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Biotechnological conversion of spent coffee grounds into lactic acid

Short running head: Bioconversion of spent coffee grounds

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Significance and Impact of the Study:

Spent coffee grounds (SCG) represent solid waste generated in millions of tons by coffee processing industries. Their disposal represents a serious environmental problem; however, SCG could be valorized within a biorefinery concept yielding various valuable products. Herein, we suggest that SCG can be used as a complex carbon source for the lactic acid production.

Abstract

This work investigates the potential conversion of spent coffee grounds (SCG) into lactic acid (LA). SCG were hydrolysed by a combination of dilute acid treatment and subsequent application of cellulase. The SCG hydrolysate contained a considerable amount of reducing sugars (9.02 ± 0.03 g l^{-1}, glucose; 26.49 ± 0.10 g l^{-1} galactose and 2.81 ± 0.07 g l^{-1} arabinose) and it was used as a substrate for

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cultivation of several lactic acid bacteria (LAB) and also lactic acid producing *Bacillus coagulans.* Among the screened microorganisms, *Lactobacillus rhamnosus,* CCM 1825 was identified as the most promising producer of LA on SCG hydrolysate. Despite the inhibitory effect exerted by furfural and phenolic compounds in the medium, reasonably high lactic acid concentrations (25.69 ± 1.45 g l^{-1}) and yields (98 %) were gained. Therefore, it could be demonstrated that SCG is a promising raw material for the production of lactic acid and could serve as a feedstock for the sustainable large-scale production of lactic acid.

Keywords

Lactic acid bacteria, fermentation, *Lactobacillus*, waste, biotechnology, spent coffee grounds, lactic acid

Introduction

Lactic acid (LA) is valuable substance widely used in food, pharmaceutical, cosmetic and chemical industries. One of the main applications of LA is as a monomer for the production of the biodegradable polymer polylactic acid (PLA), which represents a promising alternative to traditional petroleum-based plastics (Abdel-Rahman 2016). Industrial LA production is predominantly performed by microbial fermentation (Lu 2010), nevertheless, high substrate costs remain a major economic factor for the large-scale production of LA. Moreover, widely used refined sugars and starch compete with food resources and feed supply. Hence, alternative inexpensive substrates such as lignocellulosic biomass are recommended for the reasonable and sustainable process of LA production (Kuo et al. 2015). However, utilization of these complex materials usually requires pre-treatment and hydrolysis prior to fermentation (Jönsson and Martin 2016).

SCG are solid residues generated during the preparation of coffee beverage or the manufacturing of instant coffee. On an average, about 650 kg of SCG is produced by manufacturing from one ton of green coffee (Mussatto 2011). The SCG consists mainly of hemicelluloses (30 – 40 wt.%), particularly mannans, galactans, and arabinans. The cellulose content is about 10 wt %. The other substances present in SCG are lignin (approx. 30 wt.%) and polyphenols (2.5 wt.%), which are constituted mainly by highly bioavailable and bioactive chlorogenic acids. Because of the presence of caffeine, tannins, and polyphenols, SCG can represent a pollution hazard if discharged into the environment (Silva et al. 1998, Obruca 2015). Therefore, due to their properties and also because of the huge amount of SCG produced globally, treatment or even further valorization of SCG is an important topic not only for the coffee processing industry but also for elimination of environmental pollution problem (Mussatto 2011; Obruca et al. 2014). There are reports that other some wastes of coffee processing industry such as coffee mucilage (Neu et al. 2016) or coffee pulp (Pleissner et al. 2016) can be converted into LA employing *Bacillus coagulans*, nevertheless, to our best knowledge, production of LA using SCG as a substrate has not been experimentally verified yet (Murthy et al. 2012; Karmee 2017; Mata et al. 2018). Therefore, the aim of this study was to perform a basic characterization of SCG and study hydrolysis of SCG in the context of its further biotechnological conversion into LA in accordance with current industrial and economic requirements.

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Results and discussion

Characterization and hydrolysis of SCG

The composition of the SCG is shown in Table 1. Values are expressed on a dry matter base. The presented data are comparable with those published in the other articles on SCG (Obruca 2014; Ballesteros 2014). For this study, it was not necessary to determine separate values for galactose and mannose because the selected bacterial strains were able to use both saccharides and the experiments were evaluated based on the obtained LA

Compared with other lignocellulose-based materials, hexoses are substantially dominating sugars in SCG. This may be an important factor positively influencing the intended process of LA production (Iyer et al. 2000)

Table 1

To hydrolyse SCG, the combination of dilute acid hydrolysis of hemicelluloses and enzymatic saccharification of cellulose was applied. The three different concentrations of sulfuric acid were used (0 g l^{-1}; 13.5 g l^{-1} and 27 g l^{-1}) to compare the effect of acid concentration to the fermentable sugars. The results are shown in Table 2. Generally, the highest amount of reducing sugars (mainly galactose + mannose and arabinose) was released during the initial step of the hydrolytic process – dilute acid hydrolysis of hemicellulose. The highest cumulative titer of sugars (30.93 g l^{-1}, SCG load was 50 g l^{-1}) was gained with a sulfuric acid concentration of 27 g l^{-1}. Successful degradation of hemicelluloses further enabled enzymatic hydrolysis of cellulose yielding mostly glucose. Also, in this case, higher glucose yields were achieved with higher sulfuric acid concentrations in the first step; therefore, a sulfuric acid concertation of 27 g l^{-1} was used in the subsequent experiments.

The analysis also focused on chemical compounds which might impair the fermentability of spent coffee grounds hydrolysate (SCGH). The use of acid for the hemicelluloses hydrolysis and base for subsequent neutralization of SCGH may increase the concentration of salts in solution. High concentrations of salts may induce osmotic stress and result in the inhibition of bacterial growth. Moreover, SCGH contains also high amounts of potentially antimicrobial substances such as polyphenols, 5-hydroxymethylfurfural (5HMF) and levulinic acid, which might reduce the efficiency of the following fermentation step.

Table 2

Table 1 represents the composition of the initial feedstock. Analytical values were obtained from the NREL protocol, which is based on a complete hydrolysis of the material and calculates values for cellulose and hemicelluloses from the amounts of hydrolysed sugars. From hydrolysis was obtained 7.46 g/100 g DM of glucose, 21.07 g/100 g DM of galactose + mannose and 2.4 g/100 g DM of arabinose. This indicates that the percentage of the released sugars after hydrolysis is 74.5 % of glucose, 53.2 % of galactose + mannose and 70,8 % of arabinose.

Fermentations in shaking flasks

The five different LAB strains were tested for LA production on SCGH to find a potential candidate strain for biotechnological conversion of SCG into LA. These strains were chosen as LAB

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representatives commonly used for LA bio-production (Hofvendahl, 2000; Abdel-Rahman et al. 2013). *Lactobacillus rhamnosus* and *Streptococcus thermophilus* are producing L(+) isomer, *Lactobacillus delbruckii* subsp. *bulgaricus* is producing D(-) isomer and *Lactobacillus plantarum* is producing a racemic mixture of LA (Panesar et al. 2007). *B. coagulans* was chosen because of its ability to grow and metabolize at high temperatures, simple nutrition requirements and production of highly optically pure L-lactic acid (Ma et al. 2014).

The performance of the SCGH as a substrate for LA was evaluated in shaking flasks; the results are displayed in Table 3. The LA concentration after 48 h was chosen as the response, two different cultivation settings were tested - pH was or was not adjusted to 7 prior to the cultivation. The results demonstrate that sole SCGH without any supplementation can serve as a substrate for different LAB strains. This is a very interesting observation since LAB are well known for their high requirements on amino acids, vitamins and other complex nutrients (Yadav, 2011; Mozzi, 2016). Therefore, a hydrolysate of SCG represents a promising substrate for LA production by a LAB that does not require the supplementation of expensive complex components. The highest LA concentrations after 48 h were obtained with *Lbc. rhamnosus*, which was capable to produce 10.75 g l^{-1} of LA when pH was adjusted to 7 prior to inoculation. Its LA production capacity exceeded not only all the other tested LAB but also *B. coagulans*, which is considered one of the most promising LA producers (Ma et al. 2014). Therefore, *Lbc. rhamnosus* was used for further experiments with different concentration of SCGH in media and also for the following experiments in bioreactors.

Table 3

Obruca et al. (2014) reported that appropriate dilution of SCGH substantially increased biotechnological yields of polyhydroxyalkanoates employing *Burkholderia cepacia*, since the concentration of inhibitory substances was reduced. Therefore, in a further experiment, *Lbc. rhamnosus* was cultivated in media with various volume portions of SCGH for 48 h. The results are provided in the supplement materials (Table S1). Nevertheless, the highest concentration of lactic acid (11.18 ± 0.04 g l^{-1}) was achieved when the medium contained 100 vol.% of SCGH. It appears that *Lbc. rhamonosus* is less sensitive to microbial inhibitors than *B. cepacia* and diluted SCGH probably did not provide sufficient amount of the carbon substrates.

Fermentations in bioreactors

Further experiments were performed in bioreactors with controlled pH (6.5) for maximization of LA yields. Three different media compositions were tested. At first, SCGH was used without any supplementation. Secondly, SCGH was supplemented with yeast extract (3 g l^{-1}) to test whether LA yields can be further improved when this complex component is provided. Thirdly, synthetic MRS medium supplemented with sugars according to the composition of SCGH (6.75 g l^{-1} of glucose, 19.5 g l^{-1} of galactose, and 2.25 g l^{-1} of arabinose) was used to investigate whether LA production is negatively influenced by the presence of microbial inhibitors in SCGH.

The fundamental parameters describing the efficiency of LA production for the above-mentioned media compositions (Table 4), the time courses of individual cultivations are shown in Figure 1. The pH time courses are shown in the supplement material Figure S1.

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Table 4

When considering the dynamics of LA production, it is obvious that supplementation of SCGH with yeast extract (YE) sped up the fermentation. In this case, the highest LA concentration was achieved already after 45 h (Figure 1b), while the maximal production was reached after 70 h of cultivation in non-supplemented media (Figure 1a). It is also very likely that inhibitors in SCGH partially inhibit the employed bacterial strain since the fermentation in MRS media supplemented by sugars according to SCGH was the most dynamic reaching maximal LA concentration after 24 h of cultivation. Growth in MRS media (Figure 1c) was obviously completed after this time. Since the utilizable sugars were also already consumed by that time, it is likely that the cells start to lyse after that point, which could explain the biomass depletion. From the fermentation in MRS media was also evident incomplete utilization of sugars. It was caused by the inability of *Lbc. rhamnosus* to utilise arabinose (Hedberg et al., 2008).

On the contrary, total LA titers gained on the individual substrates were very similar and the differences are not statistically significant. Thus, it can be stated that the supplementation of cultivation media by YE is not needed to achieve high LA concentrations and sugars to LA conversion rate. Within an overall economical process evaluation, it will have to be decided, whether the cost arising from the addition of YE can be compensated by a shorter duration of the fermentation process. Similarly, the presence of inhibitors in SCG does not negatively influence LA yields, since total LA concentrations, as well as $Y_{P/S}$, are comparable with non-supplemented SCGH. Therefore, it is likely that the duration of the fermentation of SCGH could also be reduced by removal of inhibitors employing appropriate detoxification methods. For instance, Obruca et al. (2014) detoxified SCG by ethanol extraction of polyphenols prior to hydrolysis. This not only enhanced yields of the biotechnological process of polyhydroxyalkanoates production about 30 % but in addition extract of SCG polyphenols was generated as a co-product with interesting potential applications.

Figure 1

The results of this work show that the SCGH is a suitable substrate for LA production with *Lbc. rhamnosus* since LA titers and especially yield coefficients obtained in this study are more than comparable with those reported by other authors who employed *Lbc. rhamnosus* and used lignocellulose based substrates for LA production (see supplement materials Table S2). Furthermore, it should be stressed out that there is no need to further supplement SCGH with YE or other expensive complex substrates, since the difference between supplemented and unsupplemented hydrolysate is not so considerable.

Generally, the valorization of SCG perfectly fits within the so-called bio-refinery concept, which is based on effective and complete utilization of biomass – in this case coffee beans. They can be used for the preparation of soluble coffee and the residual solid waste – SCG – can be further used in the same factory for the production of various valuable substances, materials, and fuels. In this work, we demonstrated that LA production on SCGH employing *Lbc. rhamnosus* can be considered a very promising and suitable process which can be incorporated into a coffee bio-refinery concept.

The yields obtained in the present study would be even higher if there was fed-batch or continuous processes used. Therefore, future research should be directed towards the development of a membrane-recycle bioreactor enabling simultaneous lactic acid recovery and recycling of bacterial

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cells, which would increase the capacity and yield by operation at higher cell densities and avoid product inhibition. Further downstream processing optimization and optical purity validation will be performed as well.

Materials and Methods

Characterization of SCG

SCG were obtained from local cafe situated in Brno, Czech Republic. The waste material was firstly dried in oven to the constant weight (80 °C for 24 h) and stored at room temperature in closed containers. The moisture content was measured by drying of 3.00 ± 0.03 g at 105 °C to constant weight and it was determined as 4.89 wt. %. Ashes were determined by incinerating the samples at 550 °C. The raw material was analysed for the sugar composition based on the NREL standard procedure (Sluiter et al. 2011; Kwon 2013). Samples for the total phenolic content were prepared by extracting 25 g of SCG with 80 ml 50 vol% solution of ethanol for 10 hours. The total phenolic content in the extracts was determined by the Folin–Ciocalteu method (Singleton et al. 1965). Each experiment was repeated thrice.

Hydrolysis of SCG

Three different concentrations (27 g l^{-1}; 13.5 g l^{-1} and 0 g l^{-1}) of H_2SO_4 were tested. 10.5 g of SCG was mixed with 90 ml of liquid (water; 13.5 g l^{-1} H_2SO_4; 27 g l^{-1} H_2SO_4) in 250 ml Schott-bottles. Subsequently, the bottles with the solutions were autoclaved at 121 °C for 20 min. After autoclaving, the pH of the suspension was adjusted to 4.5 using $CaCO_3$ (Sigma-Aldrich, Germany). For enzymatic hydrolysis, 4 vol. % of Celluclast 1.5 L (Novozymes, Denmark), 0.4 vol. % of β-glucosidase (Novozymes, Denmark) and 0.4 vol% of Viscozyme L (Novozymes, Denmark) were used. Enzymatic hydrolysis was carried out at 50 °C, with shaking at 100 rpm. After 48 h of incubation, suspensions were filtered and the filtrate was used for fermentations.

Preparation of SCGH for fermentation

Pre-treatment and enzymatic hydrolysis of SCG were performed in 5 L shaking flasks with 3L working volume. The 10 wt. % suspension of SCG and 27 g l^{-1} H_2SO_4 was autoclaved at 121 °C for 40 min. Before enzymatic hydrolysis, the pH of the suspension was adjusted to 4.5 using $CaCO_3$ (Sigma-Aldrich, Germany). Enzymatic hydrolysis was performed as described above. After the 48 h of enzymatic hydrolysis, the suspension was filtered and the filtrate was used for fermentations.

Microorganisms and inoculum preparation

Lactobacillus (Lbc.) plantarum CCM 7039^T, *Lbc. rhamnosus* CCM 1825, *Lbc. delbrueckii subsp. bulgaricus* CCM 7190, *Streptococcus thermophilus* CCM 4757 and *Bacillus coagulans* CCM 2013 were used for the fermentations. The bacterial strains were purchased from the Czech Collection of Microorganisms, Brno, Czech Republic. MRS broth (Sigma-Aldrich, Germany) was used for the preparation of inoculum and cultivation of bacteria before fermentation. The medium was sterilised by autoclaving at 121°C for 15 minutes and then cooled to laboratory temperature. Prior to inoculation, the temperature was set to the cultivation temperatures: 45 °C (for *B. coagulans*) and 37

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°C (for LAB). The first inoculum was prepared from a frozen stock culture. Medium with bacteria (2 ml) was added to 50 ml of tempered sterile MRS medium. Cultivation was performed for 48 hours. The second inoculum was prepared from the first inoculum (ratio 1:25) and after 24-hour cultivation, it was used to inoculate the production medium.

Fermentations in shake flasks

Preliminary screening experiments were run in 250 ml shake flasks with a working volume of 100 ml. Liquid SCGH without filtration and without any supplementation were used for testing LA production using different bacterial strains. All experiments were run in triplicate; results are expressed as mean values and relative standard deviations of the LA production after 48 h (response). Each group of replicates was inoculated with 10 ml of a separately prepared 16 hours pre-culture of selected strains (OD 0.70 ± 0.01). Cultivations were performed for 72 h at 37 °C.

Fermentations in bioreactors

The batch fermentations in bioreactors were run in parallel in DASGIP 1-l bioreactors (Eppendorf, Germany). Agitation (100 rpm) was provided by two six-bladed Rushton turbine impellers, working volume was 750 ml. The SCGH was prepared as described above. Cultivations in bioreactors were performed with *Lbc. rhamnosus*. 50 ml of an 18 h-old pre-culture was used as the inoculum. Throughout the process, the temperature was maintained at 37 °C and the pH-value was controlled at 6.5 with automatic addition of 5 mol l^{-1} NaOH. Samples were collected periodically during 92 hours of batch fermentation. The collected samples (10 ml) were centrifuged at 21130×g for 10 min. The supernatants were used for the determination of LA and residual reducing sugars. The sediments were used for gravimetric biomass analysis. All experiments were performed in duplicate; results are expressed as mean values and relative standard deviations.

Analysis of sugars and organic acids

Saccharides and organic acids were determined with HPLC using an Agilent 1100 system equipped with an RI detector (Agilent Technologies, USA) and ION 300 column (Transgenomic) at 45 °C. The HPLC column used in this study was not capable of separating galactose and mannose, which are both major constituents of SCG hemicellulose. HPLC analysis was carried out by isocratic elution for 105 minutes using 0.005 mol l^{-1} H_2SO_4 at a flow rate of 0.325 ml min^{-1} as the mobile phase. The results are expressed as mean ± standard deviation expressed as confidence (n = 3). Statistical significance level $\alpha = 0.05$ was used.

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Conflict of Interest

The authors declare no conflict of interest.

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Tables

Table 1 Composition of SCG

chemical components	**composition (g/100 g DM)**
glucose	10.01 ± 0.16
galactose + mannose	39.62 ± 0.66
arabinose	3.39 ± 0.68
glycerol	0.27 ± 0.10
diacetyl	0.82 ± 0.20
levulinic acid	0.14 ± 0.02
2 - propanol	0.06 ± 0.06
hydroxy-methyl-furfural	0.24 ± 0.02
furfural	0.09 ± 0.01
polyphenols	5.78 ± 0.01
ashes	1.64 ± 0.22

Results are expressed as mean ± standard deviation; n = 3.

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Table 2 Comparison of hydrolysis with different concentrations of sulfuric acid

	water and enzymes		13.5 g l^{-1} sulfuric acid and enzymes		27 g l^{-1} sulfuric acid and enzymes	
	Diluted acid hydrolysis	After enzymatic hydrolysis	Dilute acid hydrolysis	after enzymatic hydrolysis	Dilute acid hydrolysis	After enzymatic hydrolysis
Chemical components	c (g/100 g DM)		c (g/100 g DM)		c (g/100 g DM)	
glucose	0.08 ± 0.001	1.06 ± 0.04	0.42 ± 0.00	6.66 ± 0.10	0.62 ± 0.00	7.46 ± 0.04
galactose + mannose	0.23 ± 0.001	2.79 ± 0.03	10.53 ± 0.53	15.11 ± 0.81	20.48 ± 0.72	21.07 ± 0.22
arabinose	0.19 ± 0.002	0.36 ± 0.01	2.68 ± 0.03	2.30 ± 0.06	2.76 ± 0.03	2.40 ± 0.02
5-HMF	0.01 ± 0.003	0.01 ± 0.00	0.03 ± 0.01	0.02 ± 0.01	0.05 ± 0.00	0.04 ± 0.01
levulinic acid	0.01 ± 0.00	0.01 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	0.04 ± 0.01

Table 3 Comparison of lactic acid production by individual strains after 48 h of cultivation

Microorganism	pH 7			without pH adjustment		
	LA ($g\ l^{-1}$)	consumed sugars ($g\ l^{-1}$)	$Y_{P/S}$	LA ($g\ l^{-1}$)	consumed sugars ($g\ l^{-1}$)	$Y_{P/S}$
Lbc. plantarum	10.75 ± 1.17	12.3	0.87	6.06 ± 0.02	7.94	0.76
Lbc. rhamnosus	10.79 ± 0.80	12.05	0.90	3.61 ± 0.03	4.61	0.78
B. coagulans	6.09 ± 0.36	8.15	0.75	2.50 ± 0.07	4.16	0.60
Lbc. delbruckii	4.52 ± 0.01	5.42	0.83	2.25 ± 0.03	3.43	0.66
S. thermophilus	5.90 ± 0.17	7.02	0.84	0.12 ± 0.01	3.97	0.79

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Table 4 Production of LA in bioreactors

Conditions	LA (g l^{-1})	Consumed sugars (g l^{-1})	$Y_{P/S}$	Volumetric productivity (g l^{-1} h^{-1})	Hour of cultivation where maximal LA titers were achieved
SCGH	25.69 ± 1.45	26.22	0.98	0.34	76
SCGH with YE	26.26 ± 0.65	30.22	0.85	0.38	70
MRS + sugars*	27.29 ± 0.64	30.02	0.91	0.57	48

*supplementation of MRS by sugars was performed according to the composition of SCGH (6.75 g l^{-1} of glucose, 19.5 g l^{-1} of galactose, and 2.25 g l^{-1} of arabinose).

Figures

Figure 1 Time course of biomass, total sugars, and lactic acid concentration during bioreactor cultivation in SCGH (a), SCGH with YE (b) and in MRS (c).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Production of LA from SCGH by *Lbc. rhamnosus*

Table S2 Production of LA in bioreactors

Figure S1: Time course of pH and neutralizing agent addition during bioreactor cultivation in SCGH (a), SCGH with YE (b) and in MRS (c)

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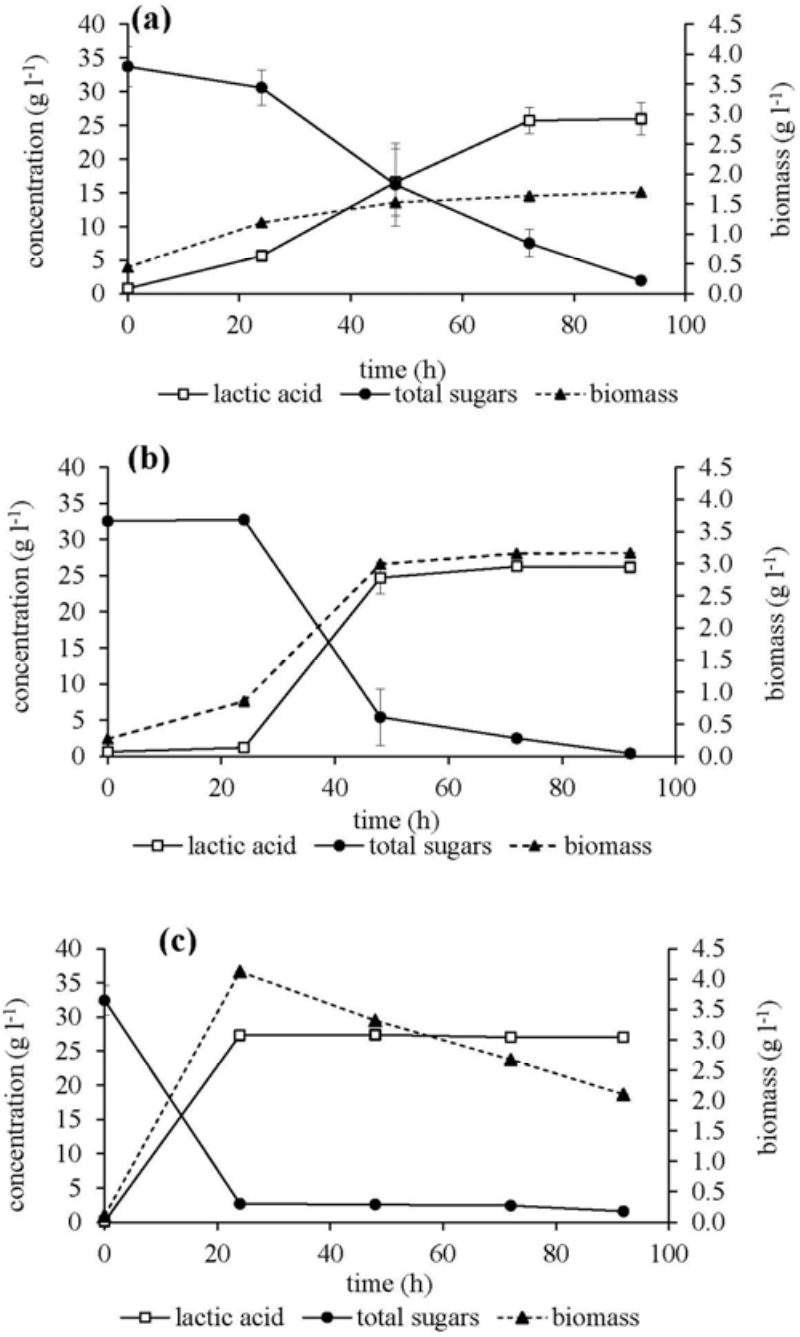

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