{-1} (Jones & Woods, 1986; Dwidar et al. 2012; Patáková et al. 2019).

He et al. (2005) reported that in batch culture fermentation of *Clostridium butyricum* ZJUCB at different pHs and under different conditions, they achieved maximum butyric acid production of 12.25 g L^{-1}. Jo et al. (2009) reached the final butyric acid concentration of 13.76 g L^{-1} by batch fermentation with *C. tyrobutyricum* strain JM1. Zig et al. (1999) in batch fermentation with *C. butyricum* S21 achieved the production of 7.3 g L^{-1} butyric acid with total productivity of 0.24 g L^{-1} h^{-1} and total yield of 24%.

We obtained a butyric acid level of 11.5 g L^{-1} with productivity of 0.40 g L^{-1} h^{-1} and total yield of 25.7%. These data suggest that our method of using the solvent strain *C. beijerinckii* NRRL B-598 may be very promising, and our results are comparable with *C. butyricum* and *C. tyrobutyricum* strains routinely used for this purpose.

CONCLUSION

This study was focused on identifying a suitable pH at which butyric acid should be produced using the solventogenic species *Clostridium beijerinckii* NRRL B-598, which is commonly used for ABE fermentation; a pH of 7 proved to be the best for acidogenic fermentation. At this pH, production of 11.5 g L^{-1} butyric acid as the main fermentation product was achieved, with almost zero lactic acid and minimal solvent production. Moreover, at this pH, cell sporulation did not occur, enabling a better yield of butyric acid.

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