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Determination of metabolites involved in fermentative succinic acid production from glucose, glycerol and crude glycerin by HPLC methodology

Determinación de los metabolitos involucrados en la producción fermentativa de ácido succínico a partir de glucosa, glicerol y glicerina bruta por metodología CLAE

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Abstract

Bio-succinic acid production process involves complex biochemical pathways in which diverse metabolites may be cogenerated. Their identification and quantification allow an adequate monitoring and understanding of the bioprocess. In this work, a HPLC methodology for simultaneous determination of glucose, glycerol, ethanol and citric, pyruvic, succinic, lactic, formic, acetic and propionic acids was validated, presenting adequate selectivity and linearity. Matrix effect was observed for citric and lactic acids, glycerol and ethanol. The limits of detection and quantification ranged from 0.006 to 0.021 $g.L^{-1}$ and 0.018 to 0.065 $g.L^{-1}$, respectively. Recovery values were between 89 and 109% and variation coefficients were less than 2.3%, using fresh samples. The intermediate precision was verified with short-term stability, after one freezing and thawing cycle, and analysis by a second analyst showing variation coefficients lower than 5% and recovery values ranged between 88 and 108%. The analysis of fermentation samples showed that *Actinobacillus succinogenes*'s metabolism was carbon source dependent, while *Basfia succiniciproducens* presented similar metabolic behavior for the carbon sources evaluated, with less variety of generated products. Succinic acid was produced in greater amount by *B. succiniciproducens*, being equivalent to 50% and 80% of metabolites produced in the fermentation of glucose and glycerol sources.

Keywords: Validation of analytical methodology, high added-value product, anaerobic fermentation, *Actinobacillus succinogenes*, *Basfia succiniciproducens*.

Resumen

El proceso de bio-producción de ácido succínico envuelve caminos metabólicos complejos, donde diversos metabolitos pueden ser cogenerados. La identificación y cuantificación de estos metabolitos permite un adecuado monitoreo y entendimiento del bioproceso. En este trabajo una metodología de CLAE para determinación simultanea de glucosa, glicerol y ácidos cítrico, pirúvico, succínico, láctico, fórmico, acético y propiónico fue validada, presentado adecuadas selectividad y linealidad. Se observó efecto de matriz para los ácidos cítrico y láctico, glicerol y etanol. Los límites de detección y cuantificación variaron respectivamente de 0.006 a 0.021 $g.L^{-1}$ y de 0.018 a 0.065 $g.L^{-1}$. Los valores de recuperación estuvieron entre 89 y 109% y los coeficientes de variación fueron menores de 2.3%, usando muestras frescas. La precisión intermedia se verificó con la estabilidad de corto tiempo, después de un ciclo de congelamiento/descongelamiento y por análisis con un segundo analista, mostrando coeficientes de variación menores de 5% y valores de recuperación en el rengo de 88 a 108%. El análisis de muestras de fermentación mostró que el metabolismo de *Actinobacillus succinogenes* fue dependiente de la fuente de carbono, mientras *Basfia succiniciproducens* presentó un comportamiento metabólico similar para las fuentes de carbono evaluadas, con menor variedad de productos generados. El ácido succínico fue producido en mayor cantidad por *B. succiniciproducens*, equivaliendo al 50% y 80% de los metabolitos producidos en las fermentaciones de glucosa y fuentes de glicerol.

Palabras clave: Validación de metodología analítica, producto de alto valor agregado, fermentación anaeróbica, *Actinobacillus succinogenes*, *Basfia succiniciproducens*.

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1 Introduction

Succinic (or butanedioic) acid is considered one of the 12 most promising bio-based platform chemicals by the US Department of Energy and the European Commission, with applications in the chemical, food, cosmetic and pharmaceutical industries; and is also able to replace benzene-derived chemicals, whose carcinogenic properties have been long recognized (Abel e Digiovanni, 2015; Biddy *et al*., 2016). Succinic acid is an important precursor to many bulk chemicals, commodity chemicals and biodegradable polymers, including 1,4-butanediol, gamma-butyrolactone, tetrahydrofuran, adipic acid, n-methylpyrrolidone, linear aliphatic esters, polybutylene succinate (PBS) and 1,3-propylene succinate, whose market demand will certainly increase (Jansen e Van Gulik, 2014; Jiang *et al*., 2017).

The natural bio-production of succinic acid is a complex process, involving the interaction of different metabolic pathways that depend on the microbiological agent used, the composition of the fermentation medium and the operational conditions. Thus, various byproducts, including other organic acids and alcohols, may be produced together with succinic acid (Song e Lee, 2006; Becker *et al*., 2013; Cao *et al*., 2013; Pateraki *et al*., 2016; Jiang *et al*., 2017).

Traditional bio-production of succinic acid is derived from glucose fermentation; yet its production from agro-industrial and waste materials, such as crude glycerin, has been gaining interest in the last years (Gargalo *et al*., 2016; Sivasankaran *et al*., 2016; Westbrook *et al*., 2018). There is a fairly large amount of crude glycerin available around the world that comes from production plants of detergents, acids and fatty esters. However, the main source of crude glycerin is the manufacture of biodiesel, which represents more than 65% of total generation. In general, for every 100 L of biodiesel produced, approximately 10 L of crude glycerin are cogenerated (Anitha *et al*., 2016; Kong *et al*., 2016; Martínez-Rico *et al*., 2018) and, according to The Food and Agriculture Organization, its global cogeneration will reach 3.9 bln L by 2027 (OECD/FAO, 2018). The chemical composition of crude glycerin is quite variable, depending on the type of raw material and manufacturing process. Typically, crude glycerin produced by the transesterification of vegetable oil contains up to 70% glycerol, impurities such as water, salts, esters, alcohol, residual fatty material, and elements such as calcium, magnesium, phosphorous, and sulfur (Quispe *et al*., 2013; Samul *et al*., 2013; Tan *et al*., 2013). From a technological point of view, the purification of crude glycerin comprises several steps, which entail higher product-related costs than gross profits (Quispe *et al*., 2013; Ardi *et al*., 2015). Thus, due to its composition and high purification costs, the accumulation of huge amounts of crude glycerin by biodiesel industries may cause serious environmental problems if the substance is not properly treated. Therefore, there is a global demand for strategies to improve the direct use of crude glycerin, mainly to supply high-value markets such as succinic acid (Babajide, 2013; Zavarize *et al*., 2014; Gargalo *et al*., 2016; Luo *et al*., 2016; Espinel-Ríos e Ruiz-Espinoza, 2019).

In this scenario, the first and fundamental stage to develop a biosuccinic process is generating useful and reliable data related to the monitoring of substrate consumption from purified and alternative forms, and possible metabolites cogeneration. This data is fundamental to expand the understanding of the bioprocess and to adopt strategies to improve its yield and productivity.

Among instrumental analyses, high-performance liquid chromatography (HPLC) is recognized as a quick and precise analytical technique that allows direct, rapid and mainly accurate measurement of multiple analytes. Suitable use of this technique requires establishing the type of detector, column, and operating conditions (Moldoveanu e David, 2013; Petrova e Sauer, 2017). Furthermore, validation of the HPLC methodology is fundamental to guarantee an appropriate detection and quantification, and therefore to certify the quality of the results obtained in order to indicate the strategies to be adopted to make processes more efficient (EURACHEM, 2014).

This work deals with the heterofermentative production of succinic acid by two natural succinic acid producing bacteria - *Actinobacillus succinogenes* 130Z and *Basfia succiniciproducens* JF4016 - by comparatively using crude glycerin, pure glycerol or glucose as carbon sources. According to the biochemical characteristics of both strains, the analytes selected to be quantified in the fermenting media were glucose, glycerol, ethanol and succinic, citric, pyruvic, lactic, formic, acetic, and propionic acids. The methodology was based on dual-mode detection with UV and refractive index (RI) sensors, and it was validated following

national and international guidelines, considering selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, repeatability and intermediate precision as performance parameters. Once the validation steps were satisfied in order to ensure reliable data, the methodology was applied to analyze complex samples from fermentation tests with both species. Data obtained allows for the comparison of the effect of feedstock on the distribution of metabolites for two bacterial strains recognized as natural producers of succinic acid.

2 Materials and methods

2.1 *Chemicals and solutions*

All chemicals used in this work were of analytical grade and purchased from Sigma-Aldrich (Brazil). Two mixture standard solutions containing all analytes of interest (glucose, glycerol, ethanol and citric, pyruvic, succinic, lactic, formic, acetic and propionic acids) were prepared in the concentration of 20.0 g.L^{-1} for each analyte. One standard solution was prepared by diluting all analytes with ultrapure water; and the other with the initial fermentation medium (composition in g.L^{-1}: yeast extract, 5.0; K_2HPO_4, 10.0; NaH_2PO_4, 5.0; $MgSO_4$, 0.2; NaCl, 1.0; $MgCl_2$, 0.2; $CaCl_2$, 0.2 and $NaHCO_3$, 10.0), hereinafter referred as the initial matrix. Before the use of the initial matrix, its pH was adjusted to 7.0 and autoclave-sterilized at 121 ºC for 20 min.

The sulfuric acid solution used as mobile phase for HPLC analyses was prepared in ultrapure water. The mobile phase was filtered through a 0.45 μm Millipore membrane and degassed by sonication for 15 min before use.

2.2 *Equipment and chromatographic conditions and solutions*

All analyses were performed using a Thermo Scientific Ultimate 3000 liquid chromatograph (Massachusetts, USA) equipped with: solvent pump (LPG-3400SD), degasser, auto-sampler (WPS-3000SL), column oven controller (TCC-3000RS), diode array detector (DAD-3000RS) and refractive index detector (Dionex). All chromatographic data were obtained using Chromeleon 7.0 software.

An Aminex HPX-87H ion exchange column (300 mm x 7.8 mm ID, 9 μm particle size; Bio-Rad, CA, USA) was used for the simultaneous separation of analytes. The operating conditions were: mobile phase of 5.0 mmol.L^{-1} H_2SO_4, isocratic elution mode, oven temperature 50 °C, eluent flow rate of 0.6 mL.min^{-1}, sample injection volume 20 μL and detection by simultaneous use of refractive index detector (RI) at 50 °C and DAD at UV-210 nm, with a run time of 25 min. These conditions were defined by preliminary studies to ensure a good peak separation of the compounds.

2.3 *Validation process*

The HPLC methodology for simultaneous detection of glycerol, glucose, organic acids and ethanol was validated by evaluating the following performance parameters: selectivity, linearity/working range, matrix effect, limit of detection, limit of quantification, recovery, repeatability and intermediate precision, as suggested by international and Brazilian guidelines (FDA, 1996; Thompson *et al*., 2002; ICH, 2005; INMETRO, 2016, ANVISA, 2017).

Selectivity was evaluated by comparing the retention times obtained for individual standard solutions of analytes and in the working standard mixture solution containing all analytes in the initial matrix. Additionally, the selectivity index (b_{an}/b_{int}) was determined for each analyte, where b_{an} is the slope of the analytical curve prepared in ultrapure water and b_{int} the slope of the analytical curve prepared in the initial matrix (Thompson *et al*., 2002).

Analytical curves were prepared in the ultrapure water matrix and in the initial matrix, using samples of standard mixture solutions at 8 different concentrations (0.1; 0.5; 1.0; 3.0; 5.0; 7.0; 10.0 and 15.0 g.L^{-1}) for all analytes in three independent replicates. Chromatographic data was analyzed to verify the absence of outliers for each concentration applying the Grubbs test (Grubbs, 1969) and the homoscedasticity condition was verified for each curve by the Cochran test (Cochran, 1941). Subsequently, analytical curves for each analyte were estimated by linear regression using the least squares method. Lack of fit of the models was verified by applying the analysis of variance (ANOVA) (ICH, 2005; INMETRO, 2016). Matrix effect was verified for each analyte by comparing the slopes of the analytical curves from the ultrapure water matrix and the initial matrix using the hypothesis test (t-test) performed at α=0.05 (Thompson *et al*., 2002; ANVISA, 2017).

The limit of detection (LOD) and the limit of quantification (LOQ) for each analyte were estimated considering the signal-to-noise ratio. Both were determined using the parameters of the analytical curves by LOD= 3.3 s/S and LOQ= 10 s/S, where s is the standard deviation of the intercept of regression line and S the intercept of the analytical curve (ICH, 2005; INMETRO, 2016).

Recovery and repeatability were evaluated analyzing samples of standard mixture solutions at three concentrations, which were different from those used to construct the analytical curves (0.7, 4.0 and 8.0 $g.L^{-1}$). The samples were analyzed in triplicate under the same conditions. The recovery was determined as percent recovery (R%), by comparing the result obtained analytically with the nominal concentration by R%= [(measured concentration / nominal concentration] x 100). Repeatability was expressed as variation coefficient (CV%), determined by CV% = [(standard deviation/mean) x 100] (ICH, 2005; ANVISA, 2017). Intermediate precision was evaluated studying the short-term stability of samples (24 h at environmental conditions of the laboratory) and stability after one freezing and thawing cycle (30 days of freezing), using samples prepared during the evaluation of recovery. Additionally, independent analyses were carried out by a second analyst, including all steps of sample preparation, analytical curves, and statistical analysis to obtain the results. For the intermediate precision, the recovery and variation coefficients were determined, and the results obtained were compared with values recommended by national and international guidelines (AOAC, 2016; ANVISA, 2017).

All samples used at the validation process steps were filtered through a 0.22 μm PVDF (polyvinylidene fluoride) membrane before being injected automatically into the chromatographic system.

2.4 *Application of the methodology for simultaneous measurement of substrate and metabolites produced in fermentation assays for succinic acid production*

Fermentations by two natural succinic acid producing bacteria - *Actinobacillus succinogenes* and *Basfia succiniciproducens* - were carried out comparatively using different feedstocks (glucose, pure glycerol and crude glycerin). In this regard and to ensure reliable results, the validated HPLC methodology was applied to determine the analytes in real samples from fermentation broths.

2.4.1 Microorganism and inoculum preparation

The strains *Actinobacillus succinogenes* 130Z and *Basfia succiniciproducens* JF4016 were purchased from the Leibniz Institute DSMZ - German Collection of Microorganisms. Both cultures were cryopreserved at -80 °C. Cells were activated in sealed anaerobic bottles containing 30 mL of tryptic soy broth (TSB) medium previously purged with nitrogen gas for 15 min and autoclaved at 121 °C for 20 min. Each bottle was inoculated with 1 mL of cell suspension from a cryopreservation vial of the stock cultures and incubated on a rotatory shaker (130 rpm) at 37 °C for 15 h.

The inoculum was prepared in 100 mL anaerobic bottles containing 80 mL of medium with the following composition ($g.L^{-1}$): glucose, 6.0; yeast extract, 5.0; $NaHCO_3$, 8.0; NaH_2PO_4, 8.5; K_2HPO_4, 15.5; $MgCl_2.6H_2O$, 0.2. The initial pH was adjusted to 7.0 and the culture media were flushed with nitrogen gas for 15 min to remove oxygen. After distribution, the media was autoclaved at 121 °C for 20 min. Glucose and $NaHCO_3$ solutions were prepared separately and aseptically mixed prior to use. Cells from the activation step (10% v/v) were inoculated into the medium and the cultures were incubated on a rotatory shaker (130 rpm) at 37 °C during 7 h for *A. succinogenes* and and 8 h for *B. succiniciproducens*.

2.4.2 Shake flask fermentation

Fermentations were carried out in 100 mL anaerobic flasks containing 80 mL of producing medium with the following composition ($g.L^{-1}$): yeast extract, 10.0; K_2HPO_4, 8.5; NaH_2PO_4, 4.5; $MgSO_4.7H_2O$, 0.525; NaCl, 0.775; $CaCl_2$, 0.185; $NaHCO_3$, 8.3, carbon source 10.0 (glucose, glycerol or crude glycerin), and $MgCO_3$, 10.0. The initial pH was adjusted to 7.0 and the producing media were flushed with nitrogen gas for 15 min to achieve anaerobiosis. After distribution, the producing media were autoclaved at 121 °C for 20 min. Carbon sources, $CaCl_2$ and $NaHCO_3$ solutions were prepared separately and added aseptically to the medium prior to use.

All experiments were inoculated with cells at the exponential growth phase using inoculum size 10% (v/v). Fermentations were performed in triplicate for each substrate at 37 °C and 130 rpm for 12 h.

Fermentation broth samples collected at the start and end of the process were centrifuged at 10,000 rpm for 10 min. The supernatants were filtered through a 0.22 μm PVDF membrane before automatic injection into the chromatographic system for the analysis of substrate consumption and metabolite production. All samples were analyzed in triplicate.

3 Results and discussion

3.1 Methodology validation

3.1.1 Selectivity

The retention time (t_R) of each analyte for the individual standard solution and the standard mixture solution are presented in Table 1. Values showed no significant changes in retention time of the individual standard and mixture solutions. In both cases, retention times presented variation coefficients inferior to the repeatability criteria indicated by AOAC (2012). Figure 1 presents the chromatograms of the standard mixture solution prepared in the initial matrix using dual detection (UV and RI). The chromatograms (Fig. 1) showed a good resolution of all peaks, indicating that the methodology allowed for the separation and identification of all analytes. Hence, no peak was attributable to more than one analyte and the peaks associated with the ten analytes could be distinguished clearly.

The identification of some analytes can be done by either RI or UV detector, or by both detectors (Fig. 1). In this work, each analyte was assigned a single detector in order to continue with the validation process, considering good signal sensitivity without the possible interference of complex matrices from fermentative processes. Glucose and glycerol substrates were measured by the RI detector. Regarding the fermentation products, the pyruvic, acetic and propionic acids, as well as ethanol, were measured by the RI detector. Citric, succinic, lactic and formic acids were measured using the UV detector.

3.1.2 Linearity and working range

Considering the variability characteristics of fermentation processes, the working range of the methodology corresponds to the range 0.1-15.0 g.L^{-1} for all the analytes evaluated in this study. The use of a large working range allows to increase the number of samples analyzed due to the reduction of the dilution steps and the time required for sample preparation. However, the verification of the homoscedasticity condition is mandatory. Different authors, such as Eyéghé-Bickong *et al.*(2012), Ivanova *et al.* (2016) and Coelho *et al.* (2018) presented lower working ranges for some of the analytes studied in this work. On the other hand, Zhang *et al.* (2011) and Zaky *et al.* (2017) considered working ranges close to those presented in this work.

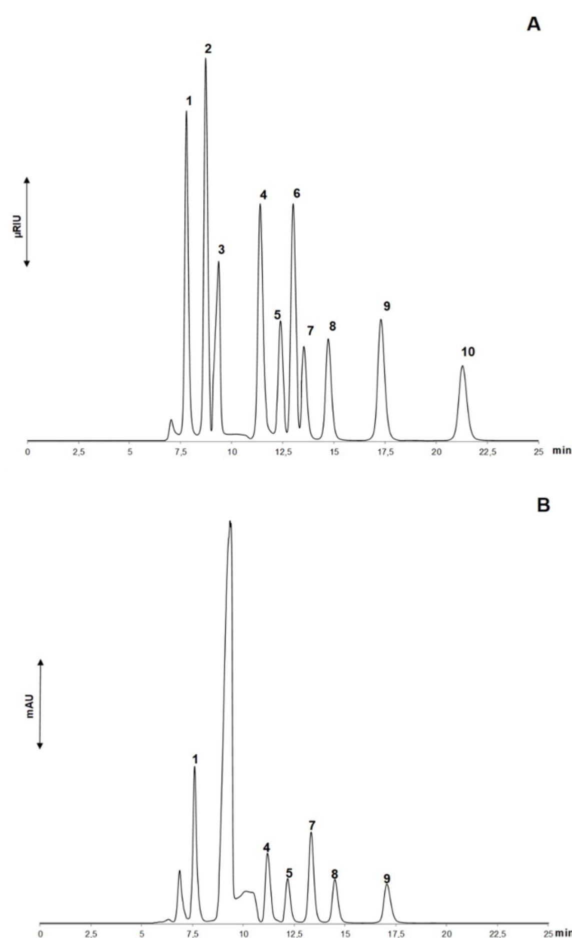

Fig. 1. HPLC chromatograms of a standard mixture solution using an Aminex HPX-87H column at 50 °C with injection volume 20 μL, flow rate of 0.6 mL.min^{-1} using 5.0 mM isocratic H_2SO_4 mobile phase. (A) RI detection at 50 °C and (B) DAD detection UV at 210 nm. Peaks: citric acid (1), glucose (2), pyruvic acid (3), succinic acid (4), lactic acid (5), glycerol (6), formic acid (7), acetic acid (8), propionic acid (9) and ethanol (10).

Table 1. Retention time (t_R) of each analyte for the individual standard solution and standard mixture.

Analyte	Individual standard*		Standard mixture*	
	t_R (min)	CV (%)	t_R (min)	CV (%)
Citric acid	7.61	0.27	7.64	0.1
Glucose	8.72	0.09	8.74	0.13
Pyruvic acid	9.44	0.69	9.47	0.58
Succinic acid	11.2	0.08	11.19	0.37
Lactic acid	12.19	0.03	12.21	0.01
Glycerol	13	0.07	13.02	0.08
Formic acid	13.34	0.02	13.37	0.01
Acetic acid	14.71	0.12	14.73	0.12
Propionic acid	17.28	0.2	17.29	0.13
Ethanol	21.26	0.13	21.3	0.09

* Individual standard solution prepared in ultrapure water

** Standard mixture solution of ten analytes prepared in initial matrix

CV (%): variation coefficient in percentage.

Analytical curves were built considering the working range for the ultrapure water matrix and the initial matrix. For all analytes, at eight levels of concentrations, outliers were not observed by Grubbs test (Grubbs's $G_{calculated}$ < Grubbs's $G_{tabulated}$). All of Grubbs's $G_{calculated}$ values were lower than Grubbs's $G_{tabulated}$ value (1.155 for n=3 and significance level of 95% or α=0.05).

Analytical methods that use linear regression must ensure that the variances at different concentration levels are homogeneous. In this sense, the uniform variance condition - called homoscedasticity - must be observed. The homoscedasticity condition was observed for all analytes by the Cochran test (Cochran's $C_{calculated}$ < Cochran's $C_{tabulated}$). All of Cochran's $C_{calculated}$ values were lower than Cochran's $C_{tabulated}$ value (0.516 for 8 levels of concentration, 3 replicates and α=0.05). This way, it is possible to indicate that in the working range established, the precision of measurements using the analytical curves is independent of the concentration level.

After the verification of homoscedasticity, analytical curves were built by linear regression. The regression data, including slope, intercept and correlation coefficient (r) for each analyte are presented in Table 2. The selectivity indexes were between 0.96 and 1.16, indicating that the methodology presents good selectivity for the simultaneous detection of all analytes (Thompson *et al*., 2002). The correlation coefficients for all evaluated analytes were around 0.999 (Table 2), suggesting that the linear model is well fitted to the experimental data. These correlation coefficient values are in accordance with the criteria proposed by a Brazilian guide (ANVISA, 2017).

Considering that the use of the correlation coefficient as an indicator of linearity can be misleading, linearity was evaluated by analysis of variance (ANOVA) of the analytical curves (Thompson *et al*., 2002; INMETRO, 2016). Table 2 shows all Snedecor's $F_{calculated}$ values from one-way ANOVA for data groups in different concentrations and having as initial hypothesis the non-relationship between detector response (peak area) and analyte concentration. As it can be surmised from Table 2, all of Snedecor's $F_{calculated}$ values are higher than Snedecor's $F_{tabulated}$ value (F7;16; 0.05 is equal to 2.657 for a confidence interval of 95%. Once $F_{calculated}$ > $F_{tabulated}$, the initial hypothesis is rejected, concluding that there is a direct relationship between peak area and analyte concentration.

3.1.3 *Matrix effect*

Considering the complex and dynamic matrices which stem from fermentation processes and the traditional laboratory routine, where ultrapure water solutions are used for the construction of analytical curves, a detailed statistical analysis was carried out to verify if analyte quantification could present some significant variation caused by the complexity of the initial matrix. This way, it is possible to ensure an adequate quantification through the selection of the most appropriate analytical curve for quantification of each analyte. It is worth mentioning that these types of studies are often omitted in validation processes, although they are mainly recommended when working with complex matrices (ICH, 2005; INMETRO, 2016).

Table 2. Data obtained from regression by the least squares method and comparison of analytical curves.

Analyte	Slope	Intercept	r	$F_{calculated}$[c]	$t_{calculated}$
Citric acid[a]	50.165	1.828	0.9998	30679.6	27.664[e]
Citric acid[b]	43.048	7.228	0.9999	85293.8	
Glucose[a]	4.909	0.014	0.9999	25530.2	1.480[d]
Glucose[b]	4.854	0.298	0.9998	87171.7	
Pyruvic acid[a]	2.822	0.079	0.9999	41380.4	1.737[d]
Pyruvic acid[b]	2.773	0.515	0.9996	46728	
Succinic acid[a]	26.781	0.304	0.9998	10201.2	2.160[d]
Succinic acid[b]	27.683	16.951	0.9992	34551.2	
Lactic acid[a]	16.295	0.121	0.9999	23861.1	3.479[d]
Lactic acid[b]	15.654	4.318	0.9995	25941.7	
Glycerol[a]	4.071	-0.023	0.9999	34339.2	3.076[d]
Glycerol[b]	4.157	0.133	0.9998	57509.8	
Formic acid[a]	32.222	-0.291	0.9999	70712.8	0.202[d]
Formic acid[b]	32.171	6.133	0.9997	52961.9	
Acetic acid[a]	1.947	-0.07	0.9999	38278.9	0.914[d]
Acetic acid[b]	1.935	0.124	0.9998	64560.5	
Propionic acid[a]	2.814	-0.008	0.9998	80701.3	1.903[e]
Propionic acid[b]	2.848	0.632	0.9999	57581.9	
Ethanol[a]	2.014	0.082	0.9997	32470.3	4.047[d]
Ethanol[b]	2.097	0.036	0.9998	64560.5	

[a] Analytical curve in ultrapure water matrix, [b] Analytical curve in initial matrix, r: correlation coefficient, [c] $F_{tabulated}$ (v1=7; v2=16; α= 0.05) = 2.657 for lack of fit evaluation by ANOVA, [d] Student Value, different residual variances $t_{tabulated}$ (v2T=6; α= 0.05) = 2.447, [e] Student Value, equal residual variances $t_{tabulated}$ (v2T=12; α= 0.05) = 2.179

Table 3. Limits of detection (LOD) and quantification (LOQ) estimates.

Analyte	LOD ($g.L^{-1}$)	LOQ ($g.L^{-1}$)
Citric acid	0.01	0.03
Glucose	0.016	0.048
Pyruvic acid	0.006	0.018
Succinic acid	0.019	0.057
Lactic acid	0.021	0.065
Glycerol	0.006	0.019
Formic acid	0.017	0.053
Acetic acid	0.013	0.04
Propionic acid	0.011	0.034
Ethanol	0.006	0.017

Analytical curves were compared applying Student's t-test with a significance of 95% and the use of initial matrix analytical curves was employed in order to compensate any matrix effects in the estimation of analyte concentrations (INMETRO, 2016; ANVISA, 2017). For citric acid, lactic acid, glycerol, and ethanol, Student's $t_{calculated}$ values were higher than Student's $t_{tabulate}$ corresponding value (Table 2). Thus, the use of analytical curves built with the initial matrix is mandatory to determine the concentration of these analytes. On the other hand, for glucose and pyruvic, succinic, formic, acetic and propionic acids, Student's $t_{calculated}$ values were lower than Student's $t_{tabulate}$ corresponding value (Table 2). Thereby, it is possible to use both analytical curves to determine the concentration of these analytes. This way, it is possible to recommend the preparation of analytical curves in water for glucose and pyruvic, succinic, formic, acetic and propionic acids to develop similar methodologies, simplifying the work of analysts without compromising the quality of results.

Although it is possible to determine the concentration of some analytes using ultrapure water analytical curves, in the present work the quantification of all the analytes was carried out using the analytical curves built with the initial matrix. This was defined to standardize the quantification step.

3.1.4 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ represent the lower amount of an analyte that can be detected and quantified, respectively, under certain analysis conditions. Table 3 shows the LOD and LOQ values obtained for each of the analytes, which ranged from 0.006 to 0.021 $g.L^{-1}$

and from 0.018 to 0.065 g.L^{-1}, respectively.

LOD and LOQ values are characteristic of the analytical methodology, including the equipment. For this reason, there are no reference values established as acceptance criteria for these parameters. It is possible to find in the literature several LOD and LOQ values for the same analytes with similar methodologies. In this sense, different authors presented various LOD and LOQ values for their methodologies to determine organic acids. For example, De Sá *et al.* (2011) reported LOD and LOQ values higher for acetic acid (0.028 g.L^{-1}; 0.093 g.L^{-1}) propionic acid (0,024 g.L^{-1}; 0.079 g.L^{-1}) and glucose (0.058 g.L^{-1}; 0.191 g.L^{-1}) in their study performed on a Shimadzu system, using an Aminex HPX-87H column with dual detection, mobile phase of 5 mM H_2SO_4, flow rate of 1.0 mL.min^{-1} and temperature of 55 ºC. Eyéghé-Bickong *et al.* (2012) reported higher LOD values for citric acid (0.03 g.L^{-1}) and succinic acid (0.02 g.L^{-1}) using an Agilent system with dual detection, in which the separation was achieved with an Aminex HPX-87H, mobile phase of 5 mM H_2SO_4, flow rate of 0.5 mL.min^{-1} and temperature of 55 ºC.

Ivanova *et al.* (2016), in their work using a Varian Pro Star system, PDA detection, LiChrosorb RP-18 column with a mobile phase of an aqueous solution of 5mM H_3PO_4/ acetonitrile 1% and flow rate of 1 mL.min^{-1}, presented LOD and LOQ values similar to those obtained in the present work for lactic acid (0.014 g.L^{-1}; 0.045 g.L^{-1}) and succinic acid (0.012 g.L^{-1}; 0.041 g.L^{-1}). Finally, Coelho *et al.*(2018) presented lower LOQ values for lactic (0.056 g.L^{-1}) and acetic acids (0.008 g.L^{-1}) using an Agilent 1260 Infinity system with an Agilent Hi-Plex H column, dual detection, mobile phase of 4mM H_2SO_4, flow rate of 0.5 mL.min^{-1} and temperature of 70 ºC.

3.1.5 Recovery

Recovery, expressed as a percentage, is used as a parameter to evaluate the accuracy in relation to systematic error. Table 4 shows the percentage of recovery (R %) values for each analyte in the different concentration levels. The recovery values obtained were in the range of 89-109%. These values are in agreement with the range of 80-110% recommended by Brazilian and international validation guides (AOAC, 2016; INMETRO, 2017), demonstrating that the methodology has as acceptable accuracy.

In a similar way, different authors, such as Qureshi *et al.* (2011), de Sena Aquino *et al.* (2015) and Ivanova *et al.* (2016), presented recovery ranges within the recommended range for the analysis of several organic acids and substrates under various operating conditions. Conversely, the recovery values presented in this work could express a higher degree of accuracy when compared with other recovery values; for instance, the ones reported for acetic and propionic acids, with mean recovery of approximately 82% and 71%, respectively (De Sá *et al.*, 2011); for succinic acid, of 87% (Eyéghé-Bickong *et al.*, 2012); and for glucose and formic acid, of 78% and 89%, respectively (Costa *et al.*, 2016).

3.1.6 Precision (repeatability and intermediate precision)

Precision was evaluated in terms of repeatability and intermediate precision. Repeatability refers to the degree of agreement between repeated measurements of the same sample under the same operating conditions, while intermediate precision refers to the precision evaluated on the same sample but considering laboratory variations, which may affect the result of the analysis of a sample. These variations are related to the analysis execution, not to the development of the methodology.

Table 4 shows the variation coefficient (CV %) values obtained for repeatability, which ranged from 0.01 to 2.33%. This data is below the limit indicated for analytical methods (AOAC, 2016; INMETRO, 2016), indicating acceptable repeatability for the methodology presented.

The CV range obtained in this study was similar to the one reported by De Sá *et al.* (2011), with values between 0.2-2.3%. These CV % values were higher than the values between 0.03-1.66% and 0.1-1.4% reported by Zhang *et al.* (2011) and Coelho *et al.* (2018), respectively. On the other hand, the CV % values found here were observed to be lower in contrast to value ranges 1.65-4.68% and 2.10-4.44% reported by Eyéghé-Bickong *et al.* (2012) and Ivanova *et al.* (2016), respectively.

Table 5 presents the variation coefficient and recovery values obtained from studies of short-term stability, stability after one freezing and thawing cycle and independent analysis carried out by a second analyst. As can be seen from Table 5, all variation coefficient values were lower than 5% and all recovery values ranged between 88 and 108%.

Table 4. Recovery and variation coefficient values obtained in the evaluation of accuracy and repeatability.

Analyte	Theoretical concentration (g.L^{-1})	Estimated mean concentration (g.L^{-1})	R (%)	CV (%)
Citric acid	0.7	0.766	109.4	0.36
	4	4.316	107.9	2.03
	8	8.196	102.5	1.24
Glucose	0.7	0.673	96.2	0.43
	4	4.142	103.6	1.12
	8	8.067	100.8	0.72
Pyruvic acid	0.7	0.762	108.8	1.34
	4	4.268	106.7	1.14
	8	8.144	101.8	0.52
Succinic acid	0.7	0.68	97.1	2.33
	4	4.01	100.3	0.09
	8	8.106	101.3	0.5
Lactic acid	0.7	0.623	89	1.81
	4	4.145	103.6	0.74
	8	8.113	101.4	0.37
Glycerol	0.7	0.675	96.4	1.06
	4	4.158	103.9	1.2
	8	8.079	101	0.71
Formic acid	0.7	0.693	99	0.82
	4	4.013	100.3	0.06
	8	8.114	101.4	0.18
Acetic acid	0.7	0.686	98	0.83
	4	4.146	103.7	1.14
	8	8.076	101	0.63
Propionic acid	0.7	0.688	98.3	0.27
	4	3.979	99.5	0.06
	8	8.047	100.6	0.01
Ethanol	0.7	0.709	101.2	0.6
	4	4.046	101.2	1.03
	8	7.852	98.2	0.6

R (%): recovery in percentage; CV (%): variation coefficient in percentage.

These results are in accordance with acceptable values indicated by Brazilian and international validation guides, which provided the present methodology with the acceptable intermediate precision (AOAC, 2016; INMETRO, 2017). Thus, it is possible to certify the stability of samples under the storage conditions evaluated. The results obtained by a different analyst suggest that there is no significant interaction between results of samples and analysts when the analysts have adequate training.

In comparison to this work, some authors have presented less detailed intermediary precision studies.

Table 5. Variation coefficient and recovery values obtained in the evaluation of intermediate precision.

Analyte	**Theoretical concentration ($g.L^{-1}$)**	**Short-term stability**			**Stability after freeze/thaw cycle**			**Analysis performed by a second analyst**		
		Estimated concentration ($g.L^{-1}$)	VC(%)	R(%)	Estimated concentration ($g.L^{-1}$)	VC (%)	R(%)	Estimated concentration ($g.L^{-1}$)	VC(%)	R(%)
Citric acid	0.7	0.758± 0.013	1.57	108.3	0.761± 0.034	4.21	108.7	0.753± 0.032	4.03	107.5
	4	4.308± 0.037	0.84	107.7	4.302± 0.018	0.42	107.6	4.112± 0.043	1.04	102.8
	8	8.539± 0.080	0.94	106.7	8.164± 0.123	1.51	102.1	8.247± 0.096	1.16	103.1
Glucose	0.7	0.673± 0.015	2.19	96.1	0.682 ± 0.007	1.02	97.4	0.695± 0.021	2.97	99.2
	4	4.153± 0.016	0.38	103.8	4.215± 0.034	0.8	105.4	4.056 ± 0.011	0.28	101.4
	8	8.286± 0.073	0.88	103.6	8.276± 0.022	0.26	103.5	8.035± 0.099	1.23	100.4
Pyruvic acid	0.7	0.753± 0.011	1.4	107.6	0.678± 0.007	1.05	96.9	0.753± 0.033	4.4	107.6
	4	4.275± 0.021	0.48	106.9	4.228± 0.029	0.65	105.7	4.064± 0.022	0.53	101.6
	8	8.330± 0.064	0.77	104.1	8.647± 0.063	0.73	108.1	7.981± 0.089	1.11	99.8
Succinic acid	0.7	0.708± 0.019	2.63	101.1	0.670± 0.007	1	95.7	0.711± 0.019	2.74	101.6
	4	4.038± 0.021	0.52	100.9	4.148± 0.014	0.35	103.7	3.890± 0.069	1.77	97.3
	8	7.990± 0.067	0.84	99.9	8.229± 0.137	1.66	102.9	8.044± 0.048	0.59	100.5
Lactic acid	0.7	0.616 ± 0.009	1.45	88.1	0.630± 0.022	3.56	90.1	0.632± 0.018	2.84	90.3
	4	4.170 ± 0.024	0.58	104.2	4.185± 0.032	0.77	104.6	4.201± 0.147	3.5	105
	8	8.340 ± 0.075	0.91	104.3	8.629± 0.128	1.48	107.8	7.976± 0.103	1.29	99.7
Glycerol	0.7	0.665 ± 0.013	1.95	94.9	0.660± 0.008	1.28	94.3	0.704± 0.002	0.34	100.5
	4	4.166 ± 0.017	0.41	104.2	4.099± 0.029	0.72	102.5	4.068± 0.014	0.34	101.7
	8	8.280 ± 0.067	0.81	103.5	8.042± 0.032	0.39	100.5	8.056± 0.085	1.06	100.7
Formic acid	0.7	0.711 ± 0.006	0.91	101.5	0.616± 0.003	0.47	88	0.690± 0.017	2.51	98.6
	4	4.038 ± 0 015	0.37	100.9	3.677± 0.061	1.65	91.9	4.067± 0.019	0.48	101.7
	8	8.055 ± 0.060	0.75	100.7	7.960± 0.245	3.07	99.5	8.075± 0.047	0.59	100.9
Acetic acid	0.7	0.673 ± 0.011	1.63	96.1	0.681± 0.014	2.06	97.3	0.699± 0.005	0.66	99.5
	4	4.148 ± 0.019	0.45	103.7	4.051± 0.041	1.02	101.3	4.049± 0.021	0.52	101.2
	8	8.254 ± 0.069	0.84	103.2	8.015± 0.016	0.2	100.2	8.049± 0.054	0.67	100.6
Propionic acid	0.7	0.698 ± 0.008	1.2	99.7	0.671± 0.024	3.65	95.8	0.697± 0.011	1.56	99.5
	4	3.976 ± 0.015	0.38	99.4	4.071± 0.070	1.72	101.8	4.052± 0.004	0.1	101.3
	8	7.930 ± 0.046	0.58	99.1	7.965± 0.185	2.33	99.6	8.076± 0.015	0.19	100.9
Ethanol	0.7	0.678 ± 0.010	1.48	96.9	0.615± 0.026	4.26	87.9	0.689± 0.009	1.3	98.5
	4	4.008 ± 0.026	0.66	100.2	3.899± 0.068	1.73	97.5	3.966± 0.005	0.12	99.2
	8	7.946 ± 0.083	1.05	99.3	7.698± 0.034	0.44	96.2	7.955± 0.033	0.42	99.4

R (%): recovery in percentage; CV (%): variation coefficient in percentage.

For example, De Sá *et al*. (2011) studied the intermediate precision only by analysis of samples after a freezing and thawing cycle; Ivanova *et al*. (2016) analyzed the same samples continuously in five different days; Costa *et al*. (2016) presented the performance of the methodology in the hands of a different analyst. In all three cases, the authors presented the coefficient of variance as the only indicator of the intermediary precision.

3.2 Analysis of real samples from fermentative succinic acid production

The validated HPLC methodology was used to identify and quantify substrate and metabolic products in real samples from individual batch fermentations by *A. succinogenes* 130Z or *B. succiniciproducens* JF4016. The fermentations were developed under similar oxygen-limited conditions, using glucose, pure glycerol and biodiesel-derived crude glycerin as carbon sources.

As can be seen in Table 6, when using glucose as carbon source, substrate consumption by *A. succinogenes* 130Z was almost total (9.552 $g.L^{-1}$). According to Pateraki *et al*. (2016), the process of glucose breakdown through the glycolytic pathway by *A. succinogenes* involves glucose phosphorylation followed by other enzyme-catalyzed steps, with phosphoenolpyruvate (PEP) formation and recycling of redox equivalents, which promotes a high substrate uptake. Despite the high glucose consumption (> 95%), the production of succinic acid (3.572 $g.L^{-1}$) was accompanied by simultaneous cogeneration of acetic acid (2.327 $g.L^{-1}$) and formic acid (1.529 $g.L^{-1}$) mainly. In addition, low amounts of citric, lactic, pyruvic, propionic acids and ethanol were produced. Thus, succinic acid was only equivalent to 37% of the total generated products, indicating the low carboxylation of PEP. Therefore, the C3 pathway was the preferential metabolic route, in which PEP is converted to pyruvate (PYR), which serves as precursor of different metabolites, such as monocarboxylic acids and alcohols (Dessie *et al*., 2018).

Table 6. Substrate consumption and metabolites formation from glucose, pure glycerol and crude glycerin by *A. succinogenes* 130Z in oxygen-limiting batch fermentation.

C-source	Glucose	Pure glycerol	Crude glycerin
Substrate consumed ($g.L^{-1}$)	9.552 ±0.060	1.753 ± 0.178	1.616 ± 0.174
Succinic acid ($g.L^{-1}$)	3.572 ± 0.052	0.427 ± 0.024	0.402 ± 0.019
Acetic acid ($g.L^{-1}$)	2.327 ± 0.076	0.536 ± 0.011	0.412 ± 0.023
Formic acid ($g.L^{-1}$)	1.529 ± 0.050	0.577 ±0.010	0.541 ± 0.014
Lactic acid ($g.L^{-1}$)	0.523 ± 0.028	0.109 ± 0.008	0.074 ± 0.005
Citric acid ($g.L^{-1}$)	0.766 ± 0.052	<LOD[a]	<LOD[a]
Pyruvic acid ($g.L^{-1}$)	0.586 ± 0.008	<LOD[a]	<LOD[a]
Propionic acid ($g.L^{-1}$)	0.109 ± 0.012	0.037 ± 0.002	<LOQ[b]
Ethanol ($g.L^{-1}$)	<LOQ[b]	<LOQ[b]	<LOQ[b]
Succinic acid yield (g/g)	0.374	0.244	0.249
Succinic acid productivity ($g.L^{-1}h^{-1}$)	0.298	0.036	0.034

[a] LOD: limit of detection (see value in Table 3)

[b] LOQ: limit of quantification (see value in Table 3)

Yield: g of succinic acid formed/ g of substrate consumed

On the other hand, the consumption of substrate by *A. succinogenes* 130Z using pure glycerol and crude glycerin was 1.753 $g.L^{-1}$ and 1.616 $g.L^{-1}$, respectively (Table 6), corresponding to about 17%. The low uptake of glycerol by *A. succinogenes* is related to a redox unbalance caused by the excess of reducing equivalents produced during the metabolism of glycerol, which cannot be naturally recycled, limiting glycerol consumption according to our findings (Schindler *et al*., 2014; Carvalho *et al*., 2014). In fact, small amounts of succinic acid were produced by *A. succinogenes* under a limited glycerol intake, with production of 0.427 $g.L^{-1}$ from pure glycerol and 0.402 $g.L^{-1}$ from crude glycerin, corresponding to a yield of 0.24 g/g and productivity around 0.03 $g.L^{-1}h^{-1}$. Nevertheless, the succinic acid production obtained in this work are similar to those reported in literature: 0.49 $g.L^{-1}$ and 0.36 $g.L^{-1}$ for fermentation by natural strains of *A. succinogenes*, using crude glycerin and pure glycerol, respectively (De Barros *et al*., 2013; Carvalho *et al*., 2014).

Succinic acid represented around 25% of the total products generated by *A. succinogenes* 130Z from both glycerol sources, and equivalent amounts of acetic and formic acids were observed (Table 6). This behavior indicates that PEP was preferentially catabolized by the C3 metabolic pathway, as previously mentioned for the metabolism of glucose. Also, propionic acid and ethanol were found in amounts below their quantification limit, while pyruvic and citric acids were not detected.

Therefore, it was observed that the type of carbon source has a huge influence on the distribution of metabolites produced by *A. succinogenes* 130Z. Indeed, a relatively large variety of products was found in the glucose fermentation broth (succinic, acetic, formic, lactic, citric, pyruvic and propionic acids, as well as ethanol), in opposition to the crude glycerin fermentation broth (succinic, acetic, formic, lactic and propionic acids, as well as ethanol).

Table 7 presents the results from batch fermentations by *B. succiniciproducens* JF4016. Similarly to *A. succinogenes* 130Z, high glucose consumption (> 85%) was observed for *B. succiniciproducens*. However, succinic acid production (4.283 $g.L^{-1}$) was superior, representing around 50% of total products, even if it was accompanied by simultaneous cogeneration of acetic acid (2.650 $g.L^{-1}$) and formic acid (1.406 $g.L^{-1}$). In addition, low amounts of lactic acid and ethanol were also produced, while pyruvic and citric acids were not detected.

In comparison with *A. succinogenes* 130Z, higher substrate consumption (around 37%) and succinic acid production (2.518 $g.L^{-1}$ and 2.605 $g.L^{-1}$) were determined in fermentation broths by *B. succiniciproducens* from crude glycerin and pure glycerol, respectively. The fermentation of these substrate sources led to the formation of succinic acid as the major product, corresponding to about 80% of total products generated, with yields between 0.68 g/g and 0.73 g/g, and productivity around 0.22 $g.L^{-1}h^{-1}$. These results suggest a better capacity of *B. succiniciproducens* JF4016 to uptake glycerol and to produce succinic acid. This can be explained

Table 7. Substrate consumption and metabolites formation from glucose, pure glycerol and crude glycerin by *B. succiniciproducens* JF4016 in oxygen-limiting batch fermentation.

C-source	Glucose	Pure glycerol	Crude glycerin
Substrate consumed ($g.L^{-1}$)	8.587 ± 0.114	3.806 ± 0.074	3.458 ± 0.072
Succinic acid ($g.L^{-1}$)	4.283 ± 0.034	2.605 ± 0.091	2.518 ± 0.019
Acetic acid ($g.L^{-1}$)	2.650 ± 0.021	0.598 ± 0.013	0.478 ± 0.017
Formic acid ($g.L^{-1}$)	1.406 ± 0.075	0.336 ± 0.014	0.236 ± 0.013
Lactic acid ($g.L^{-1}$)	0.104 ± 0.025	0.115 ± 0.017	<LOQ[b]
Citric acid ($g.L^{-1}$)	<LOD[a]	<LOD[a]	<LOD[a]
Pyruvic acid ($g.L^{-1}$)	<LOD[a]	<LOD[a]	<LOD[a]
Propionic acid ($g.L^{-1}$)	<LOQ[b]	<LOQ[b]	<LOQ[b]
Ethanol ($g.L^{-1}$)	0.062 ± 0.004	<LOQ[b]	<LOQ[b]
Succinic acid yield (g/g)	0.499	0.684	0.728
Succinic acid productivity ($g.L^{-1}h^{-1}$)	0.357	0.217	0.21

[a] LOD: limit of detection (see value in Table 3)

[b] LOQ: limit of quantification (see value in Table 3)

Yield: g of succinic acid formed/ g of substrate consumed

by the presence of PEP carboxykinase and PEP carboxylase, two key enzymes in the conversion of phosphoenolpyruvate (PEP) into oxaloacetate and finally into succinic acid via the C4 pathway, whereas only PEP carboxykinase is present in *A. succinogenes* cells (Becker *et al*., 2013; Pateraki *et al*., 2016; Ahn *et al*., 2016).

Albeit succinic acid was the principal product of the fermentation of crude glycerin and pure glycerol by *B. succiniciproducens*, the production of metabolites by the C3 pathway was also observed, in particular acetic and formic acids (Table 7). Comparatively, the amount of acetic acid (0.478 $g.L^{-1}$) produced by *B. succiniciproducens* was similar to that obtained by *A. succinogenes* (0.412 $g.L^{-1}$) in the fermentation of crude glycerin. Inversely, the amount of formic acid produced decreased significantly (49%). Ethanol, lactic and propionic acids were presented below the detection limit (<LOQ), whereas pyruvic and citric acids were not detected, as observed when glucose was used. This suggests that these acids act as intermediate metabolites that are not excreted by those cells (Becker *et al*., 2013).

Similarly, fermentations of crude glycerin, pure glycerol and glucose by the *B. succiniciproducens* strain led to the same products (succinic, acetic, formic, lactic and propionic acids, as well as ethanol). Analogous distribution of metabolites was reported by Scholten e Dägele (2008) using a strain of *B. succiniciproducens* DD1. Hence, differently from *A. succinogenes*, the carbon source does not affect the synthesis of metabolites by *B. succiniciproducens*.

Conclusions

The HPLC methodology described is suitable for the simultaneous determination of glucose, glycerol, ethanol and organic acids (citric, pyruvic, succinic, lactic, formic, acetic and propionic) and the protocol used for its validation showed adequate results for the performance parameters evaluated, ensuring the quality of the data obtained. The validated methodology was successfully applied in the analysis of samples from heterofermentation of crude glycerin, pure glycerol and glucose by two natural succinic acid producing bacteria, giving support to biochemical discussions. Under the fermentative conditions tested and comparing to *A. succinogenes* 130Z, the carbon source does not affect the distribution of metabolites synthesized by *B. succiniciproducens* JF4016, and this strain showed less variety of co-products and good natural capacity to convert all substrates, mainly crude glycerin, to succinic acid.

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