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THE IMPACT OF CRUDE GLYCEROL FROM BIODIESEL PRODUCTION AND ITS TRACE ELEMENT CONTENT ON BIOMETHANE PRODUCTION IN A BATCH EXPERIMENT: MODELLING AS A STEP TOWARDS AN IMPARTIAL ROUTINE COMPARISON OF RESULTS

VPLIV SUROVEGA GLICEROLA IZ PROIZVODNJE BIODIZLA IN VSEBNOSTI NJEGOVIH ELEMENTOV V SLEDOVIH NA PROIZVODNJO BIOMETANA V ŠARŽNEM EKSPERIMENTU: MODELIRANJE KOT KORAK K ENAKOVREDNI RUTINSKI PRIMERJAVI REZULTATOV

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Abstract

In this study, crude glycerol from the biodiesel industry was tested as a co-substrate in biogas production. To investigate the influence of crude glycerol and the underlying trace element (TE) content on the efficiency of biomethane production, a batch experiment using Automatic Methane Potential Test System (AMPTS II) was carried out. The single addition of crude glycerol significantly contributed only to the total content of K (14.4%), Si (17.3%), and P (11.6%), whereas the contributions of other metals were within the range of other substrates. The addition of crude glycerol increased biomethane production, however, its utilization beyond 1% of total volume resulted in prolonged lag phase and final cessation of biomethane production. The negative effects of inorganic salts present in crude glycerol were reflected in progressively diminishing parts of glycerol and methanol being utilized in its anaerobic digestion, posing serious problems for daily routine use. A nonlinear least square regression analysis was performed to fit the Gompertz, Logistic, Transfer, and Richards models to biomethane production. The most suitable model was the Richards model, exhibiting the best fit to the experimental curves for complex substrates. Glycerol fractions remaining after biodiesel production have to be pre-tested for their negative effects on the content of TEs and inorganic salts, lag phase in biogas production, before they are used as co-substrates in biogas production phase.

Keywords: biomethane, crude glycerol, trace element, biodiesel, modelling, AMPTS II

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Izvleček

V študiji smo za proizvodnjo biometana kot kosubstrat uporabili surovi glicerol iz industrije proizvodnje biodizla. Da bi raziskali vpliv surovega glicerola in vsebnosti osnovnih elementov v sledovih (TE) na učinkovitost proizvodnje biometana, smo izvedli šaržni poskus z uporabo Automatic Methane Potential Test System (AMPTS II). Enkratno doziranje surovega glicerola je pomembno prispevalo le k skupni koncentraciji K (14,4 %), Si (17,3 %) in P (11,6 %), medtem ko so bili prispevki drugih elementov v sledeh v območju koncentracij drugih substratov. Z dodajanjem surovega glicerola se je povečala proizvodnja biometana, a ko je njegova uporaba presegla 1 % celotne prostornine, smo zaznali dolgotrajno lag-fazo in končno prenehanje proizvodnje biometana. Negativni učinki anorganskih soli, prisotnih v surovem glicerolu, so se odražali v postopnem zmanjševanju koncentracije glicerola in metanola, ki so se razgradili v procesu anaerobne presnove, to pa predstavlja resne težave pri vsakdanji rutinski uporabi surovega glicerola. Izvedena je bila analiza nelinearne regresije najmanjših kvadratov z uporabo modelov Gompertz, Logistic, Transfer in Richards za kumulativno proizvodnjo metana. Najprimernejši model je bil Richards, ki se je najbolj prilegal eksperimentalno izmerjenim krivuljam kompleksnih substratov. Frakcije glicerola, ki ostanejo po proizvodnji biodizla, je treba predhodno preizkusiti zaradi negativnih učinkov, ki jih lahko povzročajo TE, anorganske soli in lag-faze, preden se lahko uporabijo kot kosubstrati za pridobivanje bioplina.

Ključne besede: biometan, surovi glicerol, elementi v sledovih, biodizel, modeliranje, AMPTS II

1. Introduction

The production of biodiesel has greatly expanded (Janaun and Ellis, 2010; OECD-FAO, 2016), resulting in a large amount of by-products, mainly crude glycerol. The amount of crude glycerol is usually equivalent to 10 kg per 100 kg of biodiesel produced (Kolesárová et al., 2011). As global production of biodiesel reached 33 billion liters in 2016 (OECD-FAO, 2016), the excess crude glycerol is regularly used as a substrate or co-substrate in biogas production (Hutňan et al., 2013) or as other derivatives (Kong et al., 2016). It has been shown that the addition of crude glycerol (0.63 to 6% v/v) to increases the production of biogas from various agricultural, industrial or wastewater treatment plant substrates (Astals et al., 2012; Castrillón et al., 2011; Fountoulakis et al., 2010; Nghiem et al., 2014; Razaviarani et al., 2013; Siles et al., 2010). One of the limitations associated with the use of crude glycerol is its carbon content (up to 58%) (Thompson and He, 2005), which underlines the need to determine the optimal concentration of crude glycerol to be used in anaerobic digestion. On the other hand, the addition of 8% crude glycerol to cattle manure significantly reduced biomethane production under mesophilic conditions (Castrillón et al., 2011). Even more, to have a stable anaerobic digestion process, the amount of glycerol in the feed should not exceed 1% (Fountoulakis et al., 2010). Therefore each case of anaerobic digestion should be pretested, to determine the feasibility of adding the crude glycerol. Factors such as pH, temperature, redox potential, organic loading rate (OLR), hydraulic retention time (HRT), macronutrients, micronutrients, trace elements (TE), volatile fatty acids (VFA), ammonia, and electrical conductivity (EC) play an important role in the growth and activity of microorganisms (Roussel et al., 2019; Wyman et al., 2019). Recent studies have shown that microelements such as iron (Fe), zinc (Zn), chromium (Cr), copper (Cu), magnesium (Mg), nickel (Ni), manganese (Mn), barium (Ba), and others play an important role in the biogas production process (Schattauer et al., 2011; Wyman et al., 2019). Sufficient concentrations and bioavailability of various TEs can lead to stable and more efficient methane production, while a deficiency or overload can lead to imbalances or complete failure of the process (Wintsche et al., 2016; Wyman et al., 2019). The range of optimal TE concentrations in the anaerobic digestion process is very wide (van Hullebusch et al., 2016). Another major disadvantage of using raw glycerin in biogas production is that it contains the increased concentration of TE and inorganic salts (Viana et al., 2012). The availability of some TEs is necessary to achieve fully functional, stable and efficient anaerobic digestion, stimulation of microbial

growth, and biomethane production (Demirel and Scherer, 2011). On the other hand, increased concentrations of inorganic salts can be harmful and lead to inhibition and breakdown of the anaerobic digestion process (Pobeheim et al., 2010; Soto et al., 2007). The crude glycerol fraction may contain 2-3% of salts, mainly sodium and potassium (Asad-ur-Rehman et al., 2008). The salt content can reach up to 5-7% if biodiesel is produced through homogeneous alkaline catalysis (Yang et al., 2012). Low sodium concentrations are essential for the growth of methanogenic bacteria, but the effect of concentrations above 8 g/L is strongly inhibitory (Chen et al., 2008). Concentrations of potassium below 400 mg/L can improve the process under both thermophilic and mesophilic conditions, while high concentrations are more likely to inhibit the thermophilic (Chen et al., 2008). In addition, calcium, magnesium, phosphorus, or sulphur originating from the oil used for biodiesel production may also be present in significant amounts in the crude glycerol and may affect the availability of TE for microorganisms in anaerobic digestion (Kolesárová et al., 2011).

The cumulative gas production curve when assessing the methane yield in batch laboratory experiments is similar to the curve of microbial growth kinetics. Different curve shapes may indicate different substrate behavior during anaerobic digestion, biodegradability, and inhibition (Ware and Power, 2017). Simple and easily degradable substrates result in an inverted L-shaped curve of cumulative methane production, while complex substrates and interfering inhibitors result in an elongated S-shaped or stepped curve. Models can help predict more representatively the maximum specific daily methane production from novel substrates to be used in continuous anaerobic digestion (Kolbl et al., 2014; Ware and Power, 2017), but additional limitations of biomethane potential (BMP) assays must be considered (Kolbl et al., 2017; Kolbl et al., 2014). There have been several kinetic models developed for maximum methane production from different substrates. For complex substrates, models like Gompertz and Transfer (Mohamed et al., 2018) are difficult to implement, but small amounts of data are required and are very simple to use compared to complex models such as ADM1 (Anaerobic digestion model 1) (Batstone et al., 2005; Frunzo et al., 2019), which are generally used for modelling continuous processes of anaerobic digestion.

In this study, crude glycerol from the industrial scale biodiesel industry in the Republic of Serbia was tested as a co-substrate in biogas production utilizing wastewater treatment plant sludge, cow manure, and waste whey from the dairy industry. Batch experiments were performed to determine the effects of the three loading rates of crude glycerol on biogas production, to evaluate the contribution of accompanying TEs and inorganic salts on anaerobic digestion, and to determine the extent of crude glycerol degradation. The resulting methane production curves and the extent of residual methanol were investigated through modelling to determine the most appropriate organic loading of the current crude glycerol fraction, to identify the maximum daily methane production rates, and to enable further comparisons of modelled parameters as a step forward towards an impartial routine comparison of the results over a larger number of experiments (Elagroudy et al., 2020).

2. Materials and Method

2.1. Substrates

Biogas production was monitored during co-digestion of crude glycerol with wastewater sludge, cow manure, and waste whey. Wastewater sludge and inoculum from sludge thickener and anaerobic digesters of the municipal wastewater treatment plant Ljubljana, Slovenia (500 000 population equivalents) were used for the anaerobic digestion assay. Cow manure and waste whey were collected from a farm in Slovenia as described by Kolbl et al. (2014; 2017). Crude glycerol was obtained as a by-product of the production of biodiesel from sunflower oil in an industrial setting located in the Republic of Serbia, which was produced by homogeneous base-catalyzed transesterification (Nasreen et al., 2018). Pure glycerol (99.8%; Sigma Aldrich) was used as a control of crude glycerol and glucose (Sigma Aldrich) was used as a control of the quality of the inoculum.

2.2. Batch experiment set-up

The batch experiment was performed using the standardized approach, utilizing the Automatic Methane Potential Test System (AMPTS II, Bioprocess Control, Sweden) with 500 mL glass reactors as described by Kolbl et al. (2014; 2017).

The set-up of the batch experiment is described in Table 1. The 500 mL reactors were filled with 330 mL of the digestive mixture containing inoculum from the wastewater treatment plant (300 mL), wastewater sludge (7 mL), waste cheese whey (4 mL), and cow manure (1 mL). Based on the projected industrial requirements from the biogas plant for the treatment of crude glycerol, the aliquots of pure glycerol were 2 mL, 3 mL, and 4 mL, in comparison to 3 mL, 4 mL, and 13 mL for crude glycerol (Table 1). Reactors that were filled with 2 g of pure glucose were used as a control. Reactors with crude and pure glycerol received 10 mL of demineralized water, to flush the remainder of the substrates in the dosing beakers into the reactors. The reactors were placed in a water bath with a temperature of 39 °C and mixed automatically every 5 minutes for 1 minute. All flasks were flushed with N_2 for 1 minute before the start of the experiment. At the end of experiment, the methane that was produced from reactors that were filled only with inoculum was deducted from other reactors as described by Kolbl et al. (2014; 2017).

Table 1: *AMPTS II test filling of the reactors.*

Preglednica 1: *Doziranje v reaktorje AMPTS II.*

Reactor name	inoculum	Cow manure	Cheese whey	Water	Wastewater sludge	Pure glycerol	Crude glycerol	glucose
	mL							g
Inoculum	300	-	-	-	-	-	-	-
	300	-	-	-	-	-	-	-
	300	-	-	-	-	-	-	-
Control	300	-	-	-	-	-	-	2
glucose	300	-	-	-	-	-	-	2
	300	-	-	-	-	-	-	2
Pure	300	1	2	10	7	2	-	-
glycerol 2	300	1	2	10	7	2	-	-
mL	300	1	2	10	7	2	-	-
Pure	300	1	2	10	7	3	-	-
glycerol 3	300	1	2	10	7	3	-	-
mL	300	1	2	10	7	3	-	-
Pure	300	1	2	10	7	4	-	-
glycerol 4	300	1	2	10	7	4	-	-
mL	300	1	2	10	7	4	-	-
Crude	300	1	2	10	7	-	3	-
glycerol 3	300	1	2	10	7	-	3	-
mL	300	1	2	10	7	-	3	-
Crude	300	1	2	10	7	-	4	-
glycerol 4	300	1	2	10	7	-	4	-
ml	300	1	2	10	7	-	4	-
Crude	300	1	2	10	7	-	13	-
glycerol	300	1	2	10	7	-	13	-
13 mL	300	1	2	10	7	-	13	-

The TE content in substrates (WWS, chees whey, cow manure, crude glycerol) at different loading and inoculum before the experiment and digested mixtures at the end of experiment were determined with Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES) (ARCOS FHE12, SPECTRO, Germany). The following 17 TEs out of 23 detectable were quantified: Barium (Ba), Bismuth (Bi), Calcium (Ca), Chromium (Cr), Copper (Cu), Iron (Fe), Indium (In), Potassium (K), Magnesium (Mg), Manganese (Mn), Sodium (Na), Nickel (Ni), Lead (Pb), Strontium (Sr), Zinc (Zn), Silicon (Si), and Phosphorus (P). Distilled water, purified by Fisher Chemicals (HPLC grade) was used to dilute the samples. The carrier gas used was argon 5.0 (99.999% purity, Sigma Aldrich), under the conditions previously described by Petrović et al (2016). The calibration solutions were prepared from multistandard IV standard solution (Merck) and diluted until the expected concentration of test elements was reached by Petrovic et al. (2016). Substrates, the inoculum, and samples before anaerobic digestion were collected and prepared by wet digestion with nitric acid and filtered through a syringe filter with a pore size of 0.45 µm.

To determine the actual glycerol content and the presence of other organic compounds such as methanol before and after digestion, high performance liquid chromatography (HPLC) was performed using the Agilent 1100 series equipped with an RI detector. The samples from all reactors (except with glucose) were centrifuged at 5000 rpm for 15 minutes and the supernatant was filtered through a syringe filter with a pore size of 0.45 µm before analysis. An aliquot of 20 µL of the filtrate was loaded onto an Aminex HPX087H column (7.8 x300 mm, Biorad Laboratories) in isocratic conditions, and 5mM H_2SO_4 was used as eluent at a flow rate of 0.6 cm^3/min at a temperature of 50 °C. The analyses were performed in triplicate.

2.3. Kinetic modelling

To enable further comparisons of the modelled parameters (A - maximum methane yield; μ_m - a maximum rate of methane production, λ - the lag phase and υ - a shape factor) towards an impartial routine comparison of the results obtained from a larger number of experiments, the parameters of the model (Gompertz, Logistic, Transfer, and Richards) that best fit the measurements of cumulative methane production were calculated by nonlinear least-square regression analysis using the solver in MS Excel 2016. The determination coefficient R^2 and Root-mean-square-error (RMSE) were calculated using regression analysis with 95% confidence interval in MS Excel 2016 as described before (Ware and Power, 2017). For this assay, methane production was evaluated using the modified Gompertz model, Transfer function model, Logistic model and Richards model (Huiliñir et al., 2014; Ware and Power, 2017). The equations for the models are given below (Huiliñir et al., 2014):

Modified Gompertz model:

$$y = A \cdot exp\left\{-exp\left[\frac{\mu_m \cdot e}{A} \cdot (\lambda - t) + 1\right]\right\} \quad (1)$$

Logistic function model:

$$y = \frac{A}{1 + exp\left(\frac{4 \cdot \mu_m (t - \lambda)}{A} + 2\right)} \quad (2)$$

Transfer function model:

$$y = A \cdot \left(1 - exp\left(-\frac{\mu_m \cdot (t - \lambda)}{A}\right)\right) \quad (3)$$

Richards equation

$$y = A \cdot \left\{1 + \upsilon \cdot \exp(1+\upsilon) \cdot exp\left[\frac{\mu_m}{A} \cdot (1+\upsilon)^{\left(1+\frac{1}{\upsilon}\right)} \cdot (\lambda - t)\right]\right\}^{\left(\frac{-1}{\upsilon}\right)} \quad (4)$$

where μ_m is the maximum rate of methane production, A is the maximum methane yield, λ is the duration of the lag phase, y is the methane accumulated at time t, and υ is a shape factor (Huiliñir et al., 2014; Elagroudy et al., 2020).

3. Results and discussion

The characteristics of the inoculum and substrates are listed in Table 2. All selected substrates have a high COD content. Crude glycerol is 31.2 times higher than in the inoculum, while TS content is 1783% higher than in the inoculum, cheese whey, and cow manure. Cheese whey had the lowest pH, while electrical conductivity was the highest for cow manure.

Analysis of the TE content of the substrates used for anaerobic digestion revealed the presence of 17 elements. The maximum contribution of the different substrates to the total concentration of TEs in the reaction mixture at the highest loading (crude glycerol (13 mL)) is shown in Table 3. Silver (Ag), Cadmium (Cd), Cobalt (Co), Lithium (Li), and Thallium (Tl) were not detected in any substrate, while Boron (B) was only present in cow manure in a barely detectable concentration (0.14 mg/L). Crude glycerol showed a significantly higher concentration of Si and P compared to other substrates. The P concentration in crude glycerol was 411 mg/L, which is much higher than the range of 12-137 ppm (12-137 mg/l) reported by Thompson and He (2005) for crude glycerol derived from various biodiesel feedstocks. P is an essential nutrient for anaerobic microbial consortia and influences the availability of TEs and the dynamics of anaerobic digestion (Frunzo et al., 2019).

Table 2: *Characteristics of the substrates and inoculum used in the assays.*

Preglednica 2: *Karakteristike uporabljenih substratov in inokuluma.*

Substrates	pH	EC (µS/cm)	TS (%)	COD (g/L)	NH_4^+-N (mg/L)
inoculum	7.59±0.04	4750 ± 20	4.52±0.20	40.5 ± 0.1	1710 ± 10
Cheese whey	4.39±0.02	4340 ± 10	5.44±0.11	60.2 ± 0.1	-
Cow manure	7.64±0.05	23000 ± 50	4.04±0.32	402.6 ± 0.1	4257± 7
WWS	7.03±0.13	712 ± 0.3	5.67±0.13	59.2 ± 0.1	374± 7
Crude glycerol	-	-	87.74 ±0.9	1270 ±10	-

Table 3: *The content of trace elements (TEs) in different substrates and participation of crude glycerol in total quantity of TEs in the digestive mixtures.*

Preglednica 3: *Vsebnost elementov v sledovih (TEs) v različnih substratih in delež surovega glicerola v skupni količini elementov v sledovih v pripravljeni mešanici.*

Trace element (mg/L)	Wastewater sludge	Whey	Sludge	Cow manure	Crude glycerol (max)	Total in mixture	% added with crude glycerol
Ba	1.027	0.75	1	1	0.615	7.692	2.5
Bi	0.22	0.25	0.143	0	0.231	14.769	4.8
Ca	81.727	92	81.714	48	18.538	2.769	0.9
Cr	0.117	0	0	0	0	0	0
Cu	0.707	0.25	1	0	0.538	9.538	3.1
Fe	19.98	3	18.143	5	2	1.231	0.4
In	0.16	0.25	0.143	0	0.154	11.692	3.8
K	128.94	166.25	29	2433	544.538	44.308	14.4
Mg	12.627	10	11.286	51	1.615	1.538	0.5
Mn	0.057	0	0.143	0	0	0	0
Na	31.27	53.5	30.714	85	32	12.308	4
Ni	0.017	0	0	0	0	16.923	5.5
Pb	0.35	0.25	0.429	0	0.154	6.462	2.1
Sr	1.067	0.5	1	1	0.615	7.077	2.3
Zn	2.337	1	2.857	1	1.077	5.846	1.9
Si	23.627	26	24	28	118.692	53.231	17.3
P	133.61	228.75	87.143	73	418.923	35.692	11.6

The biomethane potential was determined with the AMPTS II system using crude and pure glycerol as co-substrates in separate experiments. HPLC analysis of crude glycerol from biodiesel production showed that the glycerol and methanol content was 37.6±0.9% and 39.1±0.7% respectively. The addition of either pure or crude glycerol in the same COD range generated contrasting methane production kinetics (Figure 1). Pure glycerol led to an immediate increase in methane production irrespective of its concentration compared to the controls. The pure glycerol 2 mL variant was most similar to crude glycerol 3 mL variant. In contrast, the utilization of higher concentrations of crude glycerol (4 mL and 13 mL in reaction mixture) resulted in progressively lower methane production (Figure 1; Table 1). This shows that the increasing concentrations of crude glycerol from 3 mL to 4 mL represented the tipping point of the current anaerobic system, where additional availability of both crude glycerol and methanol did not result in additional biomethane. This observation is corroborated by the fact that an increase in the volume of amended pure glycerol (2 mL, 3 mL or 4 mL) led to a linear increase in normalized methane volumes ($y = 198.51x + 406.89$; $R^2 = 0.97$). The utilization of increased volumes of crude glycerol led to a progressively decreased normalized methane volumes ($y = -43.232x + 819.33$; $R^2 = 0.98$). This shows that the increase in methane production from pure glycerol was about 1.45 to 1.92 times higher compared to methane production from crude glycerol. This gave rise to the observation that the amounts of methane produced from the 2 mL pure glycerol variant were most comparable to those from 3 mL or 4 mL crude glycerol (Figure 1). These results show that both two aliquots of crude glycerol were degradable in

reactor mixtures despite the accompanying introduction of TEs and ions.

The HPLC analyses at the end of the experiment showed that pure glycerol was effectively consumed (>78%) during the observation independent of the initial glycerol content. Moreover, as the dosage of crude glycerol (3 mL, 4 mL, or 13 mL) increased, the amount of glycerol consumed in 13 mL crude glycerol decreased to 58%, while the methanol consumption decreased to 9.6%. The progressive partitioning of glycerol and methanol consumption with increasing crude glycerol concentration is most likely related to additional changes in electrical conductivity due to the accompanying increase in inorganic ions (Table 3). This indicates that an additional increase in the dosage of crude glycerol up to 13 mL effectively leads to a decrease in relative amounts of degraded glycerol and methanol within the system and thus to inhibition of the anaerobic process (Figure 1). Although earlier studies (Chen et al. 2000; Paris and Blondeau, 1999) indicated that methanol should be easily degraded within 24 h via at least three metabolic pathways (Bhatti et al., 1996), crude glycerol containing additional salts apparently prevented the direct use of methanol by microorganisms and its subsequent conversion to methane at higher crude glycerol loadings.

This also mirrors the observed shifts in metabolic partitioning observed before in other anaerobic reactors subjected to shock-loading (Huang et al., 1999; Fernandez et al., 2000). The high anaerobic biodegradability of glycerol (Jensen et al., 2014), organic overload (Nghiem et al., 2014), subsequent accumulation of propionate (Jensen et al., 2014), and system destabilization were recently identified as the root causes of inhibition in the anaerobic degradation of glycerol. However, based on the results obtained in this study the mechanism of organic overloading by glycerol itself did not contribute significantly to the observed inhibition in biomethane production.

The introduction of crude glycerol with methanol and accompanying TEs apparently decreased the flow over the microbial metabolic network resulting in decreased biomethane production. As pure glycerol uptake was shown not to represent the rate-limiting step in glycerol anaerobic degradation (Fountoulakis et al., 2010), the introduction of crude glycerol and accompanying impurities modified the uptake of glycerol for degradation. Consequently, the amounts of crude glycerol adopted for the use as co-substrates in anaerobic co-fermentation must be deliberately limited, not surpassing 3 or 4 mL per reaction mix utilized every 6 days.

To further describe the differences between pure and crude glycerol degradation, modelling was used to derive descriptive numerical values to compare the measured cumulative methane production from different glycerol concentrations (Table 4, Figure 2a-2f). In all cases (except crude glycerol (13 mL)), the Logistic function model and the Richard equation best described the experimental data (average R^2 = 0.97, average RMSE = 29.21). The lowest reproduction of the experimental data was achieved with the Transfer model (average R^2 = 0.94). The lowest average values for R^2 and RMSE were obtained with the Transfer model. The maximum methane yields (A) were relatively accurately predicted by all models, resulting in acceptably small differences between measured (V_0) and predicted cumulative methane production (A). However, these small differences, when projected on an industrial scale, can significantly influence investment and cost decisions in the long run. The lag phase (λ) was significantly extended when crude glycerol (4 mL) was introduced. However, only the Logistic and the Richards models introduced the lag phase ($\lambda > 0$). Even more, the lag time in the Richards model was 4.95 days and the RMSE was the smallest among models, indicating the model's best fit. Moreover, the Richard equation was the only model to correctly predict the lag phase.

The lowest fit of the Gompertz and Transfer model cannot be related to any delay phase ($\lambda=0$). The introduction of the fourth parameter (υ) in the Richard equation is relevant for complex substrates and the accumulation of anaerobic digestion intermediates that can inhibit methane production (Ware and Power, 2017; Mohamed et al., 2018). In our case, the Richards equation proved that it can describe inhibitory cumulative methane production

(crude glycerol (4 mL)) much better than other models. Instead of simple comparisons of resulting biomethane production, the modeling approach can provide a step forward towards an impartial routine comparison of the results obtained from a larger number of experiments. The results of biomethane production curve models described in Table 4 can more readily be coupled and compared with results from other studies as was suggested only recently (Weinrich et al., 2019; Elagroudy et al., 2020). Significant differences between the models were also observed for maximum rate methane production, where the Transfer model's estimations were up to 196% higher than those of the logistic model. Finally, the mechanisms behind the lag phase (λ) in the case of crude glycerol (4 mL) (Figure 2; Table 2), accumulation of methanol, and the remaining glycerol described in this study can be linked to glycerol degradation kinetics.

Glycerol is readily available to acetogenic bacteria and since methanol is also an intermediate product of the acetogenic phase in anaerobic digestion, acetogenesis and methanogenesis are apparently the limiting steps in their conversion, respectively. The rate at which methanol is produced, is higher than the rate at which it is consumed or degraded by acetogenic Bacteria or methanogenic Archaea (Viana et al., 2012). This explains the lag phase in all reactors with glycerol, irrespective of quality (pure or crude). However, methanol is rapidly degraded in parallel metabolic pathways (Bhatti et al., 1996; Hashsham et al., 2000), once the microbial communities have functionally adapted to new conditions (Florencio et al., 1995; Fernández et al., 1999). The progressively reduced biomethane yields from the increasing concentration of crude glycerol testify that introduction of accompanying TEs and methanol from crude glycerol amount to a true inhibitory factor as opposed to the general organic loading. The introduction of crude glycerol from sunflower oil biodiesel production in Serbia as a novel co-substrate in an industrial scale biogas reactor should be gradually increased up to 3 mL/330 mL of crude glycerol in incoming substrate mixes (i.e. 1% of reactor volume) and introduced into reactors every 6 days based for sufficient dilution.

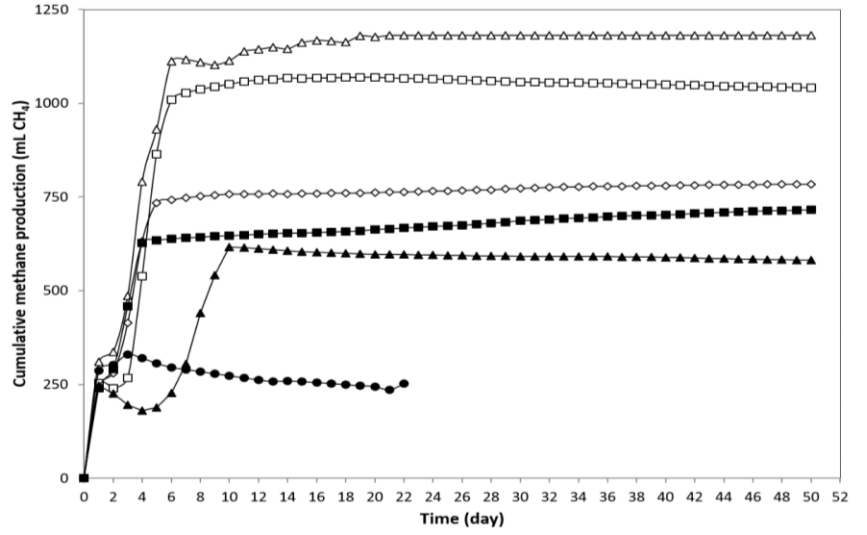

Figure 1: *The biomethane production during anaerobic digestion (39 °C, AMPTS II) of a mixture containing 2 mL (◊), 3 mL (□), and 4 mL (Δ) of pure glycerol (open symbols) and 3 mL (■), 4 mL (▲) and 13 mL (●) of crude glycerol from biodiesel production (filled symbols). Standard deviations are omitted for clarity (SD < 6%). The decreasing values within the same variant show that more biomethane was produced in the inoculum only than in experimental reactors.*

Slika 1: *Proizvodnja biometana med anaerobno presnovo (39 °C, AMPTS II) mešanice substratov, ki je vsebovala 2 mL (◊), 3 mL (□) in 4 mL (Δ) čistega glicerola (prazni simboli) in 3 mL (■), 4 mL (▲) in 13 mL (●) surovega glicerola iz proizvodnje biodizla (polni simboli). Standardne deviacije (DS) niso prikazane zaradi jasnejšega prikaza slike (SD < 6 %). Padanje vrednosti metana znotraj istih variant prikazuje, da je bila proizvodnja metana v reaktorjih z inokulumom večja kot v reaktorjih, ki smo jim dodali substrate.*

Table 4: *Overview of the kinetic parameters of average cumulative methane production curves.*

Preglednica 4: *Pregled kinetičnih parametrov krivulj povprečne kumulativne proizvodnje metana.*

Substrate	Model	Vo (mL CH_4)	A (mL CH_4)	μm (mL CH_4/day)	λ (day)	ν	R^2	RMSE	Error (%)
pure glycerol 2 mL	Gompertz	784.2 ± 5.1	773.27	166.63	0.98		0.98	21.76	-1.4
	Logistic		772.06	157.26	1.00		0.989	18.05	-1.5
	Transfer		775.06	309.49	1.35		0.97	26.69	-1.2
	Richards		769.81	216.09	1.70	8.02	0.99	12.91	-1.8
pure glycerol 3 mL	Gompertz	1069.6 ± 12	1060.41	233.14	1.24		0.97	44.15	-0.9
	Logistic		1058.29	234.78	1.44		0.98	33.39	-1.0
	Transfer		1065.26	334.06	0.77		0.93	61.29	-0.4
	Richards		1054.25	431.70	3.93	41.09	0.99	22.71	-1.4
pure glycerol 4mL	Gompertz	1181.2 ± 15	1177.60	211.67	0.16		0.98	31.37	-0.3
	Logistic		1174.56	204.75	0.24		0.99	26.10	-0.6
	Transfer		1182.63	370.71	0.45		0.97	41.25	+0.1
	Richards		1156.90	267.85	1.69	3.78	0.99	23.60	-2.0
crude glycerol 3 mL	Gompertz	716.6 ± 8	682.58	170.24	0		0.96	26.02	-4.7
	Logistic		681.53	160.5	0		0.96	25.38	-4.9
	Transfer		685.29	285.70	0.14		0.96	26.02	-4.4
	Richards		682.08	107.07	0	682.58	0.93	29.03	-4.8
crude glycerol 4 mL	Gompertz	616.5 ± 6	600.22	60.53	0		0.89	49.16	-2.6
	Logistic		598.48	58.51	0.26		0.92	43.11	-2.9
	Transfer		603.77	101.78	0		0.85	53.69	-2.1
	Richards		593.85	99.55	4.95	265.48	0.97	27.84	-3.7
crude glycerol 13 mL	Gompertz	329.5 ± 7.7	260.98	260.98	0		0.77	17.54	-20.8
	Logistic		260.98	260.98	0		0.77	17.54	-20.8
	Transfer		260.98	260.98	0		0.77	17.54	-20.8
	Richards		260.98	260.98	1	100	0.77	17.54	-20.8

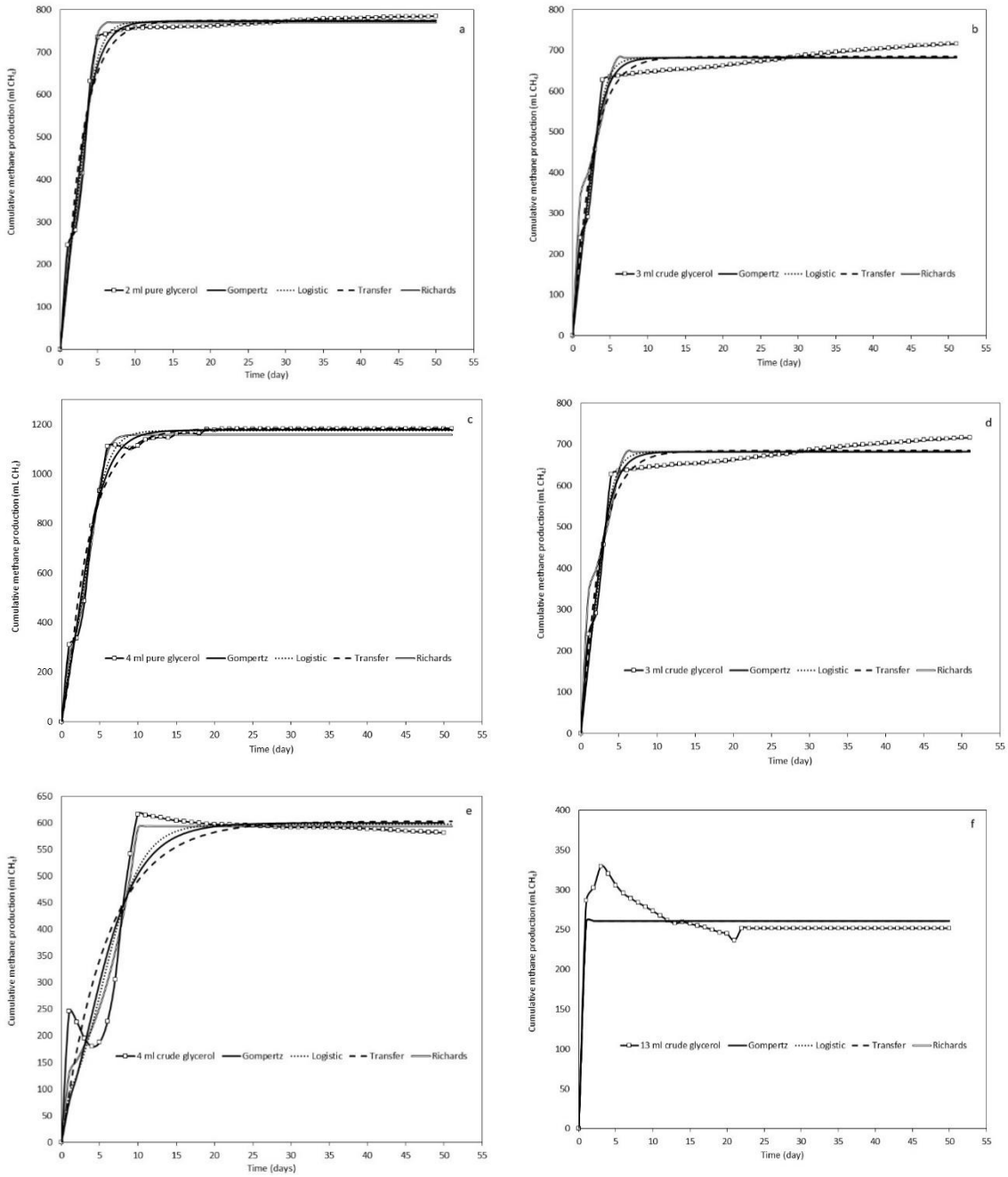

Figure 2. *The fit of the Gompertz, Logistic, Transfer, and Richards models to the methane production of pure and crude glycerol.*

Slika 2. *Ujemanje modelov Gompertz, Logistic, Transfer in Richards s proizvodnjo metana čistega in surovega glicerola.*

4. Conclusions

In summary, the introduction of crude glycerol in the anaerobic co-digestion process resulted in a prolonged initial lag phase and an increase in maximum methane production rates. Progressively diminishing proportions of glycerol and methanol were utilized in the anaerobic digestion of increasing quantities of crude glycerol, reflecting the negative effects of the inorganic salts present in crude glycerol. The lowest addition of crude glycerol contributed to the total content of K

(14.4%), Si (17.3%), and P (11.6%), while the contributions of other metals were not different from other biogas substrates. The results confirmed that the glycerol remaining after biodiesel production can be used as a co-substrate for biogas production up to 1% of the total mixture utilized every 6 days. All models successfully described the cumulative kinetics of pure glycerol, whereas modelling crude glycerol proved more difficult. The introduction of the shape factor in the Richard model ensured better adaptation to the cumulative methane curves. This shows that there is no single best model and, for best fit, all models should be applied to the specific measured cumulative methane production curves and their parameters, as compared against results from other studies.

The modeling approach shown in this study represents a step forward towards an impartial routine comparison of the results obtained from a larger number of experiments. These results extend current knowledge on mechanisms limiting crude glycerol utilization in biomethane production and provide information on sustainable quantities for its application.

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