Anaerobic Digestion of OMW: Intermittent Feeding Strategy and LCFA Oxidation Profile

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Abstract
An intermittent feeding strategy was applied to the anaerobic treatment of raw olive mill wastewater (OMW). Two reactors were operated under influent concentrations of 5 to 50 g COD L^{-1}. Two and one batch (feed-less) periods were applied to reactor R1 and R2, respectively, operating in continuous thenceforth. It was demonstrated that the intermittent feeding of OMW improved the mineralization of accumulated Long Chain Fatty Acids (LCFA) inside the reactor. Nevertheless, LCFA accumulated again when the organic loading rate was increased from 2 to 3 and 5 kg COD m^{-3} d^{-1}. The profiles of LCFA, obtained with OMW digestion, were different from previous studies with synthetic effluents. At the beginning of reactors operation, oleate was the main LCFA compound (~50%) followed by palmitate. Afterwards, a shift in the LCFA pattern accumulation was noticed for both reactors. At periods with higher OMW concentrations (30-50 g COD L^{-1}, 3-5 kg COD m^{-3} d^{-1}) palmitate was the main LCFA accumulated with 69% at R1 and 54% at R2. For real oily wastewaters, a periodically batch period could be a practical solution to maintain low values of LCFA inside the reactor. The addition of a nitrogen source was essential to enhance the methane yield.

Keywords
Long-chain fatty acids; anaerobic digestion; oleic acid; intermittent feeding; OMW; toxicity

INTRODUCTION

Microbial oxidation of long-chain fatty acids (LCFA) has been intensively studied in the recent years (Pereira *et al.* 2002; Kim *et al.*, 2004; Cirne *et al.*, 2007). The operational problems caused by LCFA, led researchers to find new strategies to treat oily wastewaters (Pereira *et al.*, 2005; Cavaleiro *et al.*, 2009) and to study unclear aspects of LCFA degradation (Sousa, 2006). However, most of these studies were accomplished with synthetic LCFA/lipidic effluents avoiding the interfering presence of other compounds (Cavaleiro *et al.*, 2009). Moreover, less attention has been given to the patterns of individual LCFA oxidation during the operation of reactors treating real/industrial complex oily wastewaters (Jeganathan *et al.*, 2006).

Olive mill wastewater (OMW) is a complex effluent with a high and variable content of lipidic compounds (4 - 25 g L^{-1}) (Hamdi, 1992; Angelidaki *et al.*, 2002). Different types of phenolic compounds ranging from highly toxic to recalcitrant are also present (Field and Lettinga 1987, Beccari *et al.*, 1999). The characteristic problems of LCFA degradation such as microbial communities inhibition, sludge flotation and biomass washout were identified during the anaerobic treatment of OMW (Boari *et al.*, 1984; Zouari *et al.*, 1996). Until now, wastewaters with high lipids content are not effectively treated by high-rate anaerobic wastewater treatment technology (Alves *et al.*, 2009). The new concepts to enhance lipids degradation based on reactors operation and feeding strategies are promising to real oily effluents depuration.

In this work, an intermittent feeding strategy was applied to the treatment of olive oil mill effluent. The degradation of individual LCFA was monitored along the reactors operation.

METHODS

Inoculum and Substrate

Two different inocula - sludge acclimated to oleate (S1) and sludge non-acclimated (S2) - were used for the batch experiments and reactors operation. The sludge acclimated to oleate was obtained as described elsewhere (Cavaleiro *et al.*, 2009). The sludge non-acclimated was obtained from a domestic wastewater treatment plant. OMW was obtained from a three-phase continuous olive oil extraction process (Amarante, Portugal). The substrate was stored at -20ºC until being used. The effluent was characterized as described in the analytical methods section and the values obtained are summarized in Table 1.

Table 1. OMW Characterization

Parameter	Average	Error*
pH	4.7	0.1
COD_t (g L^{-1})	130.1	7.4
N_t (mg L^{-1})	460.0	53.2
TP (gallic acid, g L^{-1})	4.3	0.4
oil and grease (g L^{-1})	13.6	1.5
LCFA (g L^{-1})	2.1	1.1
% oleic acid	78.0	8.8

* 95% confidence interval

Specific methanogenic activity and toxicity tests

Anaerobic batch experiments were performed as previously described (Gonçalves *et al.*, 2010). Both inocula (S1 and S2) were characterized in terms of SMA using acetate (30 mM) and H_2/CO_2 (80/20 v/v, 1 bar) as substrates. In the toxicity tests, the OMW concentration ranged from 1 to 10 g COD L^{-1}. Acetate was added as co-substrate (30 mM) in order to evaluate the influence of OMW concentration on the specific acetoclastic activity. All batch tests were incubated at 37ºC and 150 rpm. Methane production was corrected for standard temperature and pressure (STP) conditions. No nutrients were added.

Analytical methods

Total and soluble chemical oxygen demand (COD_t and COD_s) and total nitrogen (N_t) were determined using test kits (Hach Lange, Germany). Volatile solids (VS) were determined according to Standard Methods (1998). Total phenols (TP) were evaluated by a modified Folin-Ciocalteau method (Singleton and Rossi, 1965). LCFA in the solid and liquid phases of the reactors content were analysed according to Neves *et al*. (2009). Methane produced in batch experiments was analysed in a gas chromatograph (Chrompack 9000) equipped with a FID detector and a 2 m x 1/8'' Chromosorb 101 (80-120 mesh) column. Nitrogen was used as carrier gas (30 mL min^{-1}). The column, injector, and detector temperatures were 35, 110, and 220 ºC, respectively. Methane produced in the reactors was analysed in a Micro Gas Chromatograph (CP-4900, Varian), equipped with a TCD column. Helium was used as carrier gas (150kPa) and the temperatures of the column and injector were 80 and 110 ºC, respectively.

Reactors: experimental set-up and operation mode

Two up-flow anaerobic reactors of 2.5 dm^3 (useful volume) were used. They consisted in an anaerobic chamber and an external jacket which kept the temperature at 37 ºC. A solid-gas liquid separator was connected at the middle of the reactor to avoid washout and to promote the degradation of the accumulated substrate onto the biomass. The reactors R1 and R2 were inoculated

with S1 and S2, respectively. The initial biomass concentration in both reactors was approximately 10 g VS L^{-1}. OMW was diluted with tap water. Sodium bicarbonate (5 g L^{-1}) was added to provide neutral pH inside the reactor. The reactors operation was performed in two phases and the conditions are presented in Table 2. The first phase, from period I to III, was characterized by the intermittent feeding of the reactors and no nitrogen addition (COD:N of 230–270:1). Two batch periods were applied (B1 and B2) in reactor R1 and one batch period in reactor R2. In the second phase (from period IV to VII), both reactors were operated in a continuous mode. In this phase the organic loading rates (OLR) were increased by varying the COD influent from 10 to 50 g COD L^{-1} and the hydraulic retention time (HRT) from 10 to 6 days. After day 212, an additional nitrogen source (NH_4Cl) was added to provide an influent with a COD:N ratio of 100:1. Biogas was measured daily by a gas meter (Milligascounter, Ritter, Germany) and the effluents were analysed twice a week.

Table 2. OLR, Organic loading rate and HRT, hydraulic retention time applied to the reactors R1 (a) and R2 (b).

	R1				R2			
	Period	Days	HRT (d)	OLR applied (kg m^{-3} d^{-1})	Period	Days	HRT (d)	OLR applied (kg m^{-3} d^{-1})
1st phase intermittent feeding	I	0 - 50	10	0.48 ± 0.03	I	0 - 50	10	0.48 ± 0.03
	II_a	51 – 66	5	0.97 ± 0.21				
	B1-Batch	67 – 92	-	0	II	51 – 129	5	0.93 ± 0.05
	II_b	92 – 129	5	0.91 ± 0.06				
	B2-Batch	130 – 168	-	0	B1 - Batch	130 – 175	-	0
	III	169 – 211	10	0.98 ± 0.08	III	176 – 211	10	0.98 ± 0.08
2nd phase continuous feeding	IV	212 – 254	↓	0.99 ± 0.04	IV	212 – 254	↓	0.99 ± 0.04
	V	255 – 287		1.93 ± 0.22	V	255 – 287		1.93 ± 0.22
	VI	288 – 318		3.06 ± 0.21	VI	288 – 318		3.06 ± 0.21
	VII	319 – 347		4.75 ± 0.50	VII	319 – 347	6	4.49 ± 0.36

RESULTS AND DISCUSSION

Toxicity tests

The OMW toxicity towards the two different inocula was investigated. In the toxicity experiments carried out with sludge acclimated to oleate (S1) no inhibition occurred for all the concentrations tested. The initial rates of methane production in the presence of OMW were similar to the control assay (Figure 1a). Regarding to the toxicity batch experiments performed with sludge non-acclimated (S2) it was impossible to determine the intrinsic inhibition of the acetoclastic bacteria since the control assay presented a high lag-phase, induced by the lack in specific acetoclastic activity of the biomass (Table 3). However, analysing the cumulative methane production curves along the test period (Figure 1b) it is possible to detect a general inhibition of the sludge caused by the highest concentrations of OMW tested (5 and 10 g COD L^{-1}).

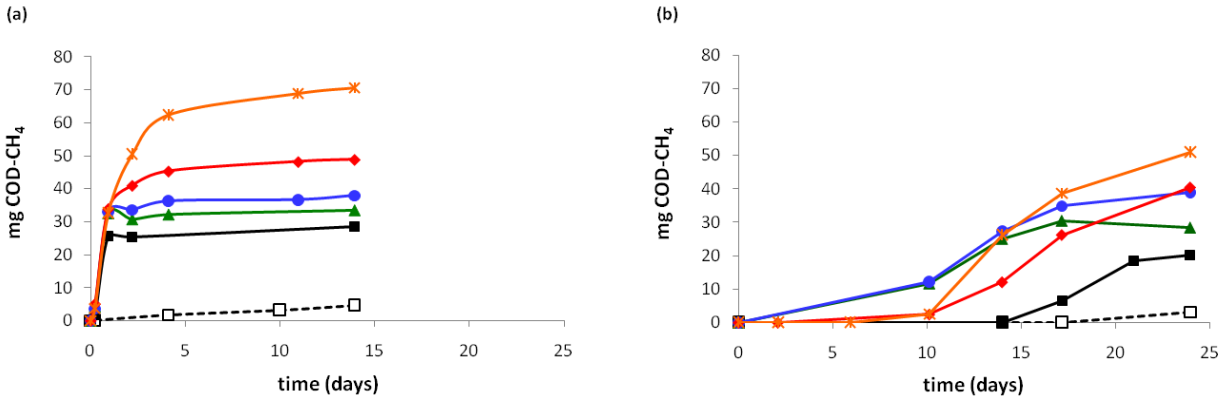

Figure 1. Cumulative methane production in the toxicity batch experiments for sludge S1 (a) and sludge S2 (b) at different OMW concentrations. □ - Blank, ■ - Control, ▲ - 1 g COD L^{-1}, ● - 2.5 g COD L^{-1}, ♦ - 5 g COD L^{-1}, * - 10 g COD L^{-1}.

Table 3. SMA ($gCOD\text{-}CH_{4\,(STP)}\ gVSS^{-1}\ d^{-1}$) of inocula

Substrate	S1	S2
Acetate	0.43 ± 0.05	<0.05
H_2/CO_2	1.43 ± 0.03	0.26 ± 0.01

Reactors operation: Intermittent feeding

The reactors start-up was performed at a HRT of 10 days and an influent with 5g COD L^{-1} along 50 days (Figure 2- Period I). In this period, both reactors showed high COD removals, good biogas quality (> 60% of CH_4) and methane production of 0.24 - 0.27 kg COD m^{-3} d^{-1}. The higher stability of R1 performance can be attributed to the use of an adapted inoculum to oleate as evidenced in the toxicity tests.

When the OLR was increased twofold by changing the HRT from 10 to 5 days, a suddenly decrease of methane production was verified in R1 (Period II-a), which after 15 days almost stopped. Furthermore, the removal efficiencies decreased, being the soluble COD removal more noteworthy attaining 50% (Figure 2-c). Consequently, the reactor feeding was stopped. During the batch period B1 the accumulated substrate was degraded and the corresponding biogas production was noticed. When the biogas production stopped, the feeding period was restarted at the same conditions (Period II-b). A recovery of biogas quality and methane production was achieved. However, the overall methane yield remained low, therefore, a new batch period was applied (B2). In reactor R2, the OLR variation provided a slower decay of the reactor performance in terms of methane production and, consequently, only one batch period was applied when the reactor was almost failing. The methane corresponding to the accumulated substrate was produced when the feed was interrupted.

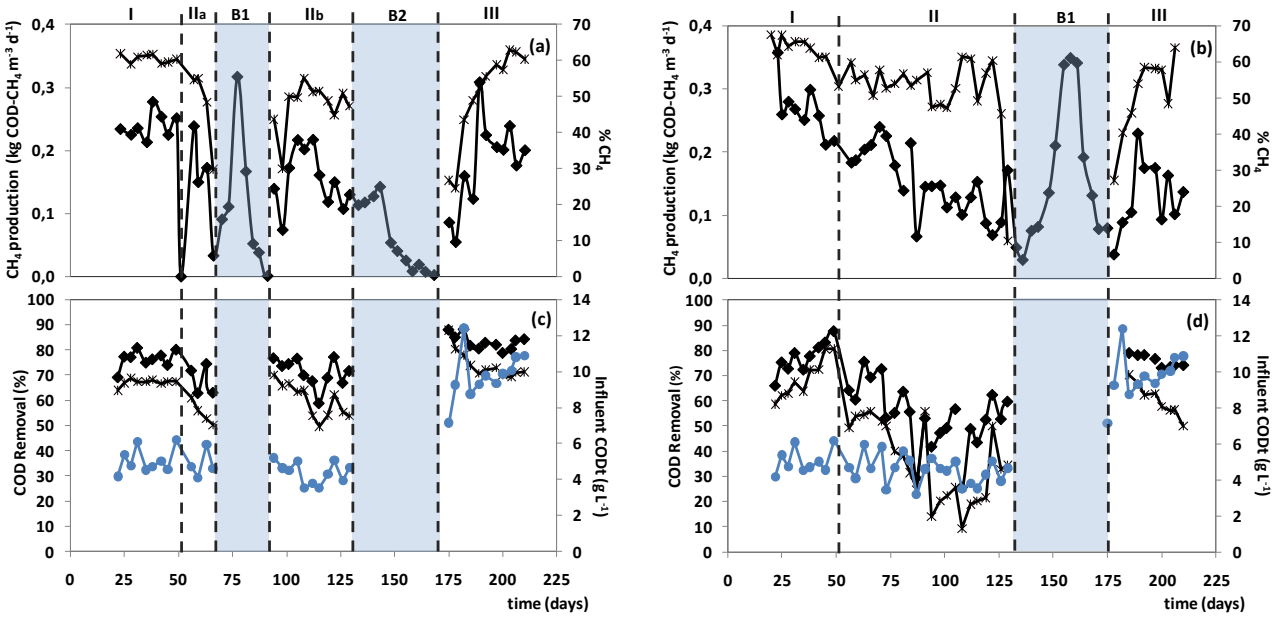

Figure 2. Reactors performance data of the 1st phase of operation. Methane production (♦) and percentage of methane in the biogas (∗) for reactor R1(a) and R2(b). Determination of removal efficiencies of total (♦) and soluble (*) COD at influent concentration of OMW (●) for reactor R1(c) and R2 (d).

After the batch period the HRT was changed to 10 days, and the OMW concentration was increased to 10 g COD L^{-1} to maintain de OLR of the previous period. In period III both reactors showed a recovery of the system performance since CH_4 increased until values of 60% and the COD removal efficiencies increased till values around 80%, suggesting that a HRT of 5 days was too small to achieve OMW treatment at these conditions. However the overall methane yields of the reactors remained low and the system did not achieve the values of the initial period (Figure 3) indicating a COD accumulation inside the reactors. At this point, additional nitrogen was added at a COD:N ratio of 100:1 to assess if nutrient limitation was occurring by the lack of nitrogen. Effectively, the addition of a nitrogen source rapidly enhanced the overall methane yield (Figure 3 - Period IV). The required COD:N:P ratio depends on the extent of loading rate and the reasonable ratio for highly loaded processes (0.8-1.2 kg COD $kgVSS^{-1}$ d^{-1}) is 250:5:1 (Droste, 1997). However, recently it has been suggested that nutrients requirements for wastewater treatment is less than previously reported since the COD removal efficiencies and biomass yield are not considered (Ammary 2004, 2005; Hussain *et al.*, 2008). Ammary (2005) reported that the OMW with a COD:N ratio of 911:5 had the sufficient nutrients to have an efficient treatment. Furthermore, Hussain *et al*. (2008) concluded that the variation of COD:N from 30:1 to 300:1 did not influenced the conversion of phenol COD to methane COD. The option of studying, in a first phase, the degradation of OMW without nitrogen addition was based in those studies, since the OMW COD/N ratio ranged from 230:1 to 270:1 (Period I to III). When the COD:N ratio was changed to 100:1 a quickly response and recovery of the system performance was obtained showing that, in this case differing from the previous reports, nitrogen addition was essential to have an efficient conversion of OMW to methane. Indeed, at this nitrogen ratio (100:1), the increase in OLR (Period V to VII) did not significantly affect the methane yield that was around 60-90% and 50-75% in R1 and R2 respectively. Although nitrogen was partially limiting the methane production (Period II and III) it was verified that the intermittent feeding promoted the degradation of the accumulated substrate inside of the reactors.

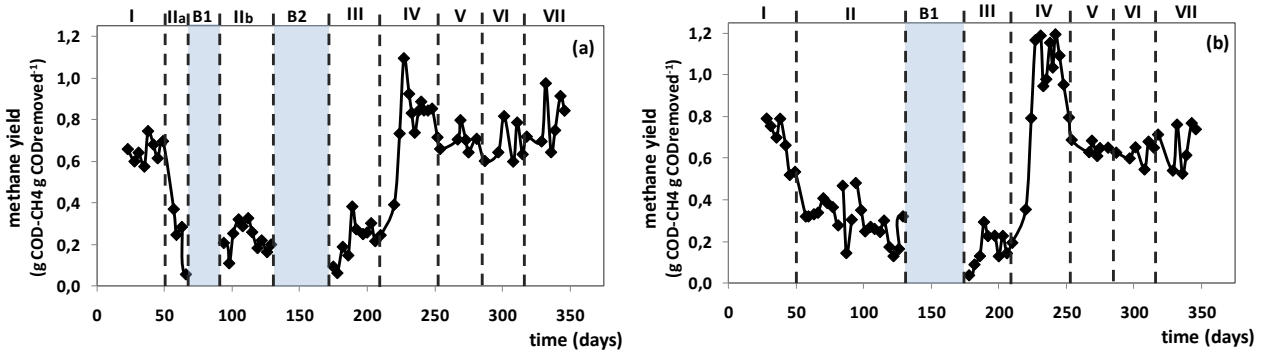

Figure 3. Overall methane yield for reactor R1 (a) and R2 (b).

Long chain fatty acids oxidation

Free LCFA in the liquid and solid phases of the reactors content were determined along the operation (Figure 4). For reactor R1 it was observed accumulation of LCFA during the periods II-a and II-b achieving a maximum value of 818 and 507 mg LCFA L^{-1}, respectively. Afterwards, accumulated LCFA were degraded during the batch periods B1 and B2. For reactor R2, the maximum value of 1869 mg L^{-1} of LCFA that was accumulated, was partially degraded during the batch period. The high accumulation of LCFA in R2 was likely due the poor inoculum quality of R2 as denoted by the batch assays of activity and toxicity of OMW. This fact supports the hypothesis that by using an acclimated sludge to oleate the reactor performance and stability are favoured (Gonçalves *et al.*, 2010).

Sequencing cycles of continuous feeding and batch degradation of accumulated LCFA was suggested by Pereira *et al.*, (2002) as a possible strategy for the treatment of effluents with high lipid content. Moreover, Cavaleiro *et al.*, (2009) demonstrated that sequencing feeding and degradation of a synthetic effluent containing 50% of COD as oleic acid was only necessary in the start-up period for the biomass acclimation. In our work, the batch degradation periods allowed the oxidation of the accumulated LCFA. However, LCFA started to accumulate again when the OLR was increased to 3 and 5 kg COD m^{-3} d^{-1} (Periods VI-VII). At this point, a new batch period could be useful to maintain low values of LCFA inside the reactor and prevent reactor failure. At the end of operation period the reactors showed similar amounts of total LCFA as well as similar individual concentrations of LCFA.

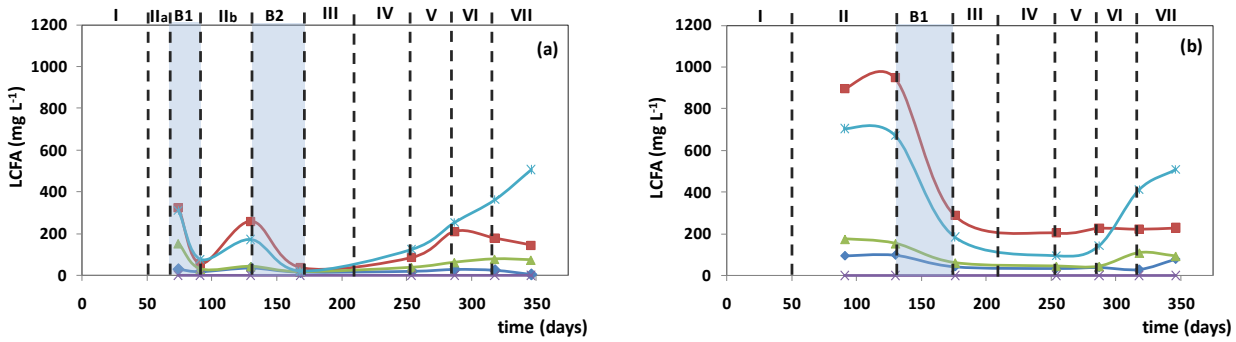

Figure 4. Long-chain fatty acids for reactor R1(a) and R2(b). (■) oleate, (*) palmitate, (▲) stearate, (♦) linoleate (×) palmitoleate.

Interestingly, in the first periods of reactors operation, oleate (C18:1) was the main LCFA compound that was accumulating with 40-51% and 48-54% for R1 and R2 respectively, followed by palmitate (C16:0) and stearate (C18:0). Afterwards, a shift in the LCFA pattern accumulation was noticed. In the final periods of the reactors operation, palmitate started to accumulate inside the

reactors with higher concentrations than oleate (Periods VI and VII).

Under anaerobic conditions, hydrolysis of fats and oils to glycerol and LCFA proceeds with less inhibition than free fatty acids resulting in the accumulation of LCFA in the wastewater (Angelidaki and Ahring, 1992). Oleic acid is the main LCFA released by the hydrolysis of vegetable oils present in olive mill wastewaters. Besides the quantity of oil and grease present in the wastewater used in this study (14 g L^{-1}), oleic acid was identified as the main free LCFA compound (78%) (Table 1). Oleate accumulation was observed as well by Cirne *et al.* in the treatment of a lipid-rich (triolein) model waste in batch tests. Although palmitate was the most abundant LCFA in all tests, accumulation of oleate was observed initially in batch tests with higher concentration of lipids (Cirne *et al.*, 2007). Nevertheless, when oleate based synthetic effluents are anaerobically digested palmitic acid has been described as the main LCFA that accumulates onto the sludge (Pereira *et al.*, 2002, 2005; Cavaleiro *et al.*, 2009). Jeganathan *et al.* (2006) investigated the treatability of a real oily wastewater from a food industry and reported that 61-87% of total LCFA accumulation was attributed to palmitic acid. It should be noted that in our study, the real oily effluent is a complex and high strength wastewater that is composed by other toxic and recalcitrant compound as phenolic that can interfere in the anaerobic process providing different reaction rates.

In this study, stearate increased slightly in both reactors but was always low, suggesting that the conversion step of stearate to palmitate was not limiting. This fact is similar to other studies (Cirne *et al.*, 2007; Jeganathan *et al.*, 2006). Pamitoleate (C16:1) was not detected during all the experimental operation. The hydrogenation of the double bond of oleate to generate stearate and then palmitate by a step of ß-oxidation is more likely to occur than the direct ß-oxidation of oleate to palmitoleate (Sousa, 2006).

CONCLUSIONS

Alternating periods of continuous OMW feed and batch operation, improved the mineralization of LCFA inside the reactor and prevented its excessive accumulation. The use of an acclimated sludge was only relevant at the reactor start-up. The intermittent feeding has been revealed as the key strategy for a long-term operation, even in the absence of adapted sludge. Nitrogen is an important factor for an efficient digestion of this type of effluent since its addition boosted the reactors methane yield. The profiles of LCFA accumulation obtained with OMW were different from studies performed with oleate-based synthetic effluents. It was revealed that oleate can be an intermediate that accumulates inside the reactor when the substrate is olive oil mill effluent.

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Table 1

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% oleic acid	78.0	8.8

* 95% confidence interval

Figure 1a

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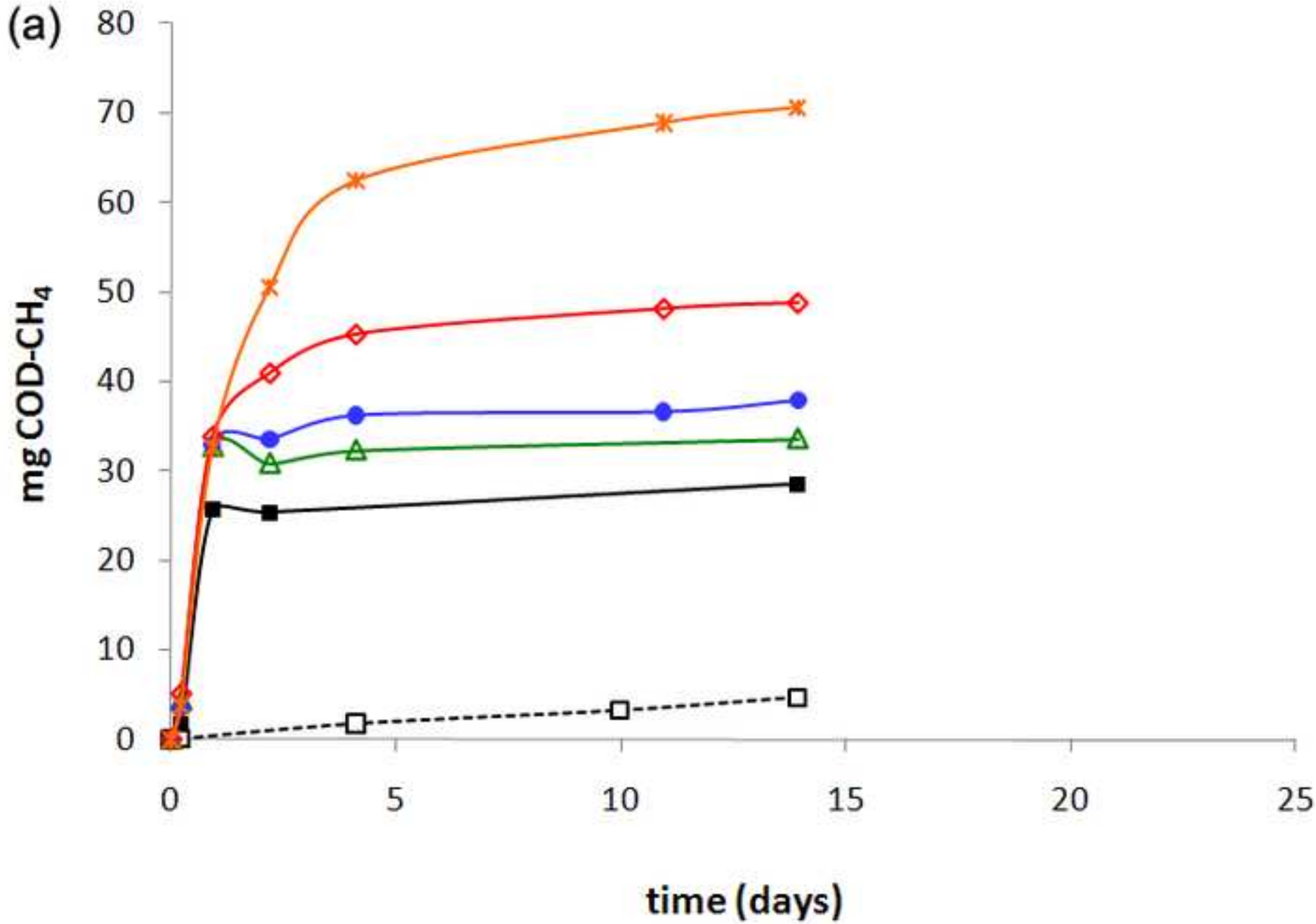

Figure 1b

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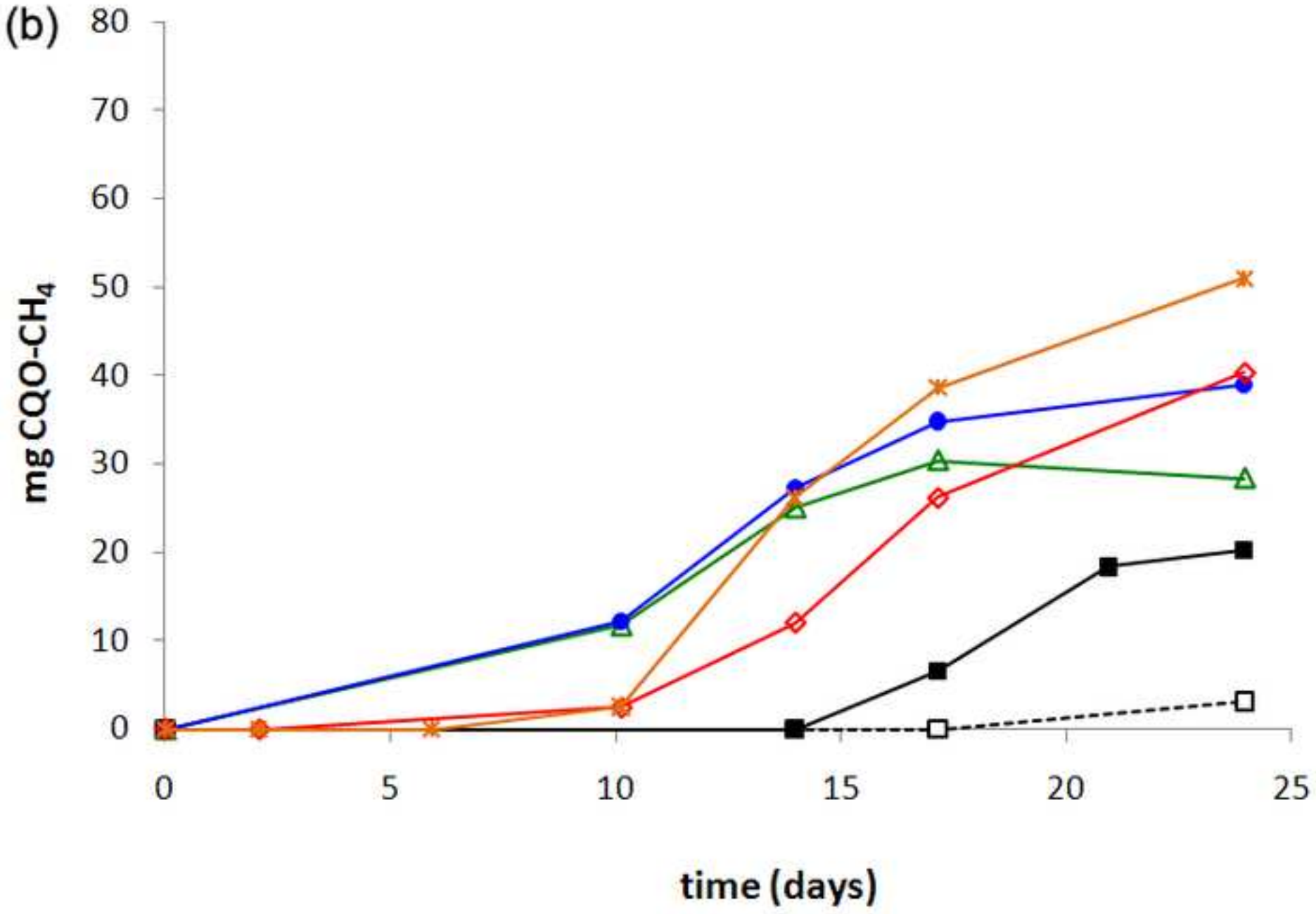

Figure 2

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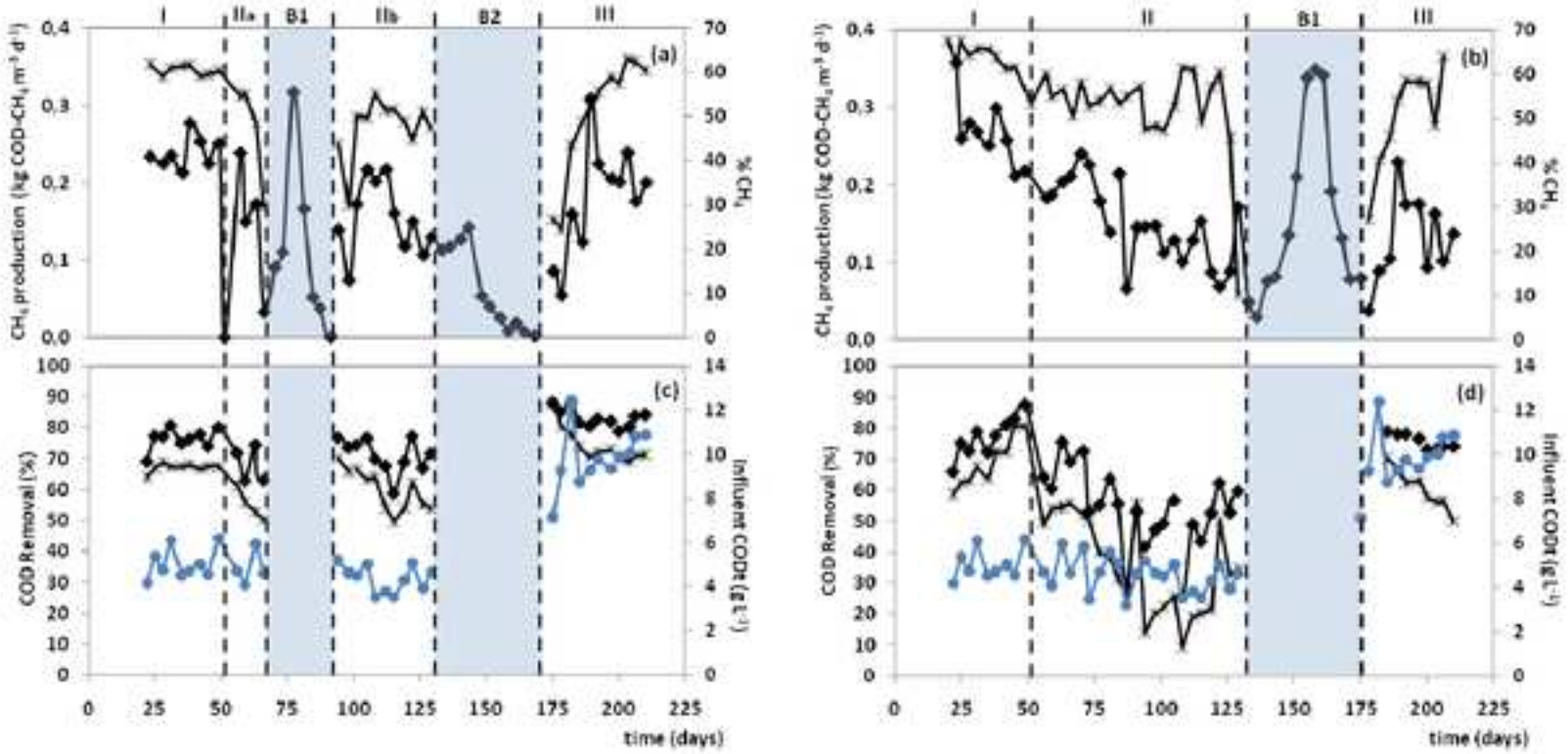

Figure 3

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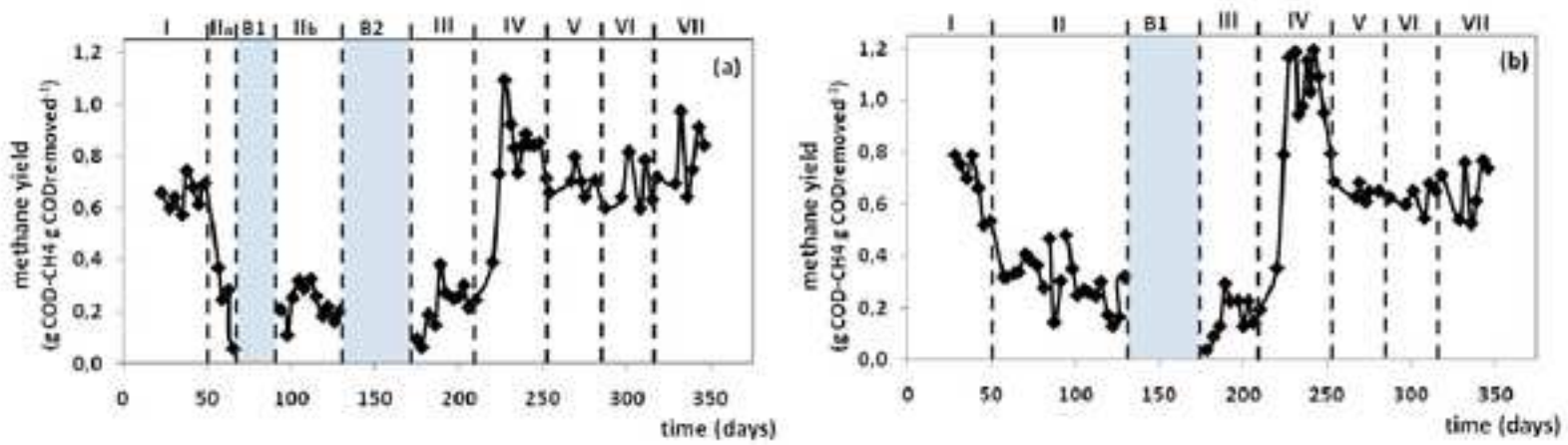

Figure 4a

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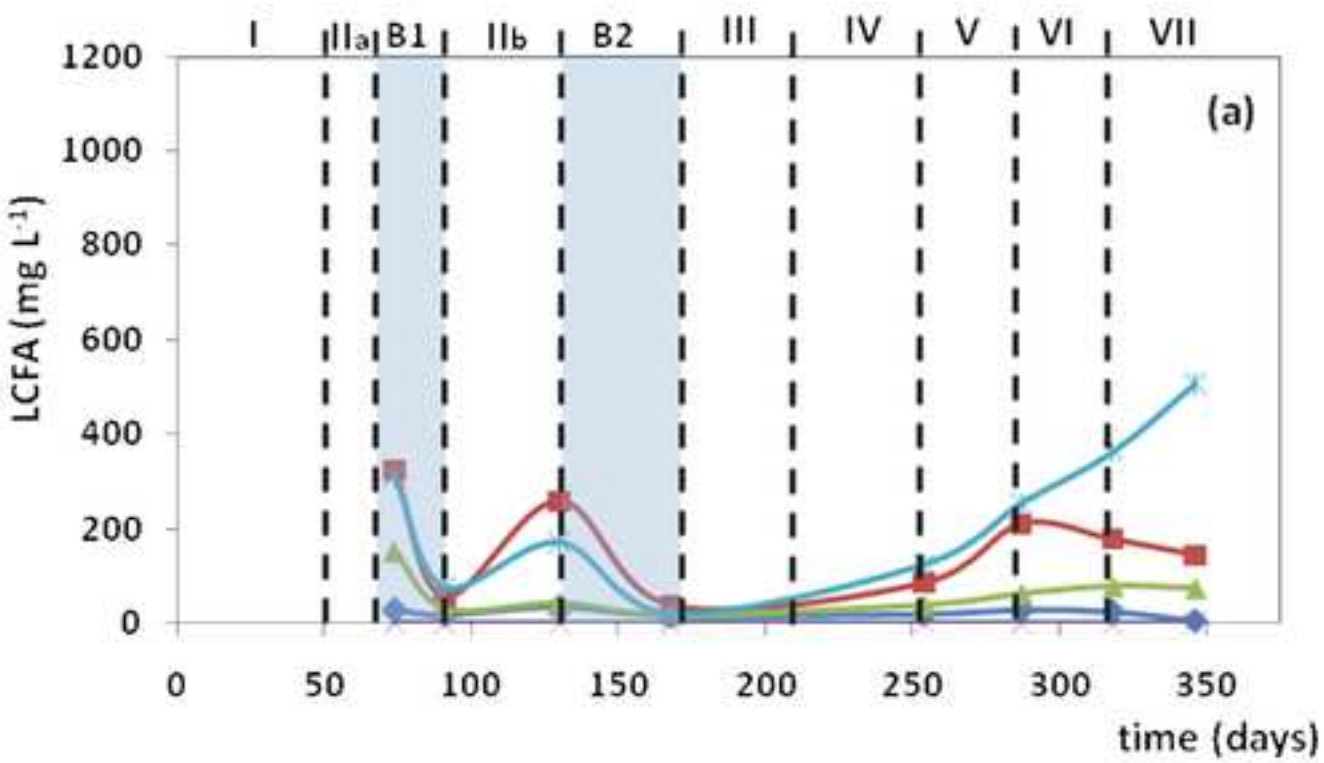

Figure 4b

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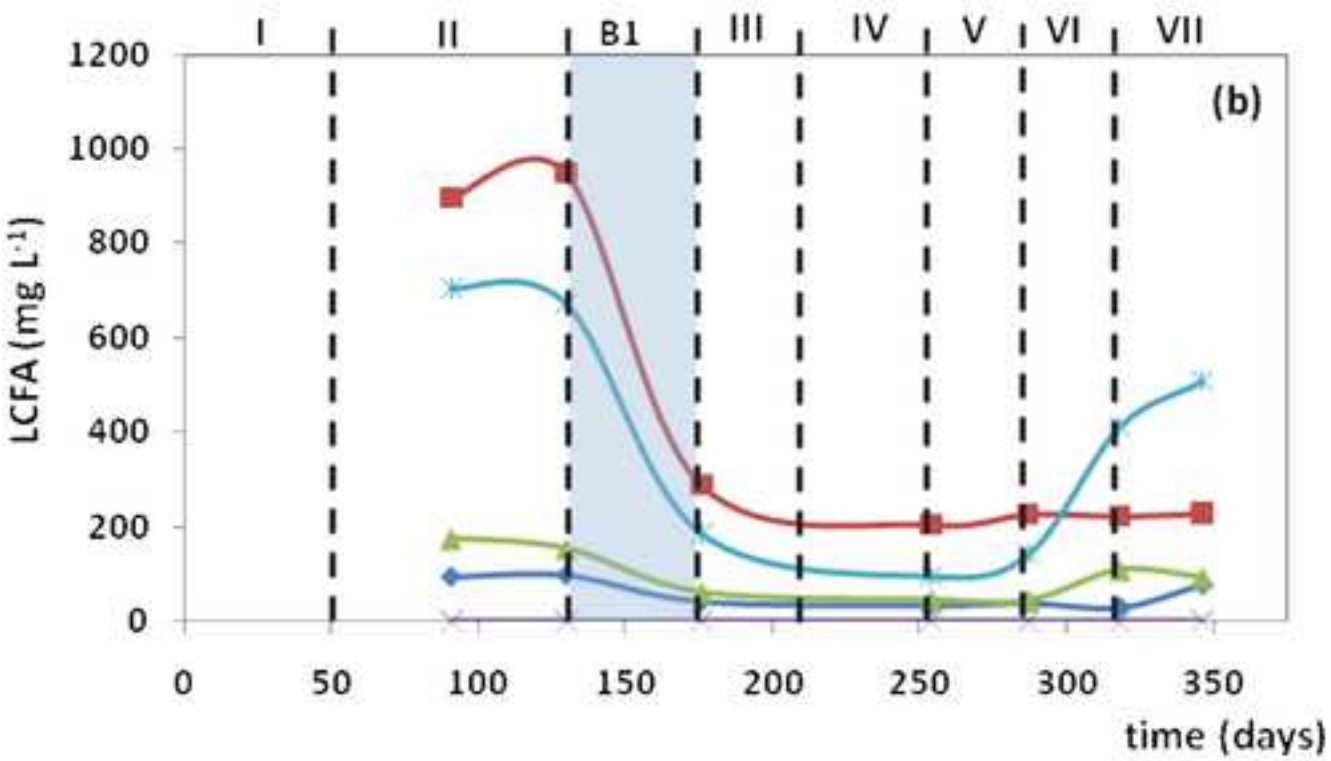

Table 3

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Substrate	S1	S2
Acetate	0.43 ± 0.05	<0.05
H_2/CO_2	1.43 ± 0.03	0.26 ± 0.01