CHEMICAL ENGINEERING TRANSACTIONS
VOL. 93, 2022
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it
Guest Editors: Marco Bravi, Alberto Brucato, Antonio Marzocchella

ISBN 978-88-95608-91-4; **ISSN** 2283-9216
DOI: 10.3303/CET2293028

Polyhydroxyalkanoates Production by Mixed Microbial Cultures in Sequencing Batch Reactors Operated under Different Feeding Conditions

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The production of polyhydroxyalkanoates (PHA) by mixed microbial cultures (MMC) requires a multistage process, whereby the microbial selection of PHA-storing microorganisms plays a key role on the overall performance. A strategy to favor the microbial selection consists in the alternance of excess (feast phase) and absence (famine phase) of the external carbon source. In this work, three runs of a lab-scale Sequencing Batch Reactor (SBR) operated under different working conditions for the establishment of the feast and famine (F/F) regime were analyzed. A fixed organic loading rate of 4.25 gCOD (Chemical Oxygen Demand)/Ld, and a fixed cycle length of 12 h were applied to the SBR. The F/F regime consisted of fully aerobic dynamic (ADF) or aerobic/anoxic (AE/ANOX) conditions. Results showed an intracellular PHA content as high as 40 ± 2 (%, w/w) when ADF conditions were applied with the organic feeding solution made of acetate (85 % on COD basis) and propionic (15%) acids. The hydroxyvalerate content in the stored polymer increased (from 25 ± 1 to 41 ± 3, % w/w) by increasing the propionic fraction (up to 35%) in the feeding solution. The AE/ANOX condition resulted in a lower PHA-storing ability which warrants further investigations.

1. Introduction

In recent years, the scientific and industrial attention is being focused on the possibility to replace plastic materials with bio-alternatives, having identical or similar properties to conventional ones, but with a lower environmental impact (Kourmentza et al., 2017). Among the others, polyhydroxyalkanoates (PHA) are particularly interesting substitutes since they are biologically produced, bio-based, and completely biodegradable in the environment. Notably, PHA are a family of polyesters with a wide range of thermal and mechanical properties, which depend on the length and composition of the side chain (Melendez-Rodriguez et al., 2021). Even though industrial PHA production involves pure microbial cultures, mixed microbial cultures (MMC)-based processes are recently being investigated at pilot scale since can be integrated into wastewater treatment plants to develop an urban biorefinery (Incocciati et al., 2020; Valentino et al., 2021). Also, novel processes integrating the side stream treatment of nitrogen removal via nitrite have been proposed (Frison et al., 2015). In general, MMC-PHA production implies multistage processes which typically include the acidogenic fermentation of waste organic feedstocks to obtain volatile fatty acids (VFA), the selection of PHA-storing microorganisms from activated sludge and the polymer accumulation to maximize the intracellular PHA content (Reis et al., 2011). The microbial selection stage plays a pivotal role on the overall process performance. A common strategy to favour the microbial selection consists in the establishment of dynamic feeding conditions, such as the alternance of excess (feast phase) and deficiency (famine phase) of external carbon substrates, that induce growth kinetics limitations favoring the accumulation of PHA. In this way, a selective pressure is imposed on the microbial culture triggering competition between bacteria that are able to store the externally fed carbon source in the form of polymer granules and bacteria that are unable to do so. The former would grow during the famine phase using the stored PHA, hence outcompeting non-PHA-storing bacteria (Daigger and Grady, 1982; Frigon et al., 2006). The F/F conditions can be easily established in Sequencing Bach Reactors

Paper Received: 20 January 2022; Revised: 2 March 2022; Accepted: 28 April 2022
Please cite this article as: Marchetti A., Lorini L., Salvatori G., Gianico A., Majone M., Villano M., 2022, Polyhydroxyalkanoates Production by Mixed Microbial Cultures in Sequencing Batch Reactors Operated Under Different Feeding Conditions, Chemical Engineering Transactions, 93, 163-168 DOI:10.3303/CET2293028

(SBR) by applying proper operating conditions (Kourmentza et al., 2017). Here, the performance of a lab-scale SBR on the microbial selection of PHA-storing microorganisms has been investigated by using a synthetic mixture of VFA containing acetic and propionic acid as carbon source, with the F/F regime established under either fully aerobic (ADF) or aerobic/anoxic (AE/ANOX) dynamic conditions.

2. Material and Methods

2.1 SBR operative conditions

This study was performed by using an SBR operated at an applied organic load rate (OLR) of 4.25 gCOD (Chemical Oxygen Demand)/Ld, made of a synthetic mixture of acetic (HAc) and propionic (HPr) acid, and at a fixed cycle length of 12 h, corresponding to 2 cycles per day. The SBR (1 L working volume, T = 25 °C) was inoculated with activated sludge collected from a municipal wastewater treatment plant, stirred by a mechanical impeller, and aerated with O_2 supplied with air pumps connected to ceramic diffusors. The hydraulic retention time (HRT) was set at 1 day and corresponded to the sludge retention time (SRT), since no settling phase was performed. Three working conditions (referred to as run 1, run 2, and run 3) were investigated by changing the ratio between the organic acids in the carbon feeding solution and the applied feast and famine (F/F) strategy. In all cases, an uncoupled carbon and nitrogen supply was applied, with a COD/N ratio between 33 and 35 gCOD/gN-NH_4^+. As shown in Figure 1, the phases of each SBR cycle consisted of carbon feeding (C feed, 0.42 L/cycle), reaction I, withdrawal of the mixed liquor (W, 0.50 L/cycle), nitrogen feeding (N feed, 0.08 L/cycle), and reaction II. In run 1, fully aerobic dynamic conditions (referred to as ADF) were imposed to guarantee the FF regime, with HAc and HPr accounting for 85% and 15% of the overall COD (Lorini et al., 2020).

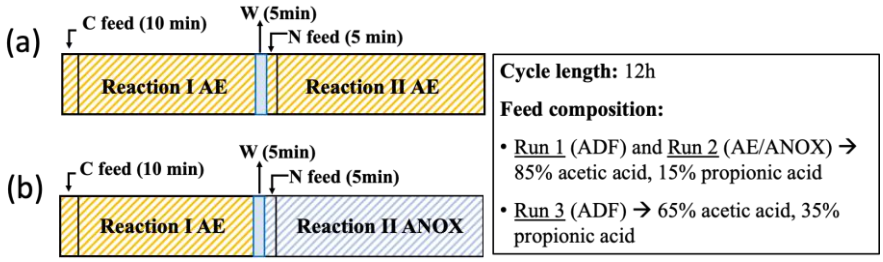

Figure 1: Scheme of the SBR cycle during either the (a) ADF strategy; (b) or the AE/ANOX feeding strategy

This feed composition was maintained in run 2 in which, however, the F/F regime consisted of the alternance of an aerobic feast phase and an anoxic famine phase (referred to as AE/ANOX). The anoxic conditions were established by the absence of oxygen and the presence of nitrite (supplied as sodium nitrite) as electron acceptor. The nitrite load accounted for 2 g NO_2^-/Ld (corresponding to about 0.61 gN/Ld) and this was supplied simultaneously with ammonium (as ammonium sulphate). Run 3 was performed under ADF conditions, but with the carbon source consisting of 65% and 35% (on COD basis) of HAc and HPr, respectively.

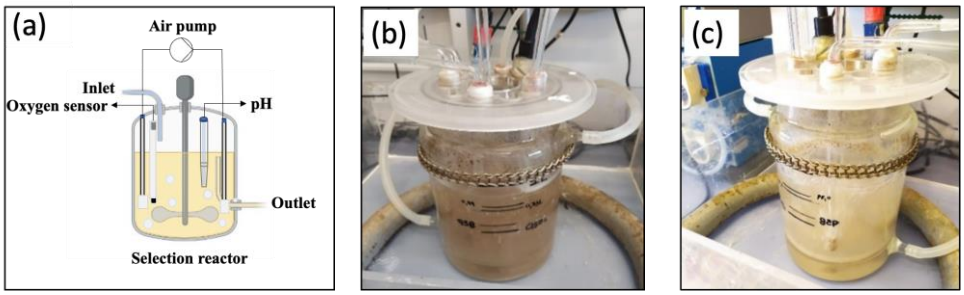

Figure 2: (a) Schematic representation of the SBR aimed at the selection PHA-producing microorganisms; (b) pictures of the reactor on the first day of operation; (c) and after the establishment of the F/F regime

The feast phase was aerobic during all the SBR runs and comprised both the C feed phase and the Reaction I phase. The reaction II phase was either aerobic (run 1 and run 3) or anoxic (run 2). The duration of each phase of the SBR cycles was controlled by a software or digital timers. Nevertheless, the feast phase duration is strictly dependent on the rise in the dissolved oxygen concentration in the medium, which consequently determines the

duration of the famine phase. A schematic representation of the SBR is reported in Figure 2a, along with two pictures of the reactor in correspondence to the first day of operation (after inoculation, Figure 2b) and after the establishment of steady state conditions (Figure 2c). The change in the colour of the mixed liquor is linked to the occurrence of the microbial selection due to the establishment of the F/F regime.

2.2 Analytical Methods

The measurement of biomass dry weight was determined as volatile suspended solids (VSS) according to standard methods (APHA, 2005). The oxygen profile was measured and registered by an Oxi 3310 (WTW) sensor equipped with a DO probe (CellOx 325, WTW). Ammonia quantification was carried out on filtered liquid samples (0.45 µm porosity) by using the Nessler spectrophotometric method (APHA, 2005), with the absorbance of colored reacted samples measured at 420 nm wavelength (SHIMADZU Spectrophotometer UV-1800). Nitrite was quantified by using ion chromatography (Dionex ICS-1000 instrument). Liquid samples for the determination of organic acids were filtered (0.45 µm porosity) and injected (1 µL) into a gas chromatograph (Dani Master, Milan, Italy) equipped with a flame ionization detector (FID). Organic acids concentrations were converted into COD according to the oxidation stoichiometry of 1.07 gCOD/g acetate and 1.51 gCOD/g propionate. Analytical determination of PHA was made on 5.0 mL of sample of mixed liquor (without filtration) immediately treated with 1.0 mL of a NaClO solution (5 % active Cl_2) and stored at −20 °C for the following analysis. For the PHA quantification samples were extracted and hydrolyzed and the released monomers were esterified into 3-hydroxyacyl methyl esters using an acidified methanol solution (3% v/v H_2SO_4) to be quantified by gas-chromatography (GC-FID Perkin Elmer 8410), as reported elsewhere (Lorini et al., 2020). The relative abundance of 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV) monomers was made by using a commercial P(HB/HV) copolymer with a HV content of 5 % (w/w) (Sigma–Aldrich, Milan, Italy). The stoichiometry conversion factors of 1.67 gCOD/gHB monomer and 1.92 gCOD/gHV monomer were used to convert the PHA concentration in terms of COD. The characterization of the SBR performance was evaluated using the data obtained from these analyses. In particular, the intracellular PHA content was calculated as the ratio between polymer and VSS concentration (%, w/w) determined in correspondence to the end of the feast phase and the PHA composition (%, w/w) was determined as the ratio between HV and (HB + HV) monomers.

3. Results and discussion

3.1 SBR operation under ADF and AE/ANOX conditions

In this study, an SBR aimed at the selection of PHA-storing microorganisms from an activated sludge has been operated under feast and famine (F/F) conditions, with uncoupled carbon (C) and nitrogen (N) supply. The obtained results (run 2 and run 3) have been compared to the results of a previous study (run 1) (Lorini et al., 2020). During all the SBR runs, the C source consisted of a synthetic mixture of HAc and HPr. The N source (as ammonium sulphate) was fed at the beginning of the famine phase (i.e., after VFA depletion) to be used for microbial growth both in fully aerobic (ADF) conditions (run 1 and run 3) and in aerobic/anoxic (AE/ANOX) conditions (run 2), wherein N in the form of sodium nitrite was also supplied to serve as the electron acceptor during the famine phase. This uncoupled strategy provides an additive means (with respect to the traditional F/F regime) to favour the selection of PHA-accumulating bacteria (Lorini et al., 2020). Indeed, only the bacteria able to store the carbon source as intracellular PHA during the feast phase can grow in the following famine phase, using the provided nutrients and the stored polymer as C and N sources. During each SBR run, the exact time in correspondence to the end of the feast phase was identified from the recorded profiles of the dissolved oxygen (DO) concentration in the reaction medium. The trend of the DO concentration during a typical SBR cycle is reported in Figure 3, for both the ADF (run 3) and AE/ANOX (run 2) conditions (Figure 3a and Figure 3b, respectively). From the DO profile, it was possible to recognise the various phases of the SBR cycle characterizing the microbial selection process through the F/F regime, numbered (in red) from 1 to 5 in the graphs. At the beginning of each cycle, a sudden decrease of the DO concentration occurred from values ranging between 6 and 7 mgO_2/L (characteristic of the end of the previous cycle) to values of approximately 1 mgO_2/L. This decrease was due to the increased microbial activity triggered by the C source supply during the feast phase. When the organic substrate was depleted, a sudden increase in the DO concentration to values close to the initial ones occurred, indicating the end of the feast phase, that represents a characteristic moment of the SBR working cycle in which the maximum value of PHA concentration in the reactor is typically reached, prior to being subsequently consumed as the only carbon source for microbial growth. Under the ADF condition (Figure 3a), with oxygen provided during the whole SBR cycle, once the feast phase was over the oxygen concentration resumed to the initial value of about 7 mgO_2/L and remained approximately constant during the entire famine phase. This because the oxygen request for microbial growth by using the intracellular stored PHA is lower than that required for the removal of the external substrate during polymer accumulation. As for the AE/ANOX condition (Figure 3b), after a sudden increase in its value in correspondence to the end of the feast

phase, the DO profile presented an abrupt decrease until 0 mgO_2/L due to the interruption of aeration in the SBR to guarantee anoxic conditions in the famine phase. Indeed, in this case, nitrate replaced oxygen as electron acceptor to support the microbial growth on the stored polymer (Frison et al., 2015). However, as shown in Figure 3b, the oxygen supply was resumed 10 minutes before the end of the cycle to establish the aerobic conditions required for the feast phase in the following cycle.

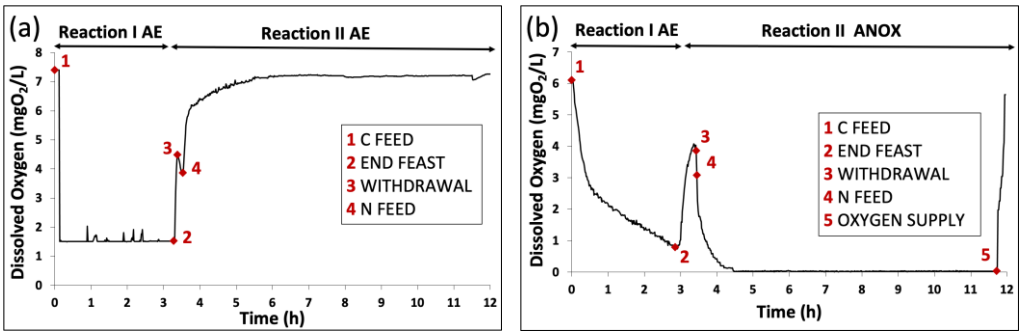

Figure 3: Typical profile of dissolved oxygen (DO) concentration in the SBR operated under: (a) aerobic dynamic feeding (ADF, run 3); (b) or aerobic/anoxic (AE/ANOX, run 2) feeding strategy

3.2 SBR performance under different working conditions

The performance of the three SBR runs has been evaluated in terms of average values of main process parameters (e.g., duration of the feast phase, intracellular PHA content, and polymer composition). As for the duration of the feast phase, average data are reported in Figure 4a as the ratio between the length of the feast phase and the length of the whole SBR cycle (set at 12 hours). The lower obtained value (equal to 21 ± 0.6 %, h/h) falls within the range that guarantees an optimal performance in the microbial selection process under ADF conditions (run 1). Indeed, as reported in the literature, the threshold value for this ratio corresponds to approximately 25 % for ADF processes with coupled feeding of carbon and nitrogen sources (Reis et al., 2011) and to 29% for ADF processes with uncoupled C and N feeding (Lorini et al., 2020). Data obtained for run 2 and run 3 (i.e., 31 ± 2 % and 33 ± 1 %, respectively) are close but slightly higher that the abovementioned threshold value, suggesting a low reduction in the selective pressure required to enrich MMC in PHA-producing microorganisms. However, it should be mentioned that in run 2 the end of the aerobic phase (imposed to a maximum value of 33% of the whole cycle length) was referred to as the end of the feast phase, even though organic acids were not always completely depleted in the reaction medium. The length of the feast phase plays a pivotal role in the performance of the selection reactor. If it is too long and the following famine phase too short, the non-PHA-accumulating bacteria will be able to survive, being in contact with the external carbon source for a long time and the metabolic pathway will be more directed towards growth, negatively affecting storage performance (Reis et al., 2011). Indeed, the duration of the feast phase affected the average value of the intracellular PHA content obtained for the three runs (Figure 4b). In run 1 the obtained lower length of the feast phase reflected in a higher PHA content (in correspondence to the end of the feast phase) with respect to the other runs, accounting for 40 ± 2 (%, w/w) (Lorini et al., 2020). Lower values were observed for run 2 and run 3, which resulted in 11 ± 1 and 16 ± 1 (%, w/w), respectively. The intracellular polymer content has an impact on the following biopolymer extraction steps, since an increase in the extraction costs is due to low contents as well as to a high presence of non-PHA-storing microorganisms (Tan et al., 2014). In terms of composition of the stored PHA, a poly(3-hydroxybutyrate/3-valerate) copolymer, P(HB/HV), was obtained in all the investigated conditions, due to the presence of both HAc and HPr in the synthetic carbon feeding solution. The P(HB/HV) copolymer has improved properties over the polyhydroxybutyrate (PHB) homopolymer, that is the most extensively studied PHA. As reported in the literature (Reis et al., 2011), the incorporation of short-chain monomers other than 3-hydroxybutyrate (HB), such as 3-hydroxyvalerate (HV), affects polymer crystallinity as well as the thermal and mechanical properties. The extent of the effect is related to the HV content in the copolymer (Melendez-Rodriguez et al., 2021) which, in turn, is linked to the final PHA applications (Muneer et al., 2020). Figure 4b shows the average value of the HV content in the polymer stored during the three SBR runs. In particular, run 1 and run 2 exhibited a comparable value of the HV content, accounting for 25 ± 1 and 23 ± 1 (%, w/w), respectively; whereas the maximum obtained value (equal to 41 ± 3 %, w/w) was reached during the operation of run 3. This because, even though the VFA composition remained qualitatively unchanged for the three SBR runs, the percentage of HPr was the same (15% on COD basis) for run 1 and run 2, but significantly higher (35%) for run 3. HAc is involved in the synthesis of both the HB and HV monomers, while

HPr is mainly involved in the production of the HV monomer (Serafim et al., 2008). Therefore, as expected, the higher HV content obtained in run 3 than in the other runs is primarily associated to the higher percentage of HPr in the organic feeding solution fed to the SBR, pointing out the fact that the PHA composition is independent on the strategy used to apply the F/F regime, either fully aerobic (run 1) or aerobic/anoxic (run 2). Furthermore, under ADF conditions (run 1 and run 3), a higher concentration of HPr in the VFA feeding solution likely led to a lower intracellular polymer content. The kinetics of substrate consumption, and therefore the kinetics of polymer storage during the feast phase, may have been slowed down in run 3 as PHA storing microorganisms, in contact with a higher concentration of HPr than in run 1, took longer time to synthesise the more complex HV monomer, thus affecting the length of the feast phase but also the total intracellular PHA content. Indeed, the metabolism of PHA production is dependent on the substrate composition since the amount of energy (e.g., as ATP) required for the conversion of different organic acids into PHA is different. In fact, HAc is converted to acetyl-CoA and subsequently to PHB, while HPr is converted to propionyl-CoA and then to PHV (Dias et al., 2005). Therefore, HAc needs less ATP to produce one C-mol of PHA compared to HPr (Wang et al., 2017).

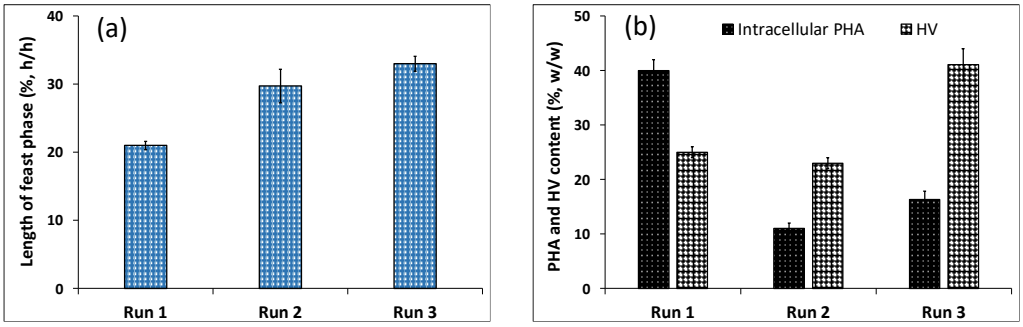

Figure 4: (a) Average value of the length of the feast phase to the overall SBR cycle length; (b) intracellular polymer content and HV content in the stored polymer at the end of the feast phase

Table 1 summarizes the average values of the main parameters characterizing the SBR performance. The amount of polymer stored during each run (ΔPHA) was calculated as the difference between the maximum (in correspondence to the end of the feast phase) and minimum (end of the famine phase) values of PHA concentration in the reactor. The greater this difference, the greater the ability of bacteria to grow on the stored polymer. In addition, once the famine phase is over the bacteria, having consumed the stored polymer, will again be ready to store the external carbon source in the form of PHA, implying a favourable microbial selection. The greater (ΔPHA) value detected for run 1 is a further indication of the good selective pressure obtained during its operation, characterized by a fully aerobic F/F regime and 15% of propionic acid in the organic feeding solution. However, by operating the F/F regime under AE/ANOX conditions without changing the VFA composition (run 2), the value of (ΔPHA) significantly decreased. Indeed, during the operation of run 2, when acids were still present in the reaction medium during the anoxic phase, they were consumed along with ammonium, that was not further consumed after VFA depletion. This resulted in a higher value of ammonium (about six-fold increase with respect to run 1) detected at the end of the cycle, that was used for microbial growth during the following aerobic phase in presence of the carbon source, negatively affecting the polymer storage.

Table 1: Main parameters characterizing the SBR performance under the different working conditions

Run	ΔPHA (mg/L)	PHA $^{\text{end feast}}$ (wt/wt, %)	HV $^{\text{end feast}}$ (wt/wt, %)	N-NH_4^+ end cycle (mgN/L)	NO_2^- end cycle ($mgNO_2^-$/L)
Run 1 (Lorini et al, 2020)	572 ± 54	40 ± 2	25 ± 1	14.0 ± 2	N/A
Run 2 (This study)	35.3 ± 7	11 ± 1	23 ± 1	83.0 ± 8	42.3 ± 20
Run 3 (This study)	232 ± 29	16 ± 1	41 ± 3	20.2 ± 3	N/A

Finally, the nitrite ion was almost completely consumed (over 95%) by the end of the cycle, being used as electron acceptor. Overall, during the operation of run 2, the SBR performance was unstable and the AE/ANOX feast and famine strategy needs to be further investigated and optimized in terms of PHA production. This also because it presents several advantages, such as the possibility to integrate the production of PHA with the nitrification/denitrification process, as well as the possibility to save, during the anoxic famine phase part of the oxygen required in the ADF processes (Frison et al., 2015).

4. Conclusions

This study compared the performance of a lab-scale SBR aimed at the selection of PHA-storing microorganisms, wherein the required feast and famine (F/F) regime was established under either fully aerobic (ADF) or aerobic/anoxic (AE/ANOX) conditions. Three SBR runs were performed at a fixed cycle length of 12 h and an applied organic load rate of 4.25 gCOD/Ld, consisting of a synthetic mixture of acetic (from 65% to 85% of the overall COD) and propionic (from 15% to 35%) acids. A higher storage ability was observed with ADF relative to the AE/ANOX conditions, and the highest obtained intracellular PHA content accounted for 40 ± 2 (%, w/w). As for the PHA composition, the obtained results highlighted that it is independent on the strategy used to apply the F/F regime. However, as expected, an increase of propionic acid in the feeding solution resulted in an increase of the HV content (up to 41 ± 3 %, w/w) in the stored polymer. In AE/ANOX conditions, over 95% of nitrite was used as electron acceptor during the anoxic famine phase, but the process was unstable thus indicating the need for further investigation and optimization in terms of PHA production.

Acknowledgements

This research was supported by Sapienza University of Rome (Ateneo 2019 grant n. RM11916B87F9FBFB).

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