# Preliminary evaluation of biopolymers production by mixed microbial culture from citrus wastewater in a MBR system using respirometric techniques

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## Abstract

This preliminary study was aimed at evaluating the feasibility to produce biopolymers (BP) from citrus wastewater by mixed microbial culture in an anaerobic/aerobic membrane bioreactor (A/O-MBR). The activated sludge of the A/O-MBR was successfully enriched in microorganisms having a good capacity in producing intracellular biopolymers. The production of BP was found to be about 0.55 mgCOD $mgCOD^{-1}$ using pure acetate at a concentration of 1000 mgCOD $L^{-1}$. When using fermented wastewater, the conversion of acetate into BP product was 0.56 mgCOD $mgCOD^{-1}$ in the test performed with C/N equal to 1000:1, whereas it was only 0.12 mgCOD $mgCOD^{-1}$ in the test with C/N of 100:5. The results achieved suggested the feasibility to use citrus wastewater as a feedstock for biopolymers production although the low biomass storage capacity (0.26 mgCOD $mgCOD_{biomass}^{-1}$) suggested the need for optimizing the operating conditions in future studies.

**Keywords:** Citrus wastewater; intracellular biopolymers; membrane bioreactor; mixed microbial culture.

## 1. Introduction

Resource recovery from wastewater (WW) has been recognized as a key factor to develop sustainable treatment processes [1]. In this light, the biopolymers (BP) production from wastewater treatment processes using mixed microbial culture (MMC) and low-value substrates as carbon source [2] was widely investigated in several studies.

For effective biopolymers production, the medium used as carbon source must have specific characteristics. First, a high availability of organic biodegradable substrate as volatile fatty acids (VFA), a high carbon to nitrogen (C/N) ratio and must not contain any substances that might affect the bacterial activity. In this context, a wide variety of effluents from the agro-food industries has been investigated in the past [3]. Because of the presence of recalcitrant organic compounds in these wastewaters, like phenols, essential oils, etc., their use as low-cost feedstock should be carefully evaluated by means of preliminary studies. Among the agro-food WW, the ones from citrus processing are very promising for biopolymers production, because the high content of organic substrates (chemical oxygen demand – COD > 5000 mg $L^{-1}$) and the high C/N ratio (> 1000) [4]. The characteristics of citrus process wastewater (CPWW), although similar to other agro-food WW (high organic load, high C/N ratio), are different in terms of chemical composition. For instance, if compared to oil-mill or cheese-way wastewaters, in CPWW the protein/carbohydrate ratio is much lower because of the greater abundance of carbohydrates. Consequently, the productivity and the characteristics of the biopolymers will be certainly different according to the peculiarities of the wastewater used as secondary feedstock. Nevertheless, to the of best authors' knowledge, no studies are reported in the literature showing the potential of citrus wastewater in producing biopolymers. Moreover, no experiences are referring the application of membrane bioreactor (MBR) in continuous flow plant as an alternative to conventional activated sludge system operating in discontinuous mode. With this aim, this preliminary study evaluated the potential production of biopolymers in a MBR simultaneously with the treatment of citrus wastewater.

## 2. Material and Methods

### 2.1 CPWW characterization

The CPWW was withdrawn from an industry that processes citrus fruits located in Palermo (Italy). The CPWW was fermented within a reactor operating in batch mode. The fermentation process was carried out with the aim to maximize the acetate production. For further details the reader is referred to the literature [5].

The average values of the main qualitative parameters of the raw CPWW are reported in Table 1:

Table 1: Characteristics of the CPWW.

| **Parameter** | **Unit** | **Value** |
|---|---|---|
| Total COD | [mg $L^{-1}$] | 4189 ± 436 |
| Total nitrogen (TN) | [mg $L^{-1}$] | 40.9 ± 10.9 |
| Total phosphorus (TP) | [mg $L^{-1}$] | 5.8 ± 4.5 |
| pH | [-] | 7.41 ± 0.38 |
| Acetate* | [mg $L^{-1}$] | 1486 ± 354 |
| Carbohydrates | [mg $L^{-1}$] | 1540 ± 181 |
| Proteins | [mg $L^{-1}$] | 161 ± 51 |
| Conductivity | [mS / cm] | 1.42 ± 0.18 |

**after fermentation*

### 2.2 MBR plant layout and operating conditions

The experimental campaign was carried out in a laboratory-scale MBR reactor realized according to an anaerobic-aerobic (A/O) scheme. The plant layout is shown in Figure 1.

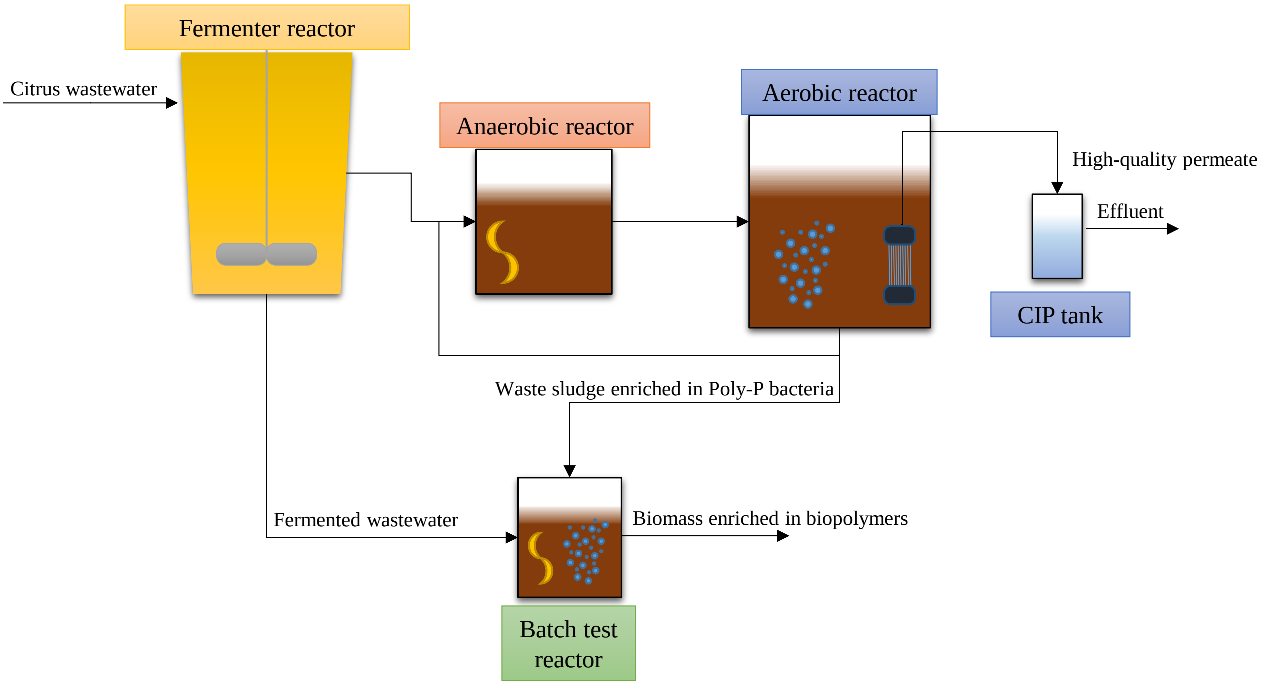

Figure 1: Layout of the A/O-MBR plant

The A/O-MBR plant was fed with a real CPWW with a flow rate of 12 L d$^{-1}$. The citrus wastewater was stored in a continuously stirred tank having a working volume of 48 L and a hydraulic retention time (HRT) of 4 days. The anaerobic reactor (volume of 2.5 L) and the aerobic reactor (volume of 7.5 L) were hydraulically connected by an internal recirculation circuit with a flow rate of 1 L h$^{-1}$. An ultrafiltration (UF) hollow fiber (HF) membrane module (Zee-Weed®01, courtesy of GE; specific area: 0.1m$^2$; nominal porosity: 0.04 μm) placed within the aerobic reactor in submerged configuration provided for the permeate extraction with flux was kept to approximately 11 L m$^{-2}$ h$^{-1}$.

The MBR plant was seeded with activated sludge with a total suspended solid (TSS) concentration of 8 g TSS L$^{-1}$. Nitrogen ($NH_4Cl$) and phosphorous ($KH_2PO_4$) were added in the feeding tank to maintain a nutrient ratio of COD: N: P = 100: 5: 1 by weight, to avoid heterotrophic growth limitation. The pH of the wastewater was adjusted to 7 by adding NaOH in the wastewater storage tank. The sludge retention time (SRT) was set at 10 days by daily withdrawing a fixed volume of activated sludge (1 L) from the system. The enriched biomass was used to perform the respirometric batch tests.

### *2.3 Accumulation batch test experiments*

Specific respirometric batch tests were performed to evaluate the ability of the enriched biomass from the A/O-MBR plant to produce biopolymers. Accumulation batch tests were performed in a 1.5 L batch reactor operating under alternative anaerobic/aerobic conditions, using pure sodium and the fermented citrus wastewater as feedstock. In the tests performed with pure acetate, five different dosages corresponding to a COD of 100 mg $L^{-1}$, 200 mg $L^{-1}$, 300 mg $L^{-1}$, 500 mg $L^{-1}$, 1000 mg $L^{-1}$, were performed. This test was aimed to evaluate the maximum conversion rate of acetate into biopolymers without any possible interference due to the real wastewater composition. In the test performed with the real wastewater, a known volume of fermented wastewater was added in the batch reactor to achieve an acetate concentration (as COD) equal to the test with pure acetate. The batch tests with citrus wastewater were replicated twice, by adding or not nitrogen, to evaluate the effect of high C/N (1000:1), or low C/N (100:5). Specifically, the C/N tested were representative of the raw CPWW (high C/N) and the one in which nitrogen and phosphorus were added to avoid growth limitation for the accumulating biomass (low C/N).

The biomass was maintained under anaerobic conditions until the whole COD added was completely depleted. Hereafter, air was supplied in batch mode by turning an air compressor on and off, keeping the DO concentration between 4.0 - 3.0 mg $L^{-1}$. The oxygen uptake rate (OUR) was calculated as the slope of the best fitting line OD/time. The trend of OUR points (respirogram) showed a concave profile, which was representative of the oxidation of the biopolymers produced by bacteria during the anaerobic phase (Fig. 2).

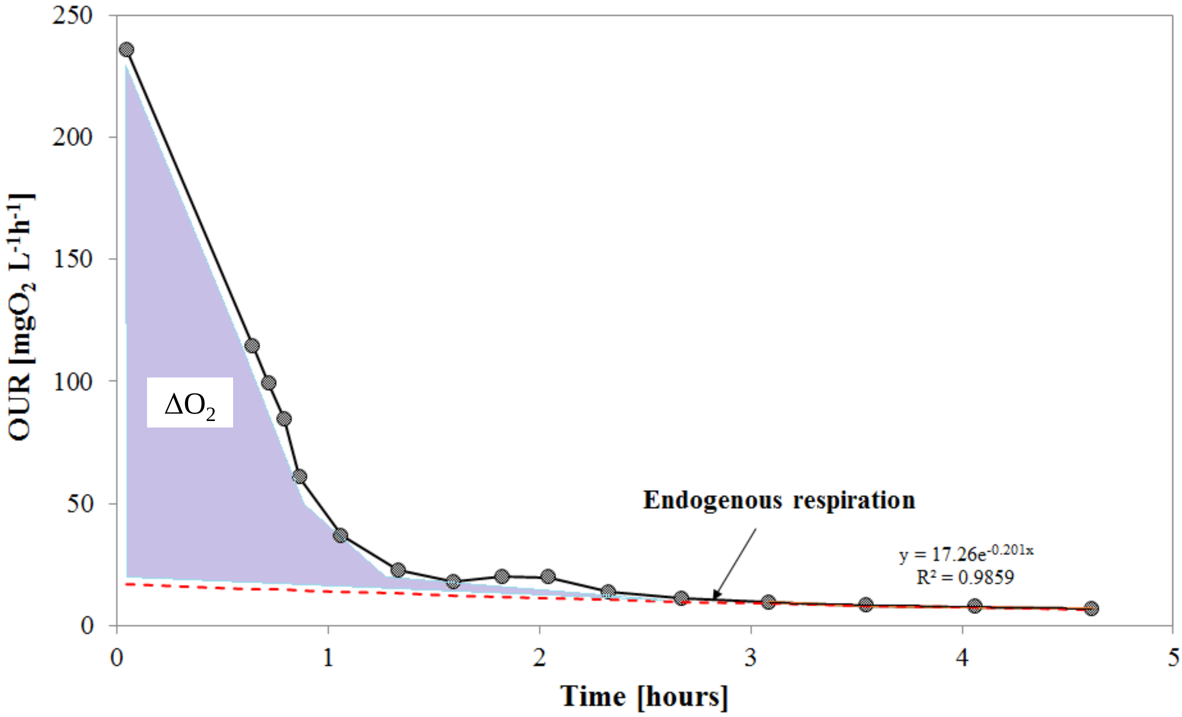

Figure 2: Typical profile of the OUR values in the batch test performed with pure acetate and fermented citrus wastewater

The biopolymers (as COD) produced was calculated through the following equation 1 derived from the literature [6]:

$$BP = \frac{\Delta O_2}{1 - Y_{sto}} \qquad [eq. 1]$$

being, $\Delta O_2$ the area under the respirogram excluding the contribution of the endogenous respiration and $Y_{sto}$ the storage yield equal to 0.85 mgCOD mgCOD$^{-1}$ according to the literature [6]. The concentration of biopolymer was than divided for the concentration of COD dosed at the beginning of the batch test, to evaluate the conversion of the organic substrate to biopolymers (mgCOD mgCOD$^{-1}$). The biopolymers content within the biomass (gCOD gCOD$_{biomass}^{-1}$) was calculated by dividing the concentration of biopolymers obtained from equation 1 for the biomass concentration expressed in terms of COD through the stoichiometric coefficient equal to 1.42 gCOD gVSS determined by direct measurements.

### 2.4 Analytical methods

All physical-chemical analysis including the TSS in the mixed liquor, the COD, total nitrogen (TN) and total phosphorous (TP) were performed according to the Standard Methods [7].

The acetate concentration in the fermented wastewater was measured by assessing the anions composition by means of an ion chromatograph (DX-120 – Dionex), using a pure acetate solution as the standard. The Gram and Neisser staining methods was used to detect the biopolymers inside the bacterial cells according to literature [8]. Biopolymers were observed as black granules inside cells by optical microscopic observation (100x and 1000x of magnification).

## 3. Results and discussion

### 3.1 Potential production of biopolymers using pure acetate

Figure 3 depicts the results achieved in the batch tests performed with pure acetate. The concentration of biopolymers produced increased linearly with the dosage of acetate (Fig. 3a). The maximum conversion rate of the acetate to biopolymers was found 0.55 mgCOD mgCOD$^{-1}$, which was comparable with results reported in previous studies carried out with agro-based wastewater [2,9].

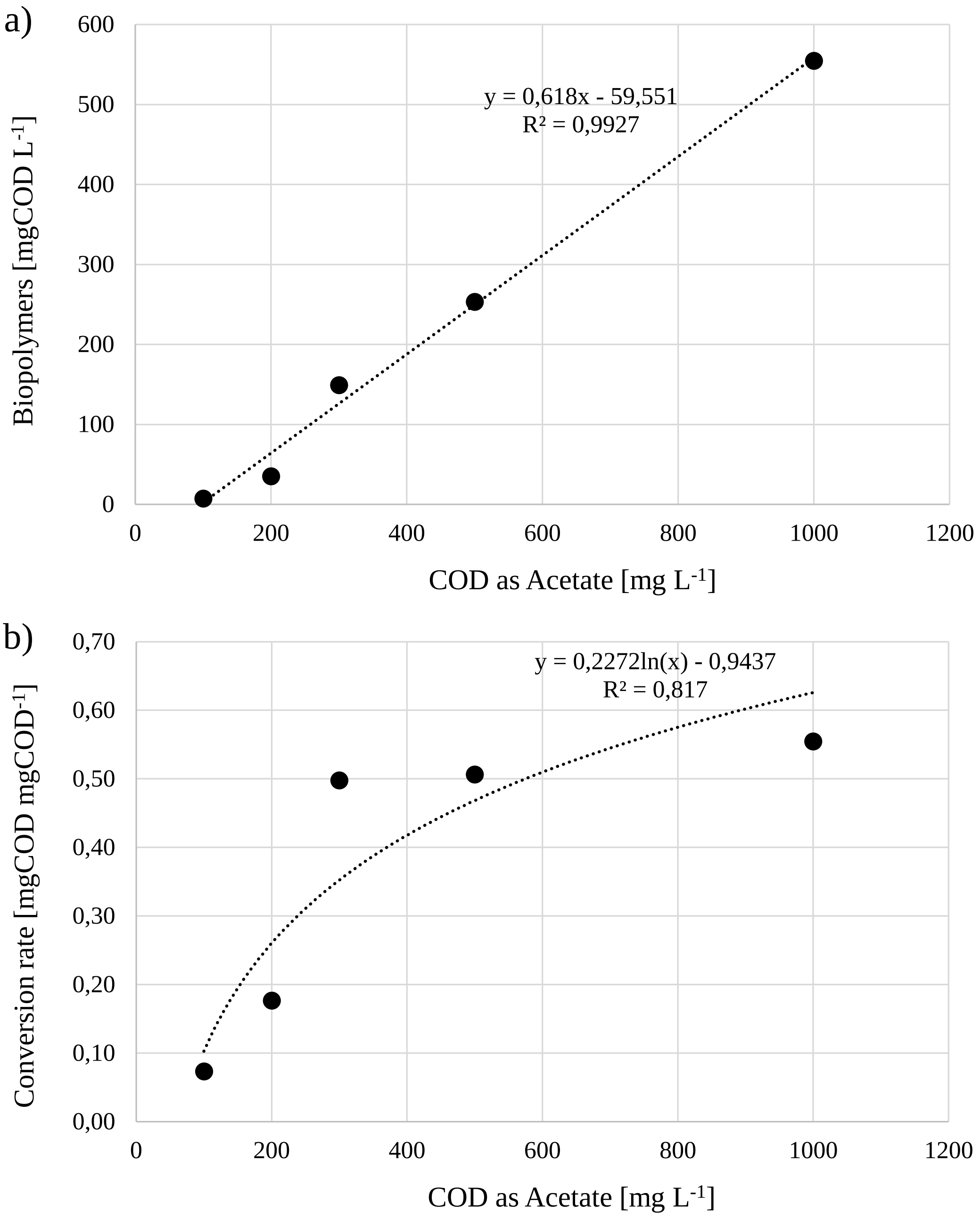

Figure 3: Concentration of intracellular biopolymers (a) and conversion rate of acetate into intracellular biopolymers (b) as a function of the acetate concentration

The relationship between the acetate concentration and its conversion to biopolymers is shown in Fig. 3b. The relationship showed an asymptotic trend suggesting the achievement of a maximum storage-capacity by bacteria as the acetate supplied was increased. Indeed, the conversion rate of acetate into biopolymers increased linearly with the acetate until a concentration of approximately 300 mgCOD $L^{-1}$, whereas for further increase of acetate its incremental rate decreased. This indicated that under high availability of organic substrate the production of biopolymers decreased. Overall, the maximum conversion rate of acetate to biopolymers (0.55 mgCOD $mgCOD^{-1}$) was obtained at a concentration equal to 1000 mgCOD $L^{-1}$. This was likely due to the achievement of the maximum capacity of biopolymer accumulation by the biomass that in turns depended on the amount of bacteria with intracellular storage capacity in the activated sludge.

The above results confirmed that under high substrate availability, the production of biopolymers by bacteria decreased according to what reported in a previous study [10]. Moreover, the supply of organic substrate at high concentration in a single pulse likely reduced the production of biopolymers by bacteria likely because of substrate inhibition as suggested by the literature [11].

### *3.2 Production of biopolymers using fermented real citrus wastewater: effect of C/N*

The results achieved in the tests performed with the fermented wastewater are shown in Figure 4.

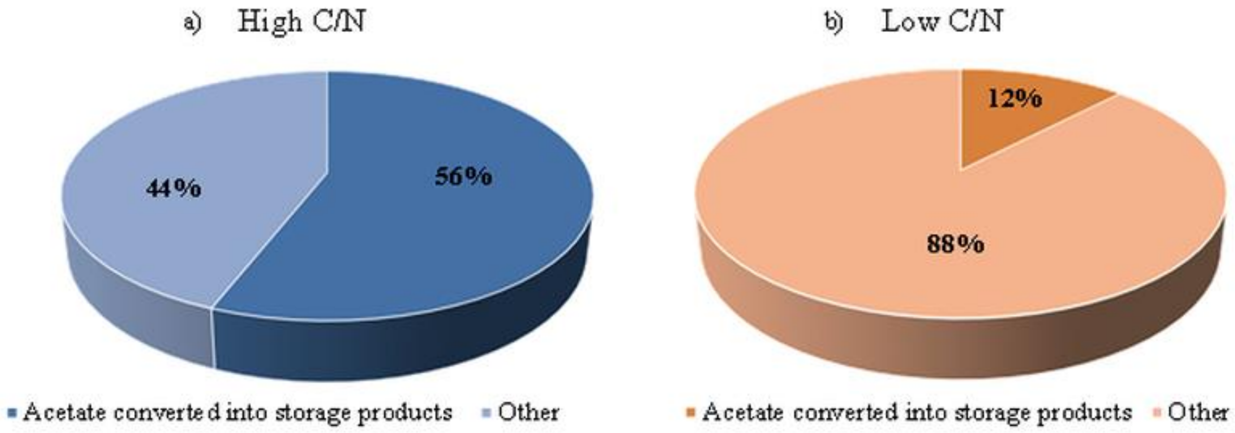

Figure 4: Utilization of the acetate in the fermented wastewater in the test with high C/N (a) and low C/N (b)

The concentration of biopolymers was higher in the test performed under high C/N, resulting close to 0.56 mgCOD $mgCOD^{-1}$, whereas that in the test performed under low C/N was only 0.12 mgCOD $mgCOD^{-1}$. Therefore, in the latter case bacteria likely used the most of acetate for cellular growth instead of biopolymers production.

The above results confirmed that high C/N is favorable to achieve the conversion of organic substrate into intracellular biopolymers as also reported in other studies [12,13]. Therefore, the limitation of synthesis phenomena driven by the low availability of nutrients represents a key factor to achieve a high conversion rate of acetate into biopolymers by bacteria. This is of meaning since citrus wastewaters are characterized by lack of nutrients, thus not requiring any pre-treatment for the increase of the C/N ratio.

Moreover, it should be stressed that the results obtained in the test with high C/N were comparable with those achieved in the test performed with pure acetate, thus suggesting the absence of process inhibiting factors in the citrus wastewater.

The maximum storage capacity by bacteria, observed in the test performed under high C/N, resulted close to 0.26 gCOD $gCOD^{-1}$ that was lower than that achieved in previous literature [14]. In previous literature, higher biopolymer accumulation capacity by bacteria was achieved when the biomass was cultivated in sequencing batch reactors (SBR) with intermittent substrate availability [15]. Indeed, the alternation of feast and famine phases, creates a competitive advantage for bacteria that quickly store substrate inside their cell during the feast phase and use this to grow during the famine phase. In the A/O MBR, the continuous supply of substrate, likely limited the selection of bacteria with intracellular storage capacity. This can explain why in this study the maximum storage capacity of biopolymers by bacteria was lower compared with that obtained in other studies.

Nevertheless, by comparing the results achieved in MBR and CAS systems both under continuous feeding mode, it was noted that MBR enabled a higher conversion rate of acetate into intracellular biopolymers [16]. Indeed, in MBR system, the selection of bacteria is strictly based on metabolic or kinetic factors rather than the ability of bacteria to aggregate in dense and settling flocs. From this point of view, MBR are characterized by a greater variety of the microbial community compared to a CAS system [17], thus resulting in a better enrichment of the accumulating biomass. Based on the above considerations, the selective enrichment of MMC in MBR system remain a promising solution to improve the overall production of biopolymers, although the operating conditions should be optimized to maximize the production yield.

### *3.3 Microscopic observation*

Neisser and Gram staining were carried out on the sludge from the MBR plant and during batch tests (Fig.5). Both analyses highlighted the presence of typical clusters of biopolymer-accumulating organisms, indicating that the alternation of anaerobic and aerobic conditions in the MBR plant enabled the selection of bacteria with intracellular storage capacity (Fig. 5a, b). This result was in good agreement with previous literature, in which is reported that biopolymer-accumulating organisms were observed in enhanced biological phosphorus removal systems, involving the alternation of anaerobic and aerobic environments [18]. Intracellular biopolymers were identified as the black granules within the cells of accumulating bacteria (Fig. 5c, d).

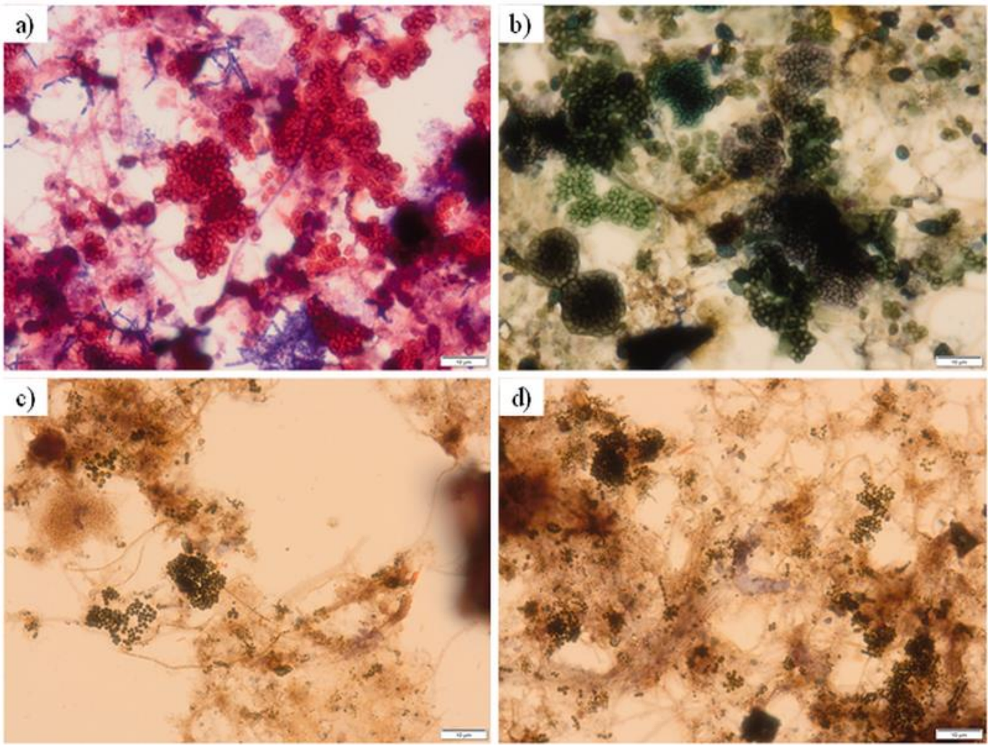

Figure 5: Microscopic images of the Gram (a) and Neisser (b) staining in the samples of the A/O-MBR plant (1000x of magnification); Neisser staining in the batch tests samples: evidence of poly-P granules within the bacterial cells at the end of anaerobic phase (c, d).

As previously discussed, the maximum storage capacity by bacteria was observed in the test performed under unbalanced nutrient condition, in which the amount of intracellular biopolymers accounted for approximately 26% of VSS. This result suggested that the enrichment of biopolymer-accumulating bacteria in the MBR plant would require proper adjustments aimed at maximizing the selection of these bacteria. It is reasonable to assess that aside the metabolic selection imposed by the alternation of anaerobic and aerobic conditions, a kinetic one, providing the alternation of feast and famine conditions, needs to be properly applied.

### *3.4 General consideration and future perspectives*

The above results highlighted the potentiality of citrus wastewaters as secondary feedstock for biopolymers production by MMC. Nevertheless, the results obtained in this study suggested that the biopolymers productivity would be significantly increased, since it resulted substantially lower than that obtained in other studies carried out with agro-based wastewaters [19]. First, since the biopolymers production is strictly related to the acetate concentration in the influent wastewater, the fermentation process needs to be optimized, focusing on the best operating parameters that allow achieving the maximum conversion rate of the organic substance into acetate. In this study, the amount of acetate in the fermented wastewater accounted for approximately 35% the COD, whereas the same value in other study is generally higher than 65-70% [20], thus suggesting that the fermentation process should be optimized. Alternatively, it should be considered to use other waste streams produced from citrus processing characterized by higher COD in order to increase the availability of acetate to supply in the accumulation reactor.

Second, a more efficient enrichment of MMC is of crucial importance to maximize the production of biopolymers. The results above discussed suggested that using MBR system is a promising approach although it should be optimized by coupling with metabolic (alternation of anaerobic/aerobic conditions) or kinetic (alternation of high/low substrate availability under aerobic conditions) selectors to achieve the enrichment of biopolymers accumulation biomass.The innovation introduced by the bacterial selection through the MBR system could insure greater microbial diversity than conventional activated sludge systems and therefore potentially greater productivity of biopolymers. Furthermore, MBRs allow tolerating severe operating conditions, which would allow to operate in process conditions more suitable for the selection of biomass and with which conventional systems would not be able to guarantee adequate purification performances.

Lastly, the feeding strategies of the batch side-stream reactor should be better investigated, evaluating the chance to implement a step-feed strategy or one based on the oxygen consumption rate.

## 4. Conclusions

The potential use of a citrus processing effluent to produce intracellular biopolymers was evaluated. The biomass enriched in bacteria with accumulation capacity of biopolymer was successfully cultivated in an A/O MBR. Biopolymer accumulating bacteria were found in the activated sludge although in moderate quantity. The conversion rate of pure acetate into intracellular biopolymers increased with the acetate concentration, reaching a maximum value of 55%. When the fermented citrus wastewater was used as organic substrate, the productivity of biopolymers was like that achieved using pure acetate (56%), indicating the suitability of citrus wastewater as low-cost substrate for biopolymers production. However, the amount of the biopolymers accounted only for 26% of the volatile suspended solids, suggesting that both the selection of the biomass and the fermentation of wastewater should be better optimized in future studies.

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