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Effect of enhancing nutrient balance in anaerobic digester feedstock by co-substrate addition on the microbial diversity and energy production from municipal sewage sludge

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Enhancement of methane production during anaerobic digestion of waste activated sludge (WAS) could improve the energy self sufficiency of the municipal wastewater treatment plants (WWTPs). Therefore, mixing WAS with organic wastes improved process performance and stability. In this work, the anaerobic co-digestion of WAS combined with the olive processing wastewater (OPW) was investigated and associated with the energetic benefits and microbial populations shifts. The bio-methane potential (BMP) of various WAS and OPW mixtures corresponding to increased phenols concentrations were tested. The anaerobic digestion of better proportions (90%/10% and 80%/20%) was performed in anaerobic sequencing batch reactors (ASBRs). The biodegradation of phenols at concentrations up to 0.76 g/L was confirmed by Sephadex gel filtration showing that ASBR, which is suspended growth reactor, can handle much higher concentration of toxic compounds. Microbial analysis showed that phenols induced significantly the archaea community dynamic, which showed highly richness and diversity in the well performed reactor. The dominant bacteria and archaea phylotypes were affiliated to *Proteobacteria* and *Methanosarcinales*, respectively. Therefore, OPW addition increased total energy production from 24.6 kWh/ton to 64.7 kWh/ton, which would provide 0.43 M€/year net benefits only from the electric power. In addition it brings a payback time on investment of 2 years for WWTPs modification, which was considered interesting.

[**Keywords:** Anaerobic co-digestion; Waste activated sludge; Agro-wastewater; Anaerobic sequencing batch reactor; Microbial diversity; Power energy]

In the last decades, the production of waste activated sludge (WAS) increased significantly in the wastewater treatment plants (WWTPs) (1,2). It was around 2 million ton per year in Tunisia due to the increased number of municipal and industrial WWTPs (3,4). Among various methods, anaerobic digestion is a promising technology for bioconversion of this organic waste into biogas (3,5). However, the biological hydrolysis of WAS is difficult making its anaerobic digestion not performing (1,6). Furthermore, it is characterized by unbalanced nutrients proportions with a low C/N ratio (from 6/1 to 16/1) (3), which is far from the optimum ratio of anaerobic digestion (from to 20/1 to 30/1) (7). In fact, the addition of olive processing wastewater (OPW) with high organic carbon concentration is a solution to adjust nutrient content of WAS. Moreover, olive oil industry produced approximately 30 million m^3 of OPW per year in the Mediterranean countries (8–10), of which about 1 million m^3 per year in Tunisia (11). The OPW has a high chemical oxygen demand (COD), which typically ranges from 50 g/L to 200 g/L and contains high levels of polyphenols up to 10 g/L (9,12,13). Mixing the OPW with WAS could also decrease the cytotoxic effect of phenolic compounds on the anaerobic bacteria (14,15).

In recent years, several reports focused on the anaerobic co-digestion of WAS with different typical of agriculture residues, agro-industrial by-products, food wastes and grass biomass (16–19). They showed that mixing different kinds of wastes is a very attractive solution for improving their digestion efficiency and increases process performance and stability. Based on these investigations, OPW could be digested successfully by combining it with other wastes like swine manure and municipal sewage sludge at small proportion of OPW (5%–30%, v/v), which increased significantly methane production compared to the anaerobic digestion of wastes separately (9,20). They showed also that when the proportion of OPW was highly increased in the mixture, the total methane production decreased, which could due to the inhibition caused by the polyphenol contained in this waste. In contrast, Rodriguez et al. (8) reported that, during anaerobic co-digestion of WAS with olive oil mill waste (OMW) at different proportions, the better COD removal and the better methane production were obtained at high proportion of OMW with mixed ratios of 6%WAS/94% OMW. Therefore, according to these previous investigations, a little information is available regarding the detailed characterization of used substrate to better study the effect of varying polyphenols concentration on the anaerobic digestion performance and the anaerobic microbial community structure.

In this work the feedstock mixtures were prepared based on the polyphenols analyses knowing that polyphenols concentration and

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quality in the OPW is variable from one process to another and from one olive variety to another. In addition, Sephadex gel technique was used to investigate whether low and/or high molecular mass polyphenols were removed from the digester. Microbial analysis was also used to monitor the behavior of microorganism and the good examination of process, especially the anaerobic biodegradation of organic wastes, which cannot be easily treated separately (21).

Application of the Anaerobic sequencing batch reactor (ASBR) in the digestion of mixed organic wastes is of interest because of its operational flexibility and the retention of slow-growing anaerobic bacteria within the reactor (22,23). The microbial analysis, by targeting 16S rRNA genes with terminal restriction length polymorphism (T-RFLP) molecular technique, enable us to understand the microbial ecosystems involved in anaerobic co-digestion over time as a function of biochemical reactions (24). In the present study an energy and economic assessment was also performed to set the basis for a process scale up. The WWTP of Chotrana I (Tunis), treating 50,000 m^3 of wastewater a day and that has adapted anaerobic digestion technology for excess sludge treatment, was considered as a model.

MATERIALS AND METHODS

Origin and characteristics of used wastes and inoculums The WAS was collected from sludge thickeners of the WWTP of Chotrana-I (Tunis, Tunisia) treating 50,000 m^3 of urban wastewater a day. This plant includes four independent semi-continuously digesters having volumes of 5250 m^3 each, making a total digesters volume of 21,000 m^3. The digesters are fed simultaneously with primary and activated sludge (3). Seed sludge, which was used in the start up of batch and ASBR experiments, was taken from within these industrial anaerobic digesters. The OPW was collected from local three-phase olive oil extraction company (Tunis, Tunisia) (25).

The physical–chemical characteristics of wastes and inoculums (anaerobic seed sludge) are shown in Table 1. The used substrates are rich in organic solids with volatile solids (VS) contents in total solids (TS) of about 44.8% and 76.3% for WAS and OPW, respectively. The WAS is rich in suspended solids in the form of microorganisms aggregates as 89.6% of TS. However, 99% of the dry matter of OPW is represented by the soluble matter, which is more easily accessible to the anaerobic bacteria. The fresh OPW was also characterized by a high total phenols content of 3.92 g/L.

Wastes were used for the preparation of different mixtures M_1, M_2, M_3, M_4, M_5 and M_6, which were composed of 100%WAS, 90%WAS/10%OPW, 80%WAS/20%OPW, 70%WAS/30%OPW, 60%WAS/40%OPW and 50%WAS/50%OPW, respectively. They were used for feeding batch reactors R_1, R_2, R_3, R_4, R_5 and R_6, respectively, with a substrate/inoculums ratio (v/v) equal to 1. They were also used for feeding R_{13}, R_{14}, R_{15}, R_{16}, R_{17} and R_{18}, respectively, with a substrate/inoculums ratio (v/v) equal to 2.

OPW was diluted by water to prepare different proportions D_1, D_2, D_3, D_4, and D_5 of Water/OPW, which were composed of 90%Water/10%OPW, 80%Water/20%OPW, 70%Water/30%OPW, 60%Water/40%OPW and 50%Water/50%OPW, respectively. They were used for feeding batch reactors R_8, R_9, R_{10}, R_{11} and R_{12}, respectively, which were considered as control experiments with a substrate/inoculums ratio (v/v) equal to 1. They were also used for feeding R_{19}, R_{20}, R_{21}, R_{22} and R_{23}, respectively, with a substrate/inoculums ratio (v/v) equal to 2. In addition, mixtures M_1, M_2 and M_3 were prepared in a second time with a concentrated VS fractions of WAS and used for feeding ASBRs at two different OLRs (Table 2).

TABLE 1. Physical–chemical characteristics of the wastes and anaerobic seeding sludge used in this work.

Parameter	OPW	WAS	Inoculum (anaerobic sludge)
TS (g/L)	57.48 ± 1.3	5.8 ± 0.21	15.58 ± 0.3
VS (g/L)	43.9 ± 0.9	2.6 ± 0.01	7.48 ± 0.05
TSS (g/L)	0.68 ± 0.03	5.2 ± 0.2	9.18 ± 0.04
VSS (g/L)	0.6 ± 0.02	2.43 ± 0.01	4.74 ± 0.02
COD total (g/L)	108 ± 2.1	5.62 ± 0.02	–
COD soluble (g/L)	93.6 ± 1.2	0.46 ± 0.01	–
C/N ratio	52.1 ± 0.7	10.4 ± 0.02	–
pH	5.07 ± 0.05	7.08 ± 0.03	7.45 ± 0.03
Conductivity (mS/cm)	16.27 ± 0.2	3.27 ± 0.1	13.2 ± 0.1
Salts (g/L)	7.98 ± 0.1	1.62 ± 0.01	1.65 ± 0.01
Phenols (mg/L)	3920 ± 22	8.45 ± 0.1	2.13 ± 0.01

TS, total solids; VS, volatile solids; TSS, total suspended solids; VSS, volatile suspended solid.

Batch experiments: bio-methane potential tests of the WAS/OPW and water/OPW mixtures The anaerobic digestion of the different mixtures of WAS/OPW and water/OPW were conducted on the basis of the bio-methane potential (BMP) approach, measuring the maximum amount of methane produced per gram of volatile solids contained in the organics used as substrates (26). This experimental device contains twenty four reactors (from R_1 to R_{24}) with a total capacity of 2.5 L and operated at controlled mesophilic temperature (37 °C). They were equipped with a magnetic stirring system.

Reactors were filled with substrates and inoculums (anaerobic seed sludge), according to two ratios equal to 1 (for R_1 to R_{12}) and 2 (for R_{13} to R_{24}) between their volumes, respectively. Tap water was added up to a 2 L working volume. R_7 and R_{24} were run as blank reactors by loading the same amount of inoculums and filling the rest of working volume with water and with no additional substrates. They were used to calculate methane production from the inoculums alone. The headspace of each reactor was flushed with nitrogen for 2 min. Biogas productions were measured via displacement method and biogas composition was determined using UniChrom gas chromatography (GC). Net methane production was calculated by subtracting the blank methane production. Experiments ended after a period of 40 days when the cumulative methane production reached a steady state. Batch experiments were duplicated and analyses were replicated to confirm the reproducibility of the work and to facilitate the comparison between the different conditions.

Anaerobic sequencing batch reactors experiments The anaerobic digestion of WAS and OPW with the mixtures M_1 (100%WAS), M_2 (90%WAS/10%OPW) and M_3 (80%WAS/20%OPW) with a concentrated WAS was also carried out in three sequencing batch reactors ($ASBR_1$, $ASBR_2$ and $ASBR_3$, respectively), which were initially seeded with anaerobic sludge (inoculums) at 15 gVS/L. The different conditions and characteristics of feeding co-substrates are presented in Table 2.

The reactors are free cells with a working cycle of 24 h including 2 h settling. The reactors have a capacity of 1.5 L, and are equipped with a magnetic stirring system. The filling and headspace volumes were 1 L and 0.5 L, respectively. The digestions were carried out in mesophilic conditions (37 °C). Reactors were kept active during four hydrolytic retention times (HRT) of 20 days. The biogas produced was measured daily by gas meter (Ritter, Bochum-Langendreer, Germany) and its composition was estimated using the CG apparatus. The volume of biogas in normal condition was converted to standard temperature pressure (STP) conditions using combine gas law. The physical–chemical analyses were performed on samples taken from the inlet and outlet of different digesters (27,28).

Physical and chemical analysis TS, VS, total suspended solids (TSS), volatile suspended solids (VSS), COD, pH, alkalinity and total volatile fatty acids (VFAs) were determined according to the APHA Standard Methods (29). The content of VFAs was determined by the potentiometric titration with 0.1 N NaOH solution and expressed as acetic acid content. Total nitrogen was determined by the Kjeldahl method. Total carbon (TC) was measured by catalytic oxidation on a TC 1200 Euro glace analyzer. Total polyphenol content was determined using the Folin–Ciocalteu method. The total polyphenol concentration was calculated from a calibration curve, using gallic acid as standard (30).

The mass distribution of the polyphenolics was determined by the gel filtration Sephadex G50. It was used to analyze the polymeric aromatic fraction present in different output of anaerobic reactors. The optic density of these fractions was measured by spectrophotometer at 280 nm. The column was calibrated with synergic acid (MM = 198 Da), lysosym (MM = 15 kDa) and the blue dextran (MM = 200 kDa) (25).

Microbial analysis: DNA extraction and T-RFLP After stabilization of ASBR processes, samples from all digesters were collected into sterile 50 mL falcon and used immediately for DNA extraction and quantification with a Fast-DNA-SPIN Kit and NanoDrop, respectively. Bacterial 16S rRNA gene fragments were PCR-amplified with the primers Eubact8F (5′-GAG TTT GAT CMT GGC TCA G-3′) and 16SR (5′-CTA CGG CTA CCT TGT TAC GA-3′). However, archaeal 16S rRNA gene fragments were PCR-amplified with the primers 958R (5′-YCC GGC GTT CAM TCC AATT-3′) and 21F (5′-TTCCGGTTGATCCYGCCGGA-3). The forward primers were labeled at the 5′-end with a fluorescent dye label, which may be 6-carboxy-fluorescine (FAM). T-RFLP analysis of 16S rRNA amplicons was performed using the restriction enzymes HaeIII and RsaI for Archeal amplicons and HaeIII, AluI for bacterial amplicons. The lengths of the fluorescent terminal restriction fragment (T-RF) were determined using the Gene Mapper V3.7 software (Applied biosystems).

Data analyses Relative abundance of bacteria and archaea populations were determined by dividing the individual T-RF area by the total area of peaks within the range from 50 pb to 800 pb to avoid detection of primers and uncertainties of size determination (31). The presence or absence and area of T-RFs were exported to Excel (Microsoft). Phylogenetic assignment was performed using a default database generated from MiCA (http://mica.ibest.uidaho.edu) (32). One-way Analysis of variance (ANOVA) was performed to compare the reactors performance and microbial diversity between runs at different conditions. When significant statistical differences were detected ($P < 0.05$), Duncan Test was used to identify significant differences between conditions. SAS 9.0 software was used for all statistical analysis.

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