Biopolymers Production from Wastes and Wastewaters by Mixed Microbial Cultures: Strategies for Microbial Selection

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REVIEW

Biopolymers Production from Wastes and Wastewaters by Mixed Microbial Cultures: Strategies for Microbial Selection

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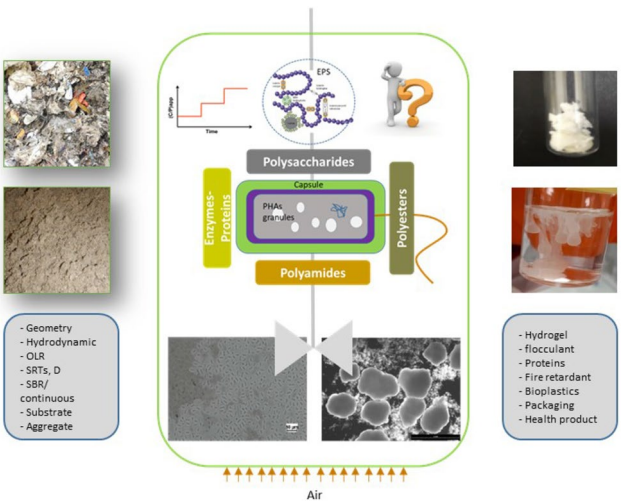
Abstract

Biopolymers are gaining attractivity for the production of both commodity and speciality chemicals. Microorganisms are able to produce a large variety of biopolymers, of which some are already produced and others need to be further characterized, and even to be discovered. This review article focuses on biopolymers such as polyesters (polyhydroxyalkanoates (PHAs), polysaccharides and proteins due to their ability to provide appealing alternatives to the already established variety of fossil-based polymers. Furthermore, these bio-proteins can also stand as alternatives to proteins from agriculture. Producing microbial biopolymers from organic wastes, and by-products, by using open mixed microbial cultures (MMC) has been suggested in order to reduce production costs as well as give the waste a new resource status. MMC strengths and weaknesses analysis has shown that this system might be relevant for producing a variety of microbial polymers in the view of complex feedstock applications. Original principles have already been developed for orientating the microbial community towards certain functionalities and the research undertaken on this topic is still very active. In this present review article, we critically examine microbial enrichment strategies, discovered these last decades, to make the biopolymer production by open MMC an industrial reality.

Graphic Abstract



Keywords Exopolysaccharides · Polyhydroxyalkanoates · Microbial proteins · Consortia engineering · Circular economy

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Statement of Novelty

If various review articles dealt with the production of PHAs, polysaccharides or proteins, none of them focused on a critical analysis of microbial selection strategies for the production of different microbial biopolymers by mixed microbial cultures. The choice done allows to specifically focus on how to implement a consortia engineering approach for biopolymer production. As this review article presents a deep analysis of an original microbial way to convert wastes into resources, we definitely think that it fits the scope of the Waste and Biomass Valorization journal, and will be appealing to a scientific audience.

Introduction

The Need for Biopolymer Production

For thousands of years, nature has provided a large variety of substances which have found numerous applications such as materials of interest in order to achieve specific functions. Biopolymers are part of those natural compounds which are highly appreciated by humans. By definition, biopolymers are macromolecules (including proteins, nucleic acids, lipids and polysaccharides) produced by living organisms such as eukaryotes and prokaryotes [1]. Microorganisms are able to synthesize a wide range of biopolymers which serve diverse biological functions. Depending on their nature, they differ from the conventional synthetic polymers by specific key properties, e.g. biofunctionality (stereoselectivity), biocompatibility and biodegradability, which makes them very useful or even irreplaceable in many areas. These bio-compounds can be used for the production of both commodity chemicals (high volume—low value products) and speciality chemicals (low volume—high value products) [2]. Based on end user industry, the market can be divided into food and beverage industry, packaging industry, bio-medical industry, agriculture and horticulture industry, electrical and electronics industry, automotive industry, textile industry, consumer goods, pharmaceutical industry, aerospace industry, and construction industry. The discovery of new biopolymers and technological developments, both expanding the field of application of biopolymers and improving the sustainability of their production, will be highly beneficial for future market developments.

The main planetary limits for the sustainable development of our societies [3] reveals that it is essential to develop solutions involving the reuse of carbon and nitrogen from our wastes and effluents. In the wastewater treatment sector, direct carbon and nitrogen upcycling, such as microbial polysaccharides, microbial polyester or microbial proteins (MPs), is re-gaining interest. For example, Matassa et al. [4] reported that MPs production showed a much lower environmental footprint compared to any other conventional plant protein production system. Similar results were found for PHAs [5]. Therefore, although the potential of producing biopolymers from microbial origin has been studied during the past century, it is highly recommended to re-assess the benefit of producing biopolymers from wastes, residues and effluents.

When considering a sustainable large-scale production system of microbial polymers, the following criteria have to be considered: economic, ethical, environmental, and engineering. Considering all these constraints, the production of biopolymers could be based on (i) the use of co-products and wastes as a substrate; (ii) inexpensive microbial cultivation conditions as for example the use of open mixed microbial cultures (MMC); (iii) tuning biopolymer composition during the production, (iv) extraction of biopolymers by using low cost and environmentally friendly processes; and (v) processing of relevant materials targeting different markets. This review will focus on different ways to successfully produce biopolymers (PHAs, polysaccharides and proteins) by using open MMC systems taking into account these different aspects.

Complex Feedstocks for Biopolymer Production

The choice of feedstock for the production of biopolymers consists in several key principles: (i) suitability for storage and transport; (ii) constant availability in the vicinity of the production plant and no competition with other applications; (iii) high content of organic matter with low toxicity to microorganisms and, if possible, high specific ratio between chemical oxygen demand (COD) and nitrogen (N) or phosphorus (P) in order to match with metabolic requirements for producing biopolymers (i.e. nutrient limitation triggers biopolymer production). Moreover, the price of substrates strongly impacts the selling price of biopolymers. For instance, prices of substrates for the production of PHB was estimated between 0.071 US$/kg for cheese whey which gave a selling price of PHB at 0.22 US$/kg, (assuming a PHB yield of 0.38 g/g) and 0.496 US$/kg for glucose which gave a selling price of PHB at 1.30 US$/kg (assuming a PHB yield of 0.33 g/g) [6]. However, these production costs should be compared to the current market value of conventional synthetic polymers, which is estimated at less than 1 US$/kg, and the current price of PHAs which ranges between 2.2 and 5.0 €/Kg [7]. Several review articles describe and critically examine the availability and relevance of different types of wastes for the production of PHAs [8] and polysaccharides [9].

Circular economy aims at establishing the concepts of consumption, in terms of reduction, reuse, recycling, and recovery, by integrating them into closed looped pathways [10]. In particular, current legislation and policies seek to promote solutions for the full recovery of organic wastes and by-products from cities, agriculture and industries (e.g. food industry, pulp and paper industry, biodiesel and bioethanol). This change of paradigm offers a new, and more attractive, status to wastes, thereby becoming a resource. Production of biopolymers could hence be entirely achieved from renewable carbon resources which do not compete with resources used for food. Pure culture uses mainly purified substrates. A variety of processing coproducts have nevertheless been tested as alternative carbon or nitrogen sources to support biopolymer synthesis with pure cultures [11, 12]. However, mainly carbohydrate hydrolysate from crops were considered and performances in terms of product yield and productivity were found much lower compared to the case where purified substrates are used. Therefore, requirement for waste valorisation might be favourable for the use of MMC to produce biopolymers, since such microbial communities can grow on diverse and complex substrates.

Organic matter in the waste is most often in the form of polymers which have to be hydrolysed before being assimilated by microorganisms [13]. This hydrolysis step can be achieved either by acidic fermentation, leading to a reduced number of simple soluble molecules such as Volatile Fatty Acids (VFAs), or by enzymatic hydrolysis; both being eventually combined with physical or chemical treatments. Direct biological transformation of some wastes into biopolymers has also been reported [11]. Since pure cultures involve strenuous and costly efforts in terms of contamination control (sterilization, microbial competition with endogenous microorganisms…) on these complex substrates, open MMC still seem an obvious choice. However, this choice is highly challenging regarding the selection of the appropriate microbial populations.

Open Mixed Microbial Cultures

Many traditional biological processing industries have, and are still, using natural microbial consortia, such as industrial fermentations (i.e. for the production of organic acids or alcohols), methanogenesis, bioconversions, extraction of metals in poor minerals, detoxification or remediation and protection of perishable foods (fermented foods and beverages) and finally industries battling against infectious diseases [14–16]. However, it is undeniable that the current development of industrial biotechnology is largely based on the use of pure cultures. It is generally accepted, and often widely justified, that to maintain product quality and high productivity, it is necessary to cultivate pure strains. In the particular case of biopolymer production, the use of pure cultures is the current rule [2]. Nevertheless, the interest in MMC has been widely debated in the past [14, 17], and this debate still continues today [11]. MMC have shown several advantages over pure cultures such as (i) the possibility of using a wide range of substrates due to the broadening of metabolic potentials; (ii) the degradation of certain recalcitrant compounds due to synergies between microbial species; (iii) the capacity to perform successive transformations such as modification of steroids or conversion of starch to ethanol; (iv) the ability to better resist to potential inhibitors, present in the feedstock or produced during the culture; (v) an increase in growth yield; (vi) a better protection against contaminants, since all ecological niches are already occupied; (vii) an increased resistance to fluctuating environmental conditions; (viii) the capacity to grow certain microorganisms which are not cultivable as a pure culture. In certain circumstances, MMC can also represent an alternative to genetic manipulation (for example the construction of long and complex metabolic pathways which can be time consuming and costly). Besides these advantages, the use of MMC also presents major challenges which have to be addressed. Indeed, difficulties to scientifically investigate such complex mixed cultures and to control the optimum balance between microorganisms involved in the community are the most often highlighted. In terms of biopolymer production, one of the biggest challenge is certainly to identify the specific environmental and technical conditions which must be applied to the culture in order to promote the relevant microbial community able to produce the targeted biopolymers, and this being completely stable over time.

MMC are open culture systems where the selection of microbial populations depends on the applied environmental conditions [17, 18]. The basic principle is that *"everything is everywhere but the environment selects*" [19]. We have capitalized knowledge on this since the well-known Winogradsky column, showing the spatial distribution of microbial metabolisms originated from sediments which depend on the local environmental conditions (light/dark and aerobic/anaerobic gradients as well as a sulphide gradient) or again the Winogradsky's culture techniques, which were based on the use of media designed to sustain only very specific metabolisms. Indeed, in the view of industrial applications of MMC, environmental conditions for microbial selection mainly result from varying operating conditions of bioreactors and feeding substrates. The stronger the selection pressure and the simpler the substrate, the lower the microbial diversity and vice versa. In other terms, the goal can either be to promote microbial diversity, for instance to increase functional resilience, or on the other hand to apply a strong selection and enable the development of a dominant strain.

This review will first describe how biopolymers can be produced by MMC, and then present the various strategies

deployed for microbial selection in the view of producing specific biopolymers.

Biopolymers Produced by MMC

Understanding biopolymer production mechanisms is essential to implement strategies for using MMC. A wide variety of microorganisms are able to produce biopolymers [2, 20, 21]. Some biopolymers are found identically in many microorganisms and thereby fulfil the same function (i.e. the PHAs), while other more specific polymers can be produced only by very few microbial species or strains and serve specialized biological functions (glycoproteins, etc.). Some biopolymers are produced and accumulate in the cell cytoplasm, e.g. polyhydroxyalkanoates, cyanophycin, glycogen, starch and polyphosphate, while far many others are excreted in the supernatant or even produced outside the cell, e.g. Poly (β-D glutamate) and many polysaccharides, such as alginates, microbial cellulose, etc. Polymers such as proteins are key components of the cells and represent a large fraction of the cell dry weight. Microorganisms are also able to produce mixtures or biochemical associations of biopolymers such as glycoproteins and lipopolysaccharides which may be of industrial or medical interest [22]. Chemical composition and properties of each biopolymer, as well as their specific use, have already been reviewed, and a detailed description of these reference studies can be found hereafter [2, 23–26]. Figure 1 presents photographs of biopolymers obtained from MMC in lab-scale reactors. In this review, the focus is directed to PHAs, polysaccharides and proteins.

Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) are polyesters produced by many prokaryotic microorganisms, both bacteria and archaea, fed on various carbon substrates. Polyhydroxyalkanoates are a class of linear polyesters composed of hydroxyl-acid monomers linked together by an ester bond. Depending on their monomer composition, they can be classified into short chain PHAs (scl-PHAs) with 3 to 5 carbons in repeating units or medium chain PHAs (mcl-PHAs) with 6 to 14 carbons in monomers. The PHAs family includes more than 150 monomers able to produce various materials with a wide variety of characteristics [13]. PHAs can be found among photosynthetic and chemotrophic microorganisms, extremophile microbes and under both aerobic and anaerobic conditions [27]. The accumulation of PHAs mainly occurs when growth conditions are unbalanced, for instance when a nutrient, such as nitrogen, phosphorus, oxygen or sulphur, is the limiting factor for cell growth [13]. For some microorganisms the production of PHAs is associated to growth. Stored PHAs can serve as a source of carbon and energy for cell development. In the case of carbon depletion, stored PHAs can be re-consumed which grants a

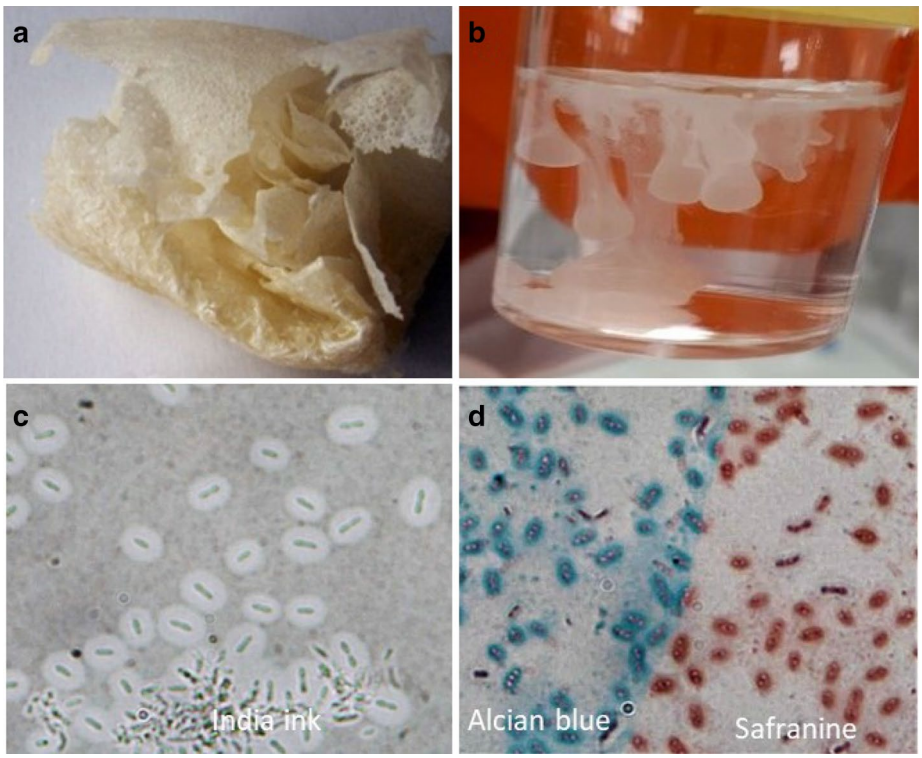

Fig. 1 Photos of biopolymers produced by open mixed microbial cultures: **a** polyhydroxybutyrate; **b** hydrogel extracted from aerobic granular sludge; **c** and **d** capsular exopolysaccharide with excellent flocculating properties. In (**c**) the capsular EPS were stained by India ink and in (**d**) by alcian blue and safranine

survival advantage to the cell. Apart from this well-known function of PHAs, other roles important for cell survival have been described. Recently, a number of underexplored functions, especially related to stress-resistance has been reviewed [28]. The fact that many microbial genera have the capacity to store PHAs gives the possibility to select PHAs-overproducer strains obtained from inoculums of very diverse origins [5].

Despite multiple properties and possibilities of application, large scale production of PHAs is still limited. Several reasons can explain this observation. The first is related to the production cost which is still too high compared to competing materials. Although the entire PHA production process should be definitely optimized, the main issue is the cost of substrates, downstream processes and energy consumption [5]. For these reasons, the use of MMC systems for microbial production has been extensively studied in the last decades, showing interesting potentials in terms of key industrial aspects, such as product yield and productivity, product quality, substrate cost and optimization of downstream processes [11]. When considering the use of open MMC for biopolymer production, PHAs production is undoubtedly the most advanced in the field, and this is due to the intensive research deployed on the topic. As it will be detailed later on, different strategies have been successfully developed to select PHA-producers and feasibility tests for producing PHAs have been undertaken by using various co-products and wastes at laboratory and pilot scales [6]. A demonstrator named "PHA2USE", designed to produce fully biodegradable bioplastics from wastewater by using MMCs, is currently being developed in the Netherlands. Producing Scl-PHAs with open MMC is presumably feasible at a lower cost compared to pure culture systems, and with an acceptable environmental impact [5].

Polysaccharides and Exo-Polymeric Substances

Polysaccharides are hydrophilic polymers composed of long chains of monosaccharide or disaccharide units covalently linked together by glycosidic bonds. These sugars can also contain some non-carbohydrate substituents (such as methyl, acetate, pyruvate, succinate, sulphate and phosphate) and can have a branched or linear molecular structure [29]. From the combination of these chemical structures, microorganisms have produced an overwhelming diversity of bacterial polysaccharides [2, 26, 30]. Some have highly electronegative or polyanionic molecules and others are neutral or cationic. Exo-polysaccharides and capsular polysaccharides are the most widespread polysaccharides in microorganisms. Cell exopolysaccharides are found in cell suspended cultures but also entangled in microbial aggregates, such as biofilms, in which are found the overwhelming majority of microorganisms living on earth.

Several authors have shown that exopolysaccharides are produced when an excess of carbohydrate is fed to the microbial culture, which implies that growth is limited by a nutrient other than the carbon and energy source [9]. A few microorganisms have been cultivated in pure cultures according to this strategy and results show that exopolysaccharide contents reached high levels ($> 40\ gL^{-1}$). Nevertheless, another study has reported that high production levels of exopolysaccharides have also been observed under limited carbon conditions, and this performed under a large variety of environmental conditions [31]. An additional point which must be considered, is that the type of substrate used for limited conditions has been proven to impact the molecular mass of exopolysaccharides [30]. For all these reasons, the best operating conditions for producing a particular microbial polysaccharide with the desired properties still need to be developed on a case-by-case basis.

As shown by the latest scientific reviews on microbial polysaccharides, and as it will be seen later in this review, the production of microbial polysaccharides is almost exclusively undertaken with pure cultures, leaving the use of MMC as relatively marginal. However, as most microorganisms are able to secrete exo-polysaccharides, open MMCs may be used as an alternative to produce these polymers at a lower cost. Moreover, using MMC offers the possibility to investigate new polysaccharides with new properties which might match better to the current and future markets [21]. Furthermore, there are several reasons for believing that the role of MMCs in the production of exopolysaccharides may increase: (i) Exopolysaccharides from MMC are still poorly characterized because of a high degree of complexity explained by a great diversity in terms of nature and combination of molecules which composes this matrix. The study of these polysaccharides is certainly only at its very beginning, but intensive research is under progress, which could bring new insights of valuable components within these exopolysaccharides [32]. (ii) A few pioneering studies have shown that the ionic fraction of Exo-Polymeric Substances (EPS) produced by granular sludge during wastewater treatment may represent a potential source of renewable polymers. Various relevant properties and applications have already been found with these extracted EPS, for instance a self-extinguishing property has recently been discovered [33]. Moreover, EPS have been suggested to serve as a sizing agent for paper coating to enhance its water-proof property or to build nanocellulose/EPS-hybrid materials [22]. Lin et al. have extracted sialoglycoproteins and proteoglycans, from EPS of microbial biofilms formed by prokaryotes [34]; (iii) Recently, a MMC was undertaken in an open continuous reactor during several months and showed excellent flocculating properties on both wastewater organic matter and inorganic minerals [35]. All these new findings are paving

the way to use open MMC systems for the production of polysaccharides and EPS with valuable properties.

Microbial Proteins

MPs, also called Single-cell proteins (SCP), are defined as dried cells of microorganisms (microalgae, yeasts, fungi or bacteria,) or eventually a microbial protein extract, often used in food and feed as a protein source. Each microorganism faces specific advantages and drawbacks regarding the production of SCP. However, most micro-organisms present similar features, such as the protein content which ranges between 43–95% of microbial cell dry weight, a high cell growth rate, the ability to use a broad spectrum of feedstocks and the ability to be produced through a continuous process. Consequently, high productivity levels can be reached, for instance a productivity of 3–4 kg MPs dry matter/m^3/h has been reported with a continuous culture of *Methylococcus Capsulatus* [36]. An overview of current production volumes and market sizes for different MPs has been published [36]. Knowing that roughly 50% of the world's fish food supply is provided by fish farming, one of the major markets for MP might hence be the aquaculture sector. A wide variety of feedstocks can be used for producing MPs. Interestingly, MPs can be produced in effluents provided by anaerobic digesters, thereby enabling nutrient and carbon recovery and converting it into valuable feed ingredients. A recent review has highlighted the advantage and drawbacks involved in the production of different types of MPs [37].

In the view of commercializing MPs for the feed sector, the produced biomass and products must comply with safety regulations meaning that they must be exempted of pathogenic microorganisms, viruses, microbial toxins, toxic heavy metals, and substrate residues, and must have safe levels of nucleic acids. It is therefore crucial to understand, not only how to select a useful protein-rich microbial population, but also how to avoid the introduction and development of pathogenic microorganisms. From such constraints arises interesting questions regarding microbial competitions.

Strategies for Microbial Selection

Generalities

Open MMC relies on a natural approach based on the selection of microorganisms in order to build a microbial community dedicated to specific targeted functions or, on the other hand, to suppress other undesired activities. Steering the function of a microbiome involves both macro-scale and meso-scale parameters [38]. Figure 2 gives a list of factors that might be considered for the selection of a MMC in an open reactor. The right balance between a set of technological and microbiological parameters must be hence chosen and maintained against time in order to enrich the population in the desired strains and get their functional stability. Ultimately, this set of parameters should give a growth advantage to the desired microbial population, such as a preferential use of substrates, a longer net residence time compared to competing strains or an appropriate specific ecological niche. As it has already been mentioned, the enrichment is sometimes not the only envisaged objective. There might be a need to implement simultaneous enrichment and production of the desired product (e.g. a biopolymer). In this case, other constraints such as high productivity and yield and product quality add up to those required for the selection. In

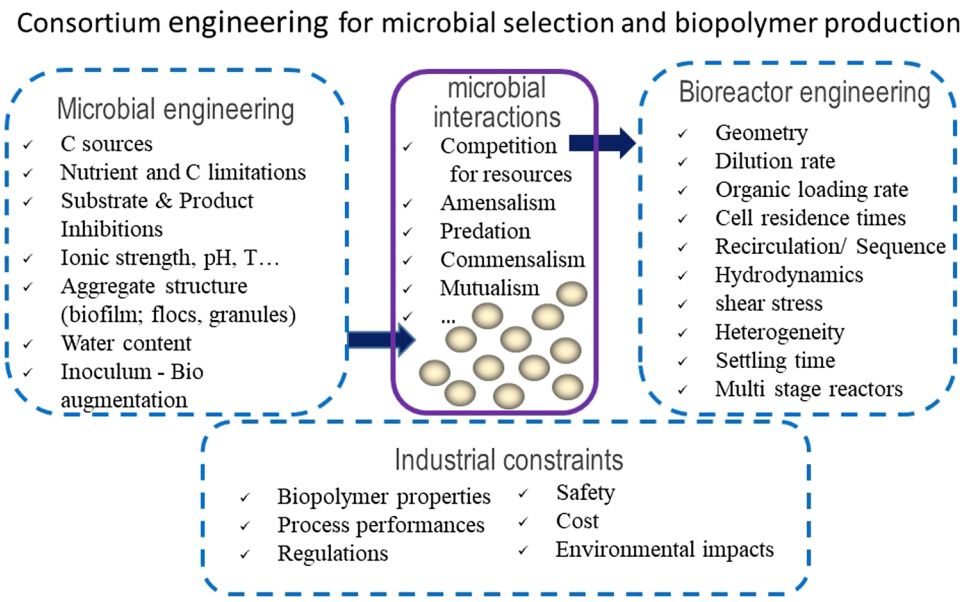

Fig. 2 Parameters to be considered for microbial community steering in an approach of consortia engineering

sectors such as bioprocessing waste or wastewater, besides the aim of enhancing the desired strain, good performance in pollutant removal must also be achieved. Functional diversity and redundancy in the microbiome may therefore be required, and a too high microbial competition might be detrimental. Facing these constraints, it is clear that profound limitation remains in our capacity to predict the behaviour of MMC during a selection process. This is specifically the case for the use of MMC for producing biopolymers.

Microbial Competition Concepts

Concepts on microbial competition were first established in the 1960ies and have not progressed much since [39], even though many omics-based methods have brought unprecedent information on who is doing what and where. Microbial interactions in MMC are commonly divided into diverse categories, where competition, amensalism commensalism and predation are the most often used to explain microbial selection (Fig. 2). Various competitive strategies are involved in the microbial population: accumulation and storage of specific nutrients (including the source of carbon and energy) depriving potential competitors, competition for space, motility, inhibitor production, production of molecules interfering with competitors signalling and so on [39]. In the resource ratio competition model, availability and consumption rates of resources are both suggested to be the main factors determining which microbes will dominate, and what will be the final diversity in a microbial community. This competition for resource is often analysed by differentiating the r-strategists (i.e. communities benefiting from a high maximum specific growth rate (μ_{max}) or a high maximum substrate consumption rate (q_{Smax})) from the the K-strategists (i.e. communities benefiting from a high affinity for the substrate) [40]. The result of competition between these two metabolic strategies depends on the concentration of substrate in the vicinity of the cells. In the case of high substrate concentrations, a race is launched for nutrients and for space which means that high μ_{max} and q_{Smax} values are described as key parameters to win the competition. Low concentrations of substrate in the vicinity of the cells, on the other hand, involve a high affinity for these substrates, meaning that low K_S values are parameters able to govern the microbial competition. This r- versus K-principle leads to an important difference in the outcomes of a selection when comparing a batch or a sequential batch reactor, where r-strategists will dominate, to a continuous reactor, where K-strategists can be preferentially enriched. Competition for resources is not the only aspect to consider when one is aiming to steer microbial communities. Indeed, in amensalism, the activity of one organism can negatively interfere with the growth of another. Therefore, the aggressor will normally dominate in the culture. Besides competition, positive interactions should also be considered. Commensalism and mutualism both involve at least one organism's conferring benefit to another. For instance, inhibitory compounds produced by one microbe and consumed by another one is often observed in MMC, and in numerous cases, these exchanges are described as key for the stability of a microbial consortia and for the overall process [17]. Moreover, the degree of specialization of a microorganism for a substrate, can allow other cells to coexist. This improves the complete use of available resources thereby potentially improving the final product yield, but can also give a more resilient culture. Finally, Quorum sensing, which involves the perception of, and response to, environmental molecular signals, might influence not only the selection but also the production and secretion of EPS by cells.

When specifically considering the production of biopolymers, the strategy for enhancing growth of the desired microbial population able to produce the targeted product, and this with high performances, must be established on the basis of the previously described microbial interactions. In the case of competition for substrates, one logical approach would consist in finding the advantage for a cell to produce a given polymer. This approach has been studied for the selection of PHAs storing microorganisms: the ability to rapidly consume the substrate and store it intracellularly allows the cell to quickly re-use the carbon substrate when needed, which offers an undeniable advantage [41]. Additional examples describing the advantage brought by the production of exopolymers can be: (i) adsorption of elements needed for growth (typically iron, phosphorus or calcium) on the EPS, thus helping the cells who are struggling to get to these elements [39, 40]; (ii) specific affinity for a surface: adhesion capacity, affinity for an area where more nutrients are available, or for an oxygen-rich region at the air–liquid interface). For instance, *Pseudomonas fluorescens* or *Azotobacter vinelandii* generated variants producing EPS which enabled the cells to float and hence improved the access to oxygen [41]; (iii) better resistance to predation, parasitism, dehydration and inhibitors; (iv) ability to trigger biofilm formation which will lead to the establishment of favourable ecological niches; (v) cell to cell interactions as described for microbe-host interactions.

Parameters to Consider for Microbial Selection

In the view of microbial community steering for biopolymer production, several parameters should be examined, see Fig. 2. The choice of a culture mode is a first parameter to be addressed. The choice can be made from a large span of systems, such as Batch, Fed-Batch, sequencing batch reactor (SBR) or continuous reactors (in parallel or in series) with or without cell recycling. Batch or Fed-batch reactors can be used if the desired microorganism is already enriched in the

inoculum. These reactors should rather be seen as production reactors, even though the selection pressure must be maintained, and although the population enrichment can pursue during the production of the polymer. SBR and continuous reactors are certainly the best reactors to produce biomass enriched in biopolymer producers. However, they strongly differ in their selection strategy when taking into account the r- versus K- principle mentioned previously in this section. Other considerations than microbial selection alone may influence the choice of the culture mode. For example, continuous culture modes guarantee constant quality of the biomass and products (if substrate composition does not vary too much) which are important criteria for downstream processes and product processing. Higher productivity levels can also be achieved compared to batch cultures, which gives the possibility to use diluted substrates and to use the aeration systems more efficiently as well as the downstream devices. When using a continuous stirred-tank reactor, the sensitivity to inhibitory substrates is reduced, which is an additional asset for continuous culture modes. The ability to reduce sensitivity towards inhibitory substrates is particularly relevant when using VFAs (especially propionate) as a substrate or when using toxic substrates, which are required for the production of mcl-PHAs. Using reactors branched in series, and with cell recycling, gives the chance to form certain compounds whose production is partially or not related to cell growth (this is mainly the case of biopolymers).

Among the diverse design parameters of reactors, SRT is one of the most efficient setting used to engineer microbial populations. Establishing a fixed SRT will determine the proportion of microorganisms which will be removed by units of time, with respect to the amount of biomass in the reactor. The SRT also determines the mean specific growth rate of cells during steady-state periods and consequently determines the selection result and also the potentiality of biopolymer production (taking into account the link between growth and biopolymer production) [42]. For heterogeneous conditions (e.g. aerobic, anoxic or anaerobic) such as occurring in granules or in heterogeneous reactors various SRTs should be considered. Furthermore, differentiating SRTs can be done by relying on structural differences between cells or between microbial aggregates, typically described as flocs, granules, suspended cells or biofilms. Therefore, using a separation technology could help to selectively withdraw undesired microbial communities. In this case, criteria, such as settling velocity, cell size, adhesion ability, cell charge and hydrophobicity, could be investigated beforehand [43].

Substrate composition is another important factor liable to influence the enrichment of specific microbial populations, and to impact microbial activities. Several studies have already shown that the more diverse the carbon and nutrients sources in the substrate, the higher the microbial diversity [44, 45]. However, although a fairly diverse microbial community can help in assimilating a complex feedstock, it might not lead to a high functional specialization (biopolymer production capacity). Moreover, from our literature analysis, it is noticeable that the relationship between the nature of the substrate and the composition of the microbial community has rarely been determined [44].

Selection Strategies Based on Extremophiles

Another strategy to guarantee a stable open MMC would be to rely on the cultivation of extremophiles [46, 47]. Indeed, by applying extreme operational conditions, the contamination risk is greatly reduced. Extreme conditions, such as high or low temperatures, high salinity, acidic or alkaline conditions and low water content, are used to select specific microbial functions. Many extremophiles have displayed the production of interesting biopolymers. The case of halophiles, for instance, has attracted attention for industrial productions. More precisely, *Haloferax mediterranei* accumulates high amounts of PHA and can excrete an anionic sulphated polysaccharide. Marine bacteria like *Bacillus*, *Halomonas*, *Planococcus*, *Enterobacter*, *Alteromonas*, *Pseudoalteromonas*, *Vibrio*, *Rhodococcus*, *Zoogloea* but also *Archaea* as *Haloferax* and *Thermococcus* showed hyperproduction capacities of biopolymers when cultivated under different conditions of salinity [46].

Selection Strategies for PHAs Production

The use of open MMC to produce PHAs is widely studied in the sectors of organic waste treatment and industrial or municipal wastewater treatment. Indeed, having access to such large amounts of waste organic matter, which can be used as a substrate source, will substantially reduce production costs. In this context, several selection methods can be suggested (Fig. 3): (i) a side stream selection where a specific process is developed, composed of a central microbial selection reactor fed sequentially by a substrate rich in VFAs with a subsequent reactor dedicated to the accumulation of PHAs and run as a fed-batch under nitrogen limitation; (ii) the production of PHAs directly branched on the wastewater treatment chain while steering, as much as possible, the selection towards a specific enrichment in PHA-accumulating bacteria; (iii) the production of PHAs from waste activated sludge (WAS); (iv) a defined selection reactor applying stringent conditions such as the use of a selective substrate (C1-carbon sources for example), extreme environments, or a continuous selection under phosphorus limitation. In fact, from a mechanism point of view, the selection principles used for these different processes are based on: (i) a selection related to the cyclic application of a feast and famine regime (or F/F regime) which promotes preferential growth of organisms able to store carbon intracellularly

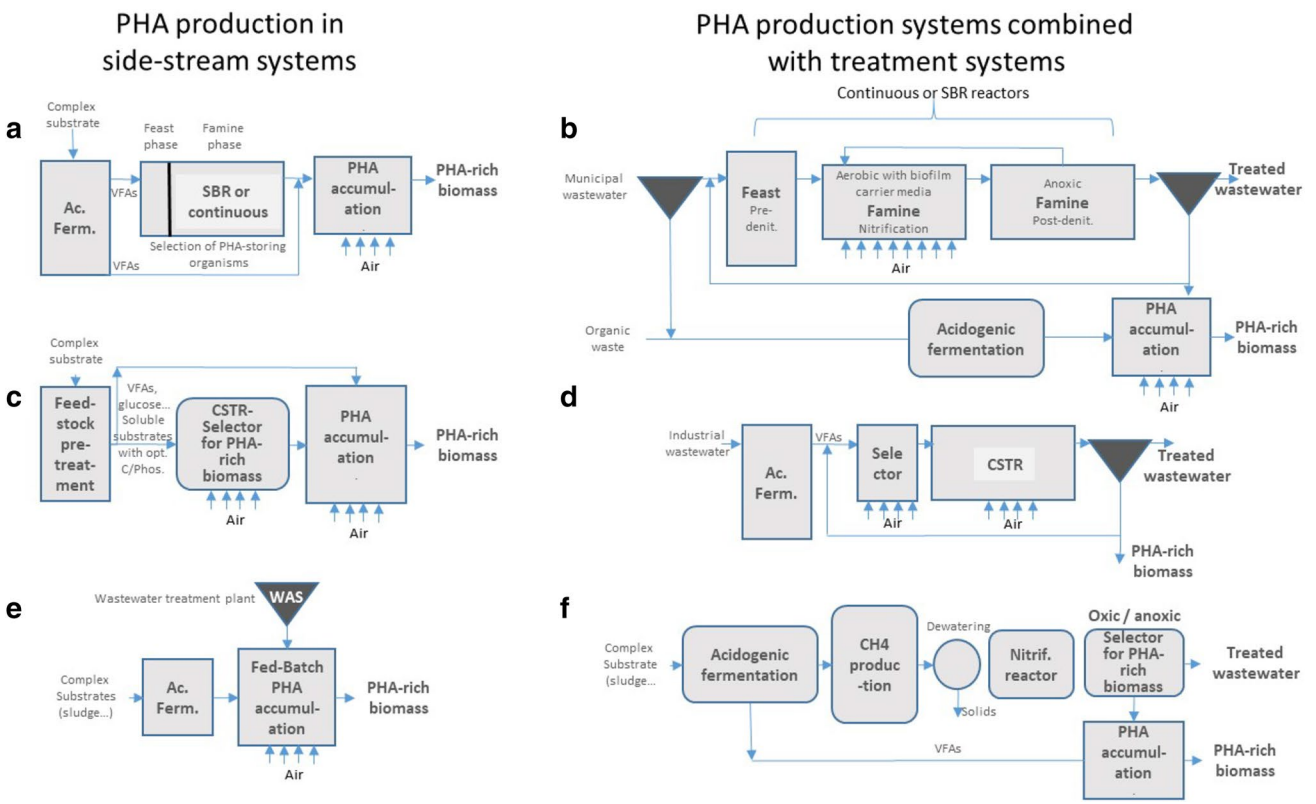

Fig. 3 Various process schemes for the selection of PHA-storing microorganisms and production of PHAs non associated (**a**, **c**, **e**) or associated (**b**, **d**, **f**) to a treatment objective. **a** Systems based on feast and famine mode considering the variations with respect to environmental conditions: aerated (AE), Anoxic (Anox), Anaerobic (AN), Phototrophic (Photo). Variations reported in the literature are (AE/AE (ADF system) [56–59], AN/AE [66], Photo/photo [84], AN/Photo [84], AE/Anox [11], Anox/AE [82]; **b** Enrichment during treatment for C&N or C&N & P removal [81]; **c** continuous production under phosphorus-growth limitation [51]; **d** Enrichment during treatment of paper mill wastewater [82]; **e** direct production from WAS using fed-batch reactor [86]; **f** enrichment during the treatment of C&N from digestate of anaerobic digester [83]

and having high potential in term of substrate specific consumption rate; (ii) a selection in continuous mode based on the affinity for phosphorus (K-strategist), (iii) selection in extreme environmental conditions. These mechanisms and corresponding enrichment systems are analysed in the following paragraphs.

Aerobic Dynamic Feeding Mode (or F/F Regime)

The Aerobic Dynamic Feeding System (ADF) described in Fig. 3a, although discovered twenty years ago [48–51], is currently the most common way to naturally select microorganisms able to store high contents of PHAs. In this process, a feast and famine regime (or F/F regime) is applied and consists in a cycle, shifting from a rather short period with an excess of organic substrate (feast) to a longer period without providing any external substrate (famine). In most studies the Sequencing Batch Reactor (SBR) is adopted, however a few experiments have been carried out in a continuous mode [52, 53]. In an ADF, the imposed dynamic feeding of substrate forces microbial competition to act on the maximum specific substrate uptake rate (q_{Smax}) rather than on the specific growth rate (μ_{max}). A significant difference in q_{Smax} is observed between PHA-producers and non-PHA-producers. This difference is explained by the biochemical pathway converting the substrate to PHB that would be much shorter and less energy-demanding than the pathway used for biomass synthesis [49, 54]. Once the PHB is stored inside the cell, it can be used for growth during the famine phase. Consequently, the storage of PHB during the feast stage confers a strong advantage to PHB-producers when entering the starvation period, and enrichment occurs as the cycles go. When applying the ADF mode, the accumulation of PHB is actually obtained from an unbalanced situation between the real growth capacity and the specific cell consumption rate (internal growth limitation). The carbon overflow is hence directed to storage. Therefore, metabolic adaptation to transient availability of external substrate may be considered as the main mechanism triggering PHA storage [51]. During the famine period, the main process limiting

the growth rate of PHB- producers is the degradation rate of intracellular PHB. The degradation of PHB can be described with a kinetic expression of first order, with respect to the PHB content of the cells [55]. A minimum time during the famine phase is required in order to metabolise the stored PHAs and hence achieve an efficient enrichment in PHA-accumulating organisms. A global carbon limitation on the whole system ensures that PHA-accumulating bacteria can fully benefit from continuous growth during the whole the feeding cycle. Since the stored PHAs are consumed during the famine period, and because a minimum time of famine is required to ensure selection, the PHA content in the ADF process is usually low. Therefore, a fed-batch reactor is added in order to increase PHA contents, which can reach up to 90% of the biomass dry weight [56, 57].

Due to the selection mode, key parameters for this ADF process are the SRT, the F/F ratio, the number of cycles per SRT, the Organic Loading Rate (OLR) and the temperature. For a given OLR, conditions to reach a high PHB content at the end of the feast period would be to increase the substrate to biomass ratio and hence reduce the number of cycles per SRT [58]. In addition, the selection of bacteria with high storage capacities requires a low F/F ratio combined with low SRTs, which makes it worth selecting bacteria with relatively high growth rates [52, 59]. Indeed, low F/F ratios, less than 20%, have already been reported [7]. Marang et al. [42] modelled and simulated the competition between PHA-producers and non PHA-producers, and showed an optimum F/F ratio of 6.5% when acetate was used as carbon source. Increasing this ratio, either by increasing the OLR or by shortening the cycles, made the selection less efficient and even led to a loss of PHA storage capacity [60]. Soluble biodegradable substrates should be depleted just at the end of the feast period in order to maximize the use of substrate for storage as well as maintain a selection pressure based on q_{Smax} and not on the substrate affinity (K_S). Authors have shown that the q_{Smax} ratio between the storing bacteria and non-storing heterotrophs is crucial, since it determines the time needed for storing bacteria to achieve a dominant position in the reactor. Jiang et al., [56] confirmed this rule by studying the effect of substrate on the microbial community and also found a change in the dominant population depending on the q_{Smax} developed on the substrate. Results showed that *Plasticicumulans acidivorans* dominated on acetate used as feed whereas *Thauera selenatis* was more specific to lactate [56]. Values of q_{Smax} as high as 4.38 Cmol/Cmol/h leading to a PHA accumulation rate of 1.74 Cmol/Cmol/h were reported for *P. acidivorans* selected on acetate feed.

In order to select a microbial community presenting high PHAs storage capacities, the optimal operating conditions to apply to the ADF technology, followed by a subsequent PHAs accumulation step (fed-batch reactor), should be a SRT of 1 day, a cycle length of 12 h (SRT/cycle length = 2), a F/F ratio less than 10% and a temperature between 25–30 °C. However, the production of PHAs with the F/F reactor alone is also possible as shown by Marang et al. [59]. Indeed, this latter study showed that, at the end of the feast period, the maximum substrate consumption rate of acetate and PHB content changed from 4.38 Cmol/Cmol/h and 52% respectively with a cycle length of 12 h to 1.34 Cmol/Cmol/h and 75% respectively for a cycle length of 20 h. Therefore, under the operational conditions of this study, increasing the cycle length from 12 to 20 h (volume exchange ratio of 0.83), increased the PHB content to 75 wt% of the produced biomass in a single-step SBR process.

The fact that ADF technology is based on a sequential process leads to strong limitations when used for industrial applications, i.e. the need for high buffer volume and elevated pumping capacity in order to adapt to discontinuous feeding as well as a high maximum oxygen demand during the feast phase [42].

A variant of the ADF process has been suggested to reduce these limitations. A microbial selection mode, based on a F/F regime, has been applied on two or more continuous stirred-tank reactors (CSTRs) branched in series [42, 52, 53]. Considering this configuration, the feast and famine periods are here separated in space, instead of time. Compared to SBRs, the CSTR mode introduces two fundamental differences: a distribution of cell residence time and a microbial competition based on substrate affinity (K_S) instead of q_{Smax}, which affects the selection rules. Consequently, even if a two-stage CSTR is able to get an enriched microbial population in PHA-producers, the selection pressure is weaker than in a SBR since it depends on both q_{Smax} and K_S. However, continuous reactors are still interesting because they facilitate the up-scaling of the process. This two-stage process also enables solid–liquid separation between the feast and famine reactor. This additional process gives more flexibility in terms of design and gives the possibility to selectively remove substrates which are unsuitable for the production of PHAs [59]. The growth of side microbial populations is hence prevented and the proportion of PHA-accumulating bacteria is enhanced. Interestingly, cells with high amounts of PHAs present higher densities and therefore can be easily separated from the rest of the biomass.

The ADF process has been applied with great success by using various substrates and complex feedstocks such as agro-industrial wastes (e.g. fruit pomaces, animal litter, cheese whey, glycerol), food wastes, pulp and paper mill effluent, olive mill wastewater, molasses, cellulose-acetate fibre manufacturing and palm oil mill effluent [11, 61, 62]. Considering that VFAs are the favourite substrates both for the selection of PHA-producers and for PHAs production, most of the studies on this topic proceed to the fermentation of the feedstock before operating the microbial selection. This has led to adopt a three-stage process, now widely used

to produce PHAs, which consists in: a first step of acidogenic fermentation to digest biodegradable organic carbon into VFAs which are precursors of PHAs; (2) a second step for the enrichment of biomass with a high PHA accumulation capacity thanks to a selective environment and specific operational conditions; (3) a third step for maximizing the cell PHAs content (Fig. 3a). This three-stage process is generally carried out in a fed-batch reactor by feeding the system with a substrate rich in organic carbon and depleted in nitrogen. Regarding microbial selection and production procedures, butyrate has been reported as a preferential substrate compared to acetate and propionate, and acetate seems to be preferred to lactate [52, 63]. VFAs preferences vary depending on metabolic pathways used and also on the microbial composition in the MMC [64, 65]. These results open up the possibility of selecting MMC with specific community members able to reach high performances in PHAs production. Besides, for ADF processes, it has been reported that the outcoming product from glucose, cellulose, starch and other carbohydrates, is mainly glycogen and not PHAs. Moreover, in complex feedstocks, a fraction of the Chemical Oxygen Demand may not be used to produce PHAs but rather other microbial biomasses which would dilute the PHAs and increase the cost of recovering the product. These results indicate that it is crucial to optimize acidogenic fermentation in order to obtain a high content of PHAs in the entire biomass [66]. Alternatively, strategies to restrict the development of side populations should be developed. In that way, settling implemented after the feast phase was used to promote the removal of carbon sources (proteins, carbohydrates) of the complex substrate that did not contribute to PHA production and the washout of non-storing bacteria, which favoured the culture enrichment [67]. The overall function robustness (such as PHA production performance and physical properties) is a key aspect for the success of a MMC at the industrial scale. The relationship between microbial community succession and overall system function should be known for various operating conditions and for various substrate mixtures. Interestingly, using three similar reactors fed with different mixtures of VFAs, Huang et al. recently showed that the selective pressure of the F/F process was strong enough to maintain the PHA accumulation function stable against time and independent of the microbial community compositional stability [68].

The possibility to control polymer composition by using specific substrates has already been proven. For example, the addition of propionate or valerate is fundamental in order to obtain the copolymer poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) with high 3-hydroxyvalerate (HV) fraction. The use of valerate-rich hydrolysate also leads to an increase in the 3HV fraction, but also possibly an increase of 3-hydroxy-2-methylvalerate (3H2MV) within the polymer [69]. Interestingly, when a valerate-rich hydrolysate was used as substrate, the fraction of *mcl*-monomers increased. Moreover, the enrichment of PHA-storing organisms showed less dependence to the F/F ratio, possibly because of the selectivity of valerate. Some studies reported the production of mcl-PHAs in addition to scl-PHAs. However, to our knowledge, no study has yet been dedicated to the selection of microbial communities able to produce only mcl-PHAs. Therefore, there is a field of research still to develop, which aim would be to orientate microbial selection towards microorganisms able produce PHAs of greater industrial interest.

The addition of a nutrient limitation during the feast period has recently been envisaged in order to boost the enrichment dynamic. This is also relevant because wastewater and organic wastes often have a low nitrogen or phosphorus content. However, Jonhsson et al. showed that a global limitation in nitrogen favours microorganisms presenting high N-specific uptake rates, which are different from populations selected under carbon limitation [70]. The consequence is an unstable selection of PHA-producers. Similar results have been obtained when considering P-growth limitation in SBR [71]. Nevertheless, when optimized, the control of nitrogen supply can bring a substantial improvement to the enrichment of PHAs-producers. This is the case when the nitrogen limitation was applied only during the feast phase [72]. Under this condition, and because the cellular plasticity for nitrogen is low, only microorganisms capable of storing carbon are selected. The growth of side populations is hence no longer possible. The authors thus observed a more efficient selection, a better storing performance of PHAs in the accumulation assays and a significant increase in product productivity (increasing by two-fold compared to the control: 6.09 gPHA/L/day versus 2.55 gPHA/L/day) [72]. This result was confirmed by other studies [73]. Thanks to this nitrogen feeding strategy, high OLR can be applied while maintaining a stable enrichment [72, 74]. An additional study has recently shown that a MMC selected in an ADF process fed by crude glycerol or synthetic substrates was able to convert 1,3-Propanediol (1,3-PDO) into PHB, but only under nitrogen limiting conditions [75]. This result has highlighted the relevance of applying a nitrogen limitation for PHA accumulation by using this type of substrate, which is not VFAs. This may also be the case for other substrates which are currently reported as unsuitable for PHA production.

Our understanding of selection drivers is still in progress. Very recently, the influence of temperature (from 20 to 40 °C) on the mechanism of microbial selection was assessed by using a F/F system fed with acetate [76]. The objective was to understand the relation between an environmental parameter such as temperature and the functional development of a microbial community. The obtained results were instructive because they were highly contrasted: depending on the temperature, three distinct strategies for

using carbon were identified: (i) direct growth on acetate, (ii) storage of acetate as PHB and subsequent growth on it (the conventional mechanism), and (iii) growth on the substrate combined with subsequent decay and cryptic growth. In the latter case, the ability to produce PHB in a subsequent batch was observed, but was not involved in the competition during the selection. Therefore, the temperature (between 20 and 40 °C) strongly influences the selected microbial community as well as the usage mode of the substrate. Further analyses are required to clearly understand the exact role of temperature in the functional response of microbial communities. Since this study used acetate as unique carbon source, this temperature-dependent selection should also be verified for more complex substrates.

Anoxic/Anaerobic Feast and Famine Regime

The F/F regime has also been carried out by applying alternatively aerobic, anoxic and anaerobic conditions (Fig. 3a) [77] [78]. Storage of PHAs has been observed in enhanced biological phosphorus removal systems or nitrification-denitrification systems (Fig. 3b). The main groups of bacteria selected are Polyphosphate-Accumulating Organisms (PAOs) and Glycogen-Accumulating Organisms (GAOs) whose metabolism has been extensively described [79]. PAOs are defined as any organism able to aerobically use stored polyphosphate in order to energize anaerobic carbon uptake. The GAOs phenotype is comparable to PAOs phenotype, except that instead of polyphosphate, glycogen is used as an energy source under anaerobic conditions. Both populations store PHAs during the anaerobic phase and grow on it in the aerobic or anoxic phase. Again, the selection of PHA-accumulating bacteria is highly dependent on the presence of VFAs and fermentable carbon because of their high affinity for these types of substrates. Therefore, particular attention must be paid on the nature of carbon substrates present in the feed. The hydraulic retention time applied for the feast anaerobic selector should be adjusted for reaching an almost complete uptake of the incoming VFAs (and fermentable COD). In the subsequent reactors, the applied SRT should also allow stored PHAs to be consumed. By developing this strategy, Bengtsson et al. [80] obtained a biomass with a PHA content of 49% (w/w of VSS) and a treatment performance in line with European standards. A relatively low SRT (2 days) was applied to obtain a higher biomass yield and a higher active fraction in the biomass which led to a better PHA productivity and higher PHA content. As a consequence, plastic carriers were used to make the nitrification more reliable. Outcomes of this work clearly showed that production of PHAs may be readily integrated with carbon and nitrogen removal from municipal wastewater and can hence take part in the resource recovery approach. Tuning the nature of PHAs has been shown possible. For instance, in the study of Bengtsson et al. [80], the produced PHAs contained both Hydroxybutyrate (HB) and Hydroxyvalerate (HV) monomers with a HV content between 53 and 69 mol%. One drawback was that the non-PHAs biomass diluted the PHA content in the final harvested biomass. Besides, Bengtsson et al. investigated the feasibility of using fermented wastewater from a paper mill to enrich the biomass with GAOs able to produce PHAs [53]. In this case, since the wastewater was poor in nitrogen, an anaerobic/aerobic sequence was suggested to enrich the community in GAOs. The PHA yield reached 0.62 Cmol PHAs/Cmol COD and considering the whole transformation, including acidogenic fermentation, 1 kg COD of wastewater influent resulted in 0.10 kg of PHAs. Applying the F/F regime in other configurations have also been investigated. For example, studying the treatment of sugar beet wastewater involved an anoxic feast period, followed by an aerobic famine phase in order to integrate PHA production as well as nitrogen removal (Fig. 3d) [81]. An original sequence was suggested with, this time, the alternation between an aerobic feast period and an anoxic famine period in order to select PHAs storing biomass while performing nitrogen removal via nitrite, and this by using an anaerobic digestate (Fig. 3f) [82]. Although these studies did not analyse in detail the selection efficiency, the F/F regime was nevertheless shown as a flexible strategy for selecting PHAs storing communities.

Phototrophic mixed cultures have recently been selected for their capacity to produce PHAs (Fig. 3a) [83]. The idea was to use light instead of oxygen in order to produce the energy necessary for cell metabolism. A SBR culture mode was carried out under 24 h cycles at a SRT (equal to HRT) of 3 days. The reactor run anaerobically, under a continuous light provided by a halogen lamp with an intensity of 127 W/m^2 (1.8 W/L of broth) and fed with acetate according to a C:N:P molar ratio of 124:20:1. Under these conditions, a culture was enriched in purple phototrophic bacteria able to accumulate PHAs. Only a low content of algae was observed, which limited oxygen production and promoted the reduced power re-oxidation with the growth of PHB. However, the PHB content in this culture remained low, with less than 5% of the dry weight biomass, mainly because of the light intensity limitation. When the light intensity was increased to 227 W/m^2 (6.1 W/L of broth), the PHB content also increased and reached 60% of the dry weight biomass. It is important to notice that phosphate depletion was observed early in the cycle. Therefore, the culture was probably P-limited during most of the time. Cavaille et al. proved that phosphorus limitation promotes the selection of PHAs-accumulating organisms [45]. Consequently, the selection factor, in the above-mentioned experiments, is likely to be the P-growth limitation. Interestingly, in the applied operational conditions and because of the SBR mode, selected

microorganisms showed a high phosphate consumption capacity (high q_{S_Phos}) and an ability to store phosphate. The selection strategy was still based on the substrate consumption rate coupled with a storage capacity, but for a compound other than the carbon source ($q_{S\_Phos\_max}$ in that case).

Selection in Chemostat Mode

Cavaille et al. obtained a reliable selection of PHB producers by imposing a dual carbon and phosphorus limitation on a continuous open culture, fed with different mixtures of VFAs (Fig. 3c) [45]. The essential role of phosphorus limitation, both on the enrichment of PHA-producers and on the storage of PHB, has been proven over a wide experimental field in terms of dilution rate and degree of phosphorus limitation. Low concentrations of phosphorus in the reactor select communities with very high affinity for phosphorus compounds. Interestingly, these communities have always shown a capacity to accumulate PHA. This trend was not only shown when VFAs were used as substrate, but also with a mixture of glucose and fructose. This is a rather interesting feature, because in an ADF mode, sugars preferentially led to the production of glycogen rather than PHAs. Moreover, if the phosphorus limitation is switched to a nitrogen limitation, then the selected bacteria lost the ability to produce PHAs and instead produced exopolysaccharides, and this even with VFAs as a substrate. The role of a phosphorus limitation on the selection of PHAs producers is therefore straightforward. However, this mechanism is not yet understood. The current assumption could be a genetic link between genes responsible for the high phosphate affinity and the capacity to store PHAs, which could have been established to give an advantage when grown in P-poor/C-rich environments. In order to prove this association, the genome of the selected bacteria grown under P-limitation should be analysed.

The PHA specific production rate was found to be dependent on the intracellular concentration of phosphorus (P_{in}). The selected cells are able to expose a great plasticity regarding their P_{in} while preserving a maximum cell growth. Cavaillé et al. studied this plasticity during experiments in a chemostat, and showed that intensity of the degree of limitation clearly played an essential role distributing the carbonaceous substrate between PHBs and the catalytic biomass [45]. Phosphorus requirements varied according to the dilution rate (D) and therefore to the μ of the microorganism. At a given D, a domain of P_{in} can be defined where the cells are both C&P limited (i.e. the DNL growth zone). Within this domain, the P_{in} value governs the redirection of carbon towards PHB storage and determines the PHA content of the cells.

From a microbiological point of view, the species which are selected and those who become dominant strongly depend on the dilution rate and on the nature of the substrate. When applying high dilution rates ($D > 0.15\ h^{-1}$), a highly dominant population is selected (up to 80% of operational taxonomic unit on simple substrate). More diversity is observed for lower D values. Selection under high D values favours the growth of strains exposing high μ_{max}. The presence of butyrate in the feed enhances the growth of PHB-producers with high q_{PHBmax}. When using acetate as a substrate, *Malikia* sp. is selected under an intermediate dilution rate ($0.21\ h^{-1}$) while with higher D values, it is the genus *Acinetobacter* which dominates. However, if butyrate is added to the feed *Acinetobacter* sp. is replaced by *Malikia*. Therefore, it was concluded that besides the role of P-limitation, bacteria have specific affinities for a given substrate which determine the outcome of the competition. Excluding bacteria of the genus *Acinetobacter*, all the other dominant bacterial genus which were selected under a double limitation of C&P, belong to the *Comamonadaceae* family (class of *β-proteobacteria*).

Maximum production capacities of PHA were determined using batch tests, inoculated with the biomass provided from the chemostat cultures. All bacteria exposed the capacity to accumulate PHAs. However, kinetic parameters diverged from one population to another. Therefore, it is important to rationally choose the operational conditions applied to the continuous process. An operating zone for producing PHB (the DNL growth zone) was determined for the continuous process. The PHB content reached 80% (w/w cell DW) with a $D = 0.023\ h^{-1}$, but this content decreased with the D value and became marginal at $D = 0.3\ h^{-1}$. This decrease is due to the competition between growth and PHB storage, since the selected cell has to maintain a μ equal to the imposed D in the chemostat culture. Therefore, at low D values, a high substrate concentration must be fed in order to obtain a high PHB productivity in a one stage reactor. At high D values, a more diluted substrate can be used, but a two-stage process must thus be applied in order to obtain a high PHB productivity while maintaining a high PHB content. The content of PHAs reached 75% with the possibility to tune the contents of HV and HB and produce a copolymer PHBV which is able to confer a higher elasticity. The maximum productivity may be tuned by varying the concentration of the feeding substrate and the dilution rate. Nevertheless, the productivity will be limited by the oxygen transfer rate which must be determined as a trade-off between productivity, cost for oxygen transfer and environmental impact due to the energy consumption. At a k_La of $100\ h^{-1}$, a productivity around 0.6 gPHA/L/h was achieved. Additionally, the successful production of PHA in continuous reactors requires to remove suspended solids from the feed in order to reduce the dilution of PHA with side biomass.

Direct Enrichment from Activated Sludge in Fed-Batch

Waste Activated Sludge (WAS) contains a significant proportion of microorganisms able to accumulate PHAs. This is explained by the significant proportion of VFAs and fermentable COD in municipal wastewater and by the operating mode which involves alternating environmental conditions. As already mentioned, the storage of organic carbon represents a survival mechanism for microorganisms experiencing such dynamics. Therefore, it was suggested to use WAS as an inoculum for the direct production of PHAs. Mengmeng et al. [84] carried out sequencing feeding of activated sludge with a sludge fermentative broth where nitrogen and phosphorus were partially removed. WAS showed the ability to directly store a large amount of PHA (up to 56.5% of dry cell) under a nitrogen limitation without acclimation. Aerobic fed-batch conditions, using acetic acid as substrate, have also been investigated (Fig. 3e) [85, 86]. Results showed that PHB production was induced by phosphorus limitation. When investigating WAS from different origins, the PHB content reached 70% gCOD_PHB/g COD_biomass, with a PHB yield of 0.35 gCOD_PHB/gCOD_S. The extent of phosphorus limitation had a direct impact on the amount of produced PHB, because this limitation determined the ratio between the specific PHB production rate and μ. However, a residual phosphorus level had to be maintained in order to allow the increase in PHB concentration in the culture (i.e. need more cells to store more PHAs). Competition according to the levels of phosphorus during the fed-batch culture was observed. Results showed that this competition enhanced the growth of PHB-accumulating organisms as it was observed in the chemostat study run by the same authors. Pyrosequencing of 16S rRNA transcripts revealed changes in the active bacteria of the microbial community against time. Therefore, the Fed-batch operational mode, with low levels of residual phosphorus, favoured the enrichment of microorganisms having a strong affinity for phosphorus as well as those able to store PHAs.

The productivity of this system depends on the concentration of WAS used in the reactor, and thus on the chosen oxygen transfer rate. The performance of the PHAs production system is highly sensitive to the proportion of PHA-accumulating organisms initially found in the WAS. This proportion results from the operational conditions applied in the wastewater treatment plant and from the wastewater characteristics itself (potential VFAs fraction of the COD). The mode of selection prevailing in the wastewater treatment plant is related to the F/F regime as it has been described earlier in this review. Any improvement of this preliminary selection would benefit to the direct production system of PHA.

Cultivation of Extremophiles

A review on that topic has been recently published [87] and only an example of the effect of substrate on the microbial selection is described hereafter. Halophiles such as *Haloferax mediterranei* accumulates high amounts of PHA in high salinity media which allows the cultivation to be operated in open continuous systems, and to use minimal fresh water [47]. Moreover, *H. mediterranei* is able to assimilate a great variety of substrates. PHA storage capacities of this species in an Aerobic Dynamic Feeding (ADF) process were compared with three different substrates (acetate, glucose and starch). Results showed that acetate and glucose gave the highest PHAs content, with 64.7% and 60.5% DW respectively [88]. In contrast, the starch-enriched MMC indicated a low q_s, which proved that the bioconversion was limited (PHA content of 27.3% DW). This suggests that starch substrate might not have selected the right microbial community, and this probably because hydrolysis was the limiting step. Techno-economic assessments have recently shown the interesting potential of Archaea for PHAs production [47].

Comparison Between Pure Cultures and MMC for PHAs Production

Comparison between pure cultures and MMC is required for choosing the best culture mode. However, this comparison is not well documented in the literature. In term of performances of pure cultures PHA concentrations of greater than 80 g/L with productivities of greater than 2 g PHA/L/h can be routinely obtained by fed-batch cultivation of several bacteria on purified substrates such as glucose [6, 89] or VFAs [90]. A substantial decrease in the cost of PHAs can be achieved if raw organic residues are used such as cheese whey or glycerol [6]. However, the performances obtained in this case decrease. For example, on waste glycerol, 68.8 g dry weight/1 with a P(3HB) accumulation of 38% resulting in a final productivity of 0.84 gPHB/L/h was obtained on waste glycerol [91]. Depending on the substrate nutrient composition, selection of a growth-associated or a non-growth-associated PHA producer must hence be performed. Though a comparison based on the volumetric productivity is not easy because of differences in production steps exist, similar performances have been reported for MMC. For example, productivities of up to 1.2 g PHA/L h, combined with high PHA yields, up to 0.8 Cmol PHA/Cmol S, have already been attained from MMCs using synthetic substrates [92]. Similarly to pure cultures, lower performances were obtained on pilot scale using real wastes as substrates [93]. In this latter study, increasing the VFA fraction after the organic substrate fermentation and minimization of acid and base consumption for pH control were identified as major bottlenecks for improvement of the process. Further data are

necessary to get a comprehensive comparison between pure cultures and MMC for PHAs production.

Strategies for Polysaccharides or EPS Production

As mentioned previously, the current industrial production of microbial polysaccharides is largely dominated by the use of pure strains cultivated in a sterile environment. However, over the past twenty years, the use of open MMC for producing polysaccharides has emerged. Studies on this topic mainly target the production of EPS, which may contain polymers with interesting features such as gelling or flocculating properties but with a complex and often unknown composition.

Production of Exopolymeric Substances with Flocculant Properties

Capsular EPS are able to present excellent flocculating properties and therefore can be a suitable alternative to synthetic polymers, such as polyacrylamides [94]. A specific literature review covering the production of microbial biopolymers with flocculating properties highlighted that, to date, EPS have either been extracted from WAS treating different types of wastewater or been produced by pure cultures, sometimes isolated from WAS [35]. However, recently, a MMC showing excellent properties of anionic flocculant (see Fig. 1c) has been successfully selected in a continuous reactor fed with a mixture of glucose and fructose [95]. In order to determine the optimal selection conditions, two steps were required and are briefly described here. In a first step, nitrogen limitation applied to a CSTR inevitably led to a microbial community dominated by *Sphaerotilus natans*. Logically, the filamentous form of *S. natans* confers to this strain a great affinity for the limiting substrate. *S. natans* outcompeted the other microorganisms, which confirms the prevalence of K-strategists over µ-strategists in the chemostat. Unfortunately, the EPS of *S. natans* did not show good flocculant properties. However, during the transient regime, before the dominant *S. natans* appeared, a production of biomass with good flocculant properties was observed. This biomass showed a lower settling velocity compared to *S. natans*. A SBR mode, equipped with a decantation phase with enough time and in a cyclic manner, selected a dominant taxon which gave the biomass an excellent flocculating property. Indeed, a better flocculating efficiency (turbidity and COD removal), compared to polyacrylamide, was obtained with wastewater and with a mineral suspension used as flocculation medium. In a third step, the selection strategy was also tested using a complex substrate, i.e. a hydrolysate obtained by enzymatic hydrolysis of biomass from refuse screening of a municipal wastewater plant. This hydrolysate contained 50% of the COD in the form of glucose (and a small amount of xylose) and 50% of other compounds. Although some microorganisms showed comparable capsules compared to those obtained after microbial selection with refined sugars, the flocculation performance was not as good and varied with time. Based on this outcome, the reactor was firstly fed with refined sugar to select the desired population, and then fed with the hydrolysate. Interestingly, the excellent flocculating properties were recovered and remained constant one month after switching the substrate, which represented more than 85 SRTs for the flocculating biomass.

Still with the aim to produce a flocculant from EPS, a complementary approach to the previously described method was developed by Ajao et al. [96]. The authors of this study developed a MMC exposing interesting flocculating properties and this while treating fresh or saline synthetic wastewaters which contained glycerol and ethanol in order to simulate wastewater from biodiesel and (bio) ethanol industries. Unfortunately, the reason for the EPS production and the nature of the selection pressure applied were not described. Though EPS could be produced by pure culture, the results exposed here show that EPS having excellent flocculating properties can be continuously produced from wastes when appropriate selection strategies are applied.

Production of Exopolymeric Substances from Aerobic Granules

According to De Kreuk et al. "Granules making up aerobic granular activated sludge are to be understood as aggregates of microbial origin, which do not coagulate under reduced hydrodynamic shear, and which settle significantly faster than activated sludge flocs" [97]. Aerobic granular sludge (AGS) can be seen as a special kind of biofilm with a spherical shape and developed without the addition of a carrier material. A part of the EPS produced by AGS has hydrogel properties which are responsible for the granule structure (see Fig. 1b) [98]. EPS are found in higher concentrations in AGS (up to 25% of the dry mass of the biomass volatile suspended solid) compared to conventional activated sludge flocs, and their properties are more attractive [99]. The very first studies performed on the characterization of AGS showed that the structural EPS were mainly composed of alginate-like materials or of a novel heteropolysaccharide named Granulan [100]. Further studies showed that structural EPS was found to be highly complex with a composition and gelling mechanisms which were variable according to their origin.[98]. Besides polysaccharides, proteins were also found in high proportions and may hence play a significant role in the gel formation and gel strength (Fig. 4) [98, 101]. Hyaluronic acid-like and sulphated glycosaminoglycans-like polymers were also found in the AGS extracellular matrix [102].

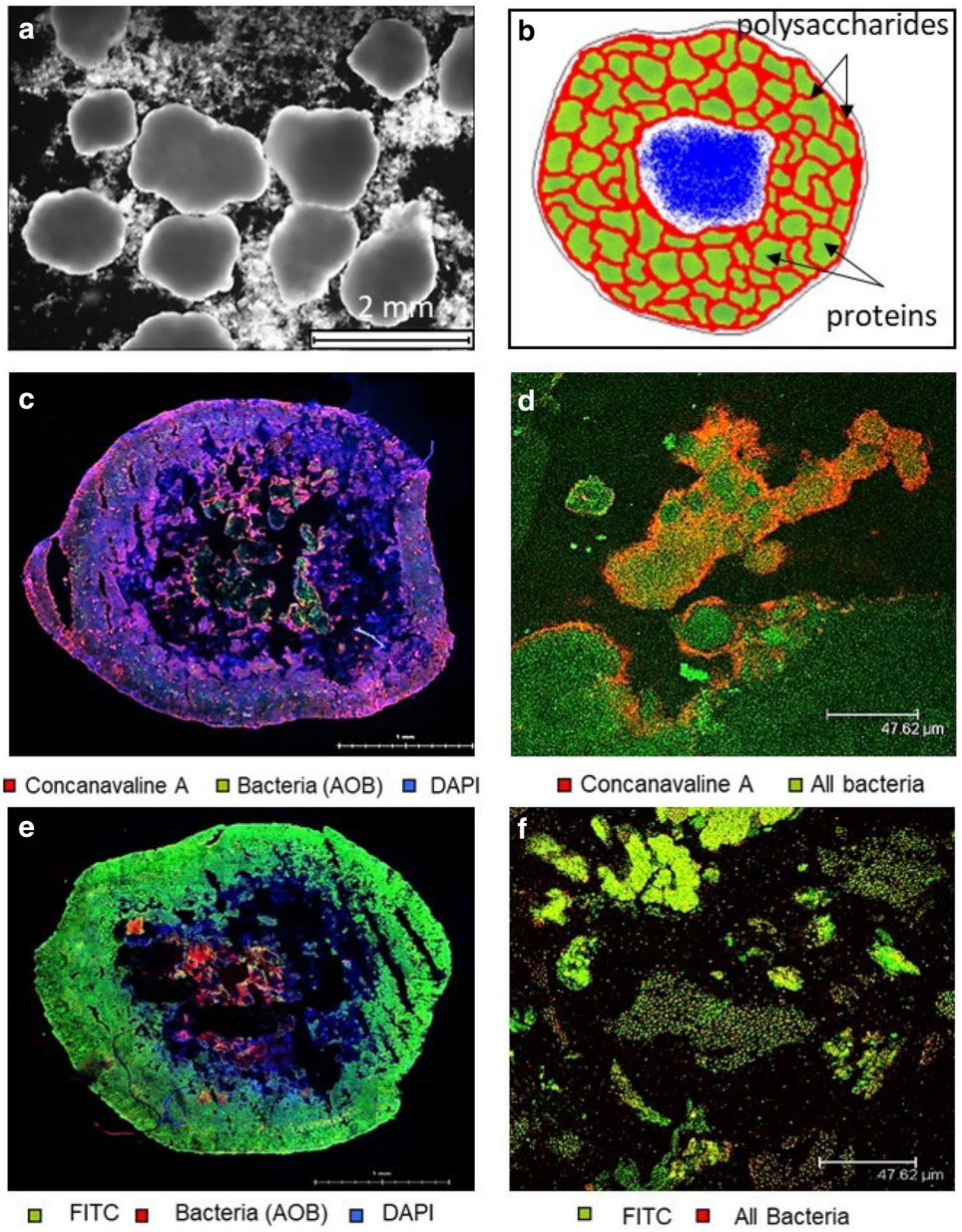

Fig. 4 Aerobic granular sludge (AGS): **a** photo of AGS using phase-contrast optical microscopy; **b** proposed structural model for AGS that includes a protein matrix embedded into a polysaccharide network; **c** and **d** CLSM images of AGS stained with concanavaline A (red) for polysaccharides detection, DAPI (blue), ammonium oxidising bacteria (AOB) (green) (**c**) and all bacteria (green, **d**); **e** and **f** CLSM images of AGS stained with FITC A (green) for protein detection, DAPI (blue), ammonium oxidising bacteria (AOB) (red) (**e**) and all bacteria (red, **f**)

EPS production during wastewater treatment and extraction for industrial applications represents an appealing route that can help to recover resources from wastewater, decrease the cost of sludge treatment and disposal as well as contribute to the implementation of a circular economy. The AGS process was initially described some 20 years ago [103] and there are now around 70 full-scale treatment plants using this technology worldwide. Pronk et al. presented the performances of a large full scale aerobic granular sludge plant treating domestic sewage [104]. The process was run at high granular biomass concentration (> 8 g/L) with SVI5 values of 45 mL/g and showed good performance of COD, N and P removal, with between 58 and 63% less energy consumption compared to an equivalent activated sludge plant. A commercialised product, Kaumera Nereda® Gum, developed by the Royal Haskoning DHV Company is already extracted from granular sludge produced in the first large-scale Kaumera unit in Zutphen (Netherlands) and a second production unit in Epe is in progress [105]. The obtained gum offers several applications such as coating material for slow-release fertilizers and paper, bio-stimulants for agriculture, fire retardant, curing agents for concrete and additives for bio-nanocomposite materials. The challenge is therefore to produce a large amount of EPS exposing the desired quality while treating the wastewater.

The formation of AGS depends on two main factors. (i) The first is the ability to select slow-growing heterotrophic bacteria at the expense of fast-growing bacteria [97]. This

selection can be achieved by implementing a F/F regime, either by integrating only an aerated reaction phase [106] or by applying a sequence between anaerobic/aerobic (anoxic) phases. In the latter condition, the readily-biodegradable carbon sources are consumed under anaerobic conditions and converted into intracellular PHAs. The storage compounds are then used in the aerobic (anoxic) phase for cell growth. The winners outcompete fast growing strict aerobic heterotrophs because they have a strong affinity for VFAs, and a unique capacity to capture soluble carbon in the anaerobic step by using polyphosphate and glycogen as an energy source. In order to maximize the selection pressure, an anaerobic period, long enough to consume the entire fraction of fermentable substrates in the feed, must be imposed. (ii) The second factor relies on a selective removal of biomass according to its settling velocity. This aspect will be discussed later in the paper.

While treating C, N and P pollution from urban wastewater, the cycle alternating anaerobic, anoxic and aerobic (anoxia) conditions promotes the selection of PAOs or GAOs which are slow-growing bacteria, which play an important role in the granulation process, but also the selection of nitrifying bacteria thereby guaranteeing full biological nutrient removal [107]. Granules should be thus seen as a heterogeneous micro reactor optimized for granule stability and pollutant removal. Limitation of oxygen transport within granules creates different ecological niches: aerobic conditions on the outskirts, anoxic and then finally anaerobic conditions when reaching the heart of the granule. Mathematical modelling showed that stable smooth and dense granules as well as good treatment performances are obtained when the aerobic volume within the granule is just sufficient for full nitrification, and when the soluble substrate can be consumed in a large fraction of the granule volume [108]. In the case of wastewater, if some easily biodegradable substrates are not consumed anaerobically and therefore promote the development of aerobic fast-growing heterotrophs negative impact on the granule structure and stability is observed. This case has been studied by Pronk et al. [109], who tested the granulation potential in the presence of carbon compounds frequently found in industrial wastewaters i.e. acetate, methanol, butanol, propanol, propionaldehyde and valeraldehyde. Different behaviours were observed. Indeed, the methanol was converted to methane but acetate was preferentially assimilated by PAOs and GAOs. Stable granules were hence obtained. Some conclusions were drawn: the capture of readily biodegradable substrate in anaerobic conditions within the whole granule volume leads to stable and smooth granules, and bacteria able to transform methanol into PHAs could not be selected. Butanol and propanol were degraded only in aerobic conditions but their absorption and diffusion throughout the granules during the anaerobic phase prevented the deterioration of the granule stability. Propionaldehyde and valeraldehyde were transformed into their corresponding carboxylic acids and alcohols which were absorbed and converted into storage polymers, respectively. Another study compared AGS produced with synthetic and with real domestic wastewater [110]. Results concluded that non diffusible substrates (colloidal and particulate organic matter) led to high proportions of flocs (20 to 40% of the total suspended solids), together with small granules, and to the promotion of fast-growing heterotrophic bacteria. PAOs and GAOs were hence outcompeted by fermentative bacteria. These studies, undertaken to evaluate the effect of substrates, confirmed the importance of preventing the development of fast-growing microorganisms in order to achieve a stable granulation, and this regardless of the substrate.

From a technical point of view, a set of well-balanced operational conditions must be implemented to obtain the local environmental conditions which are favourable for each targeted microbial population. A list of the most important factors for selecting AGS has been reviewed by Winkler et al. [37] and reveals similar parameters to those described in Fig. 2. This includes reactor design parameters, such as shear stress and hydrodynamics (best design will be a high H/D ratio, up-flow air velocities > 1.2 cm/s), SRT of flocs and granules and settling time (as low as 1 min), OLR, volumetric exchange ratio, aeration intensity, inoculum nature, type of substrate, pH and temperature or again the presence of divalent cations. In a SBR with sequential settling, AGS are enriched by introducing a long SRT for granules and a short SRT for flocs. This selection is achieved by applying a short settling time which preferentially removed the flocs with the outcoming supernatant. Maintaining the SRT at around 20 days by wasting led to effective phosphorus removal and stable overall bacterial community compositions [111]. Experiences were conducted by testing different values of OLR, ranging from 2 to 22.5 kg COD/m^3/day. Stronger hydraulic selection pressure under shorter cycle times accelerates the granulation process, however, beyond a maximum OLR, granulation appeared unstable on the long term [106]. Up-flow column reactors with large height:diameter ratios are preferred to CSTR because they ensure a more homogenous shear stress. Feeding strategies, where the substrate is fed to granules in high concentrations in order to create substrate gradients within the granule, can improve the quality of granulation. Moreover, the location chosen for discharging the effluent biomass from the reactor plays a role in microbial competition. For instance, a discharge exclusively from the top of the sludge bed allowed to enrich in PAOs instead of GAOs at high temperatures [112]. The cycle distribution was also a parameter to optimize affecting the selection of the functional populations such as PAOs and nitrifiers and denitrifiers. Recently, de Sousa Rollemberg et al. have improved the performances

of denitrification and phosphorus removal by increasing the duration of the anaerobic phase [113]. They found an increase of the SRT likely due to the enrichment of a biomass with better settleability that might be polymer-accumulating bacteria [114, 115]. The best results in terms of nutrient removal performances and AGS stability were achieved in a reactor with short anoxic phase (10% of the total cycle) and medium aerobic phase (55% of the total cycle) [116]. Finally, stable AGS can be obtained even at low oxygen concentrations which reduces costs related to aeration.

AGS technology has obviously been optimized to achieve the required treatment performance levels but has still not been improved for the production of biopolymers from EPS. AGS reactor stability requires the application of long SRTs for the formation of granules and must not exceed a certain value of OLR. These operational limits will have to be pushed back in order to increase the productivity of hydrogel biopolymers.

A key aspect in understanding AGS systems, regarding biopolymer production, is characterizing the microbial community composition and understanding how it influences the nature and quantity of biopolymers. A conceptual model of the bacterial ecosystem of AGS was developed based on physiological traits [117]. AGS can be compared to micro reactors, where various ecological niches co-exist and are colonized by specific microbes. For example, working on biological dephosphatation, Lemaire et al. showed that *Accumulibacter* sp. was mainly distributed in the outermost region of the granules, while the *Competibacter* sp. clustered in the core of granules [118]. However, it seems that in the wastewater treatment sector using the AGS technology, several bacterial families are frequently identified such as *Rhodocyclaceae*, *Xanthomonadaceae*, *Comamonadaceae*, and *Rhodobacteraceae* [119]. These bacteria certainly share similar functions (like denitrification) but also probably take part in the structure of the granule via EPS production [117]. Indeed, certain genus such as *Zoogleae* sp., *Thauera* Sp., *Pseudomonas*, *Flavobacterium*, *Chryseobacterium*, *Stenotrophomonas*, and *Acinetobacter*, which are regularly found in AGS can produce consistent amounts of EPS. In fact, many microorganisms are able to produce EPS and it is hence not surprising to observe that structural EPS can vary depending on the origin of the granule [100, 120]. Some polysaccharides are certainly strain specific, but due to their main hydrogel property, such compounds certainly share similar roles in structuring the granule. Since alginate-like polysaccharides have been identified in granules, the microbial origin of these compounds is questioned. Indeed, it is recognised that different types of alginate can be naturally secreted by a range of Pseudomonas and *Azotobacter* species. However, it has not been clearly demonstrated that this capacity is spread to other types of bacteria. Therefore, in the case of AGS, whether the produced EPS have gelling properties resulting from a majority of inherent microorganisms, or only from specific species in the granules, is not yet elucidated. Seviour et al. attempted to address this question by performing specific microbial enrichment tests with different carbon sources [100]. Results from this study showed that the synthesis of granulan occurred in granules under conditions which were favourable for the enrichment of *Competibacter* and did not occur in granules without *Competibacter*. This experience concluded that granulan production was strain-dependent. Besides, adding Mn^{2+} ions, which interfere with the cyclic-di-diguanylate, a compound involved in cell–cell interactions, Wan et al. observed a disintegration of AGS and a wash out of typical polysaccharide producers, such as *Acinetobacter* sp., *Thauera* sp., and *Bdellovibrio* sp. [121]. Some populations are hence apparently strongly involved in the production of EPS, thereby playing a key role in the granule structure. These experiences open up promising research fields aiming at how to control the microbiome of granules in order to improve the quality and quantity of EPS.

Comparison Between Pure Cultures and MMC for Polysaccharide Production

The production at the industrial scale of a few polysaccharides by pure cultures of selected microorganisms is already well established. Great effort is put into the optimization of the microorganism and culture conditions to achieve higher yields and productivity and to improve material properties. Besides, new polysaccharides exhibiting superior material properties targeting medical and industrial applications are investigated among the high diversity of polymers constituting the EPS [30]. The naturally provided EPS portfolio seems to be still massively underexplored. MMC can provide a field for discovering new biopolymers that can be produced from wastes and effluents as it has been demonstrated in the previous part of this section. As shown, a major challenge is to understand how to select and strongly enriched the microbial species able to produce EPS with interesting properties. AGS studies show we're on the right track.

Strategies for Microbial Proteins Production

In the middle of the last century, a first selection strategy adopted for MP production by using open MMC was based on the use of a single carbon substrate such as methane or methanol. For these substrates, high metabolic specificity allowed to cultivate specialist bacteria without requiring any sterilisation [17]. The corresponding MMC was found more stable than pure cultures and this was explained by the fact that, during methane catabolism, methanol which is an intermediary molecule, accumulated. Methanol inhibits methane-oxidizing bacteria if they are grown in pure

culture. This inhibition is however avoided in MMC. The natural development of *Hyphomicrobium* sp., which shows a high affinity for methanol, enables to scavenge its concentration down to a threshold level lifting the inhibition of methane oxidation. Heterotrophs are also encountered in the MMC certainly because they degrade various cell components. Although neglected for many years, MP production by using methane provided from anaerobic digestion is currently regaining interest and MMC was chosen for this conversion [122]. Besides, autotrophic hydrogen-oxidizing bacteria have already been proposed to exploit hydrogen gas obtained from water electrolysis produced in excess from wind and solar energy, or from biomass gasification and nitrogen from wastewaters [123]. A chemostat cultivation mode, carried out at a rather high dilution rate (0.1 h^{-1}), led to a microbial community strongly dominated by *Sulfuricurvum* sp. (more than 96% of OTU) although the medium did not contain any reduced sulphur. An aerobic metabolism was hence assumed. The performances achieved with this process, i.e. a high productivity reaching 0.41 kg biomass/m^3 h, a protein content of 71% of the cell dry weight and a yield of 0.29 g cell/gCOD_H_2 were comparable to those obtained with pure cultures.

MMC have also been applied successfully to other feedstocks in order to get a substantial valorisation of both their organic matter and nutrient contents. A MMC revealing a high protein content (43%) has been produced by using waste liquor from sulphite pulping and the obtained MPs were then applied in animal feed [124]. Acclimatization to the feedstock was the only requirement. More recently, production of MPs by phototrophic and photosynthetic microorganisms was proposed and compared [125]. These microbes achieved biomass yields close to unity when supplied with electron equivalents from light and reached high protein contents, up to 60% of the dry weight [126, 127]. The high growth yield and high protein content are particularly important when the aim is to remove nitrogen and phosphorus from wastewater containing high N/COD and P/COD levels, such as those found in domestic wastewaters. Nutrients could hence be recovered in the biomass instead of being oxidised. Microalgae utilizes preferentially photoautotrophic growth which is rather slow. This explains why microalgae are mainly produced in open high rate algal ponds, so the cost for the biomass product can be acceptable. Symbiotic interactions between these phototrophic organisms and heterotrophic bacteria can reveal interesting functions such as the simultaneous removal of both organics and nutrients from effluents. Purple bacteria are phototrophic anoxygenic microbes, also named Phototrophic purple bacteria (PPB) or purple non-sulphur bacteria, and are found in many natural habitats (fresh and salt water and soil) including wastewaters. PPB can be readily selected by exposing the biomass to infra-red (IR) radiations, starting with the inoculum obtained from different origins. These microbes have the ability to use different metabolic modes such as the phototrophic pathway based on H_2 as electron donor, the aerobic chemoheterotrophic pathway which does not require light and photoheterotrophic metabolism using IR light as an energy source as well as a variety of simple substrates like VFAs and sugars as electron donors and C-sources [126]. PPB are also able to accumulate intracellular inorganic polyphosphate and perform denitrification, which are both interesting features in the wastewater treatment sector and are not inhibited by O_2 production. One key aspect for implementing phototrophic culture, is the optimisation of light delivery to the bacterial cell, which requires to consider the hydrodynamics, light transfer as well as cell characteristics and needs. Thanks to anaerobic conditions and infrared irradiation, Hülsen et al. reported a rapid (2–3 days) enrichment within batch reactors of phototrophic purple bacteria (PPB) obtained from primary settled municipal wastewater [126]. Algae and cyanobacteria were outcompeted because they are not able to use the light with wave lengths above 750 nm. The selected PPB were identified as part of the order of *Rhodobacterales*. Following this first study, the selection strategy was tested in continuous reactors for treating agri-industrial wastewaters (poultry, red meat, pork, dairy and sugar wastewaters). By applying an OLR of 4 kgCOD/m^3/day and IR light irradiated at 18 W/m^2, removal efficiencies reached 90% of the COD, 90% of the total nitrogen and 45% of the total phosphorus, with a final production of 190 kg of crude protein per tonne of COD influent and an energy consumption of 7.0 kWh/ton of protein (dry weight). The constant dominance of PPB species in the system through time has been rapidly achieved [125], but a syntrophic community was necessary to obtain good treatment performances. For instance, acid-producing bacteria produced VFAs which were assimilated by PPB by using light as the energy source. Since PPB have a limited capacity to assimilate amino acids, the presence of *Bacteriodiotes* sp. was needed to hydrolyse proteins and amino acids. Predation has also been observed and should be further studied in order to evaluate the consequences on the selection of microbial communities.

The studies reported in this paper are pioneering in the area and optimization of the selection of PPB in MMC relies mainly on the use of IR light. However, interactions with other communities are crucial to comply with both effluent treatment and MP production objectives. More insights on these interactions are necessary in order to optimise both objectives and maintain a high quality of the MP product. One remaining utmost question related to the direct use of wastes for feed (animals) and feed additives, is the health security aspect. Indeed, the need to prevent the development of pathogens is crucial for developing this new MP production value chain. In terms of future perspectives, the need for optimization will obviously bring more scientific questions

on how to improve the enrichment of a given population or to controlling the growth of undesirable populations.

Conclusion and Outlook

Since ages, we have been inspired by natural systems in order to develop biological transformations which are of interest, and this is what has been done when developing processes based on MMC (i.e. alcoholic, lactic fermentations, antibiotics etc.).. More recently, a new challenge has risen with the need to produce biobased polymers at a low cost. These biopolymers should provide an alternative to some conventional polymers produced from fossil resources but also an alternative to proteins currently produced from agriculture. One great advantage of MMC relies on their capacity to use complex feedstocks and to work in a continuous process without requiring a sterile environment. One main difficulty in implementing MMC for biopolymer production is the need to enrich a microbial population with efficient polymer producers and to control the quality of the final product. As it has been mentioned in this review, microbial enrichment requires a deep understanding of interactions between communities and an advanced knowledge about microbial cultivation methods and microbial metabolisms. The degree of this challenge increases when complex feedstocks are used and when the additional treatment of the waste is targeted. The field of PHAs has played a leading role in the understanding of MMC dedicated to the production of biopolymers. However, scientists were also interested in the use of wastes as a resource for MMC to produce other types of biopolymers such as polysaccharides and proteins. These new fields of research have opened up numerous potential applications requiring a better control of the composition of microbial communities.

The development of processes based on F/F cycles has given way to many original PHAs production systems which are currently being scaled up. Significant progresses have been made in the production of scl-PHAs, with a main focus on PHB or PHBV. Obtaining a wider range of products would be beneficial for the industrial use of these polymers. These issues will raise new scientific questions on how to obtain specific microbial communities and how to control their functions.

Similar F/F selection approaches were implemented to generate granules during wastewater treatment offering the possibility to extract different compounds, of which some have interesting hydrogel properties for many industrial sectors. Here again, the technologies are being developed on an industrial scale. In this area, the complexity of EPS adds up to the microbiological complexity. More than ever, it will be necessary to make the link between selected microbial communities and biopolymer production activities. The key populations involved in the production of EPS, in particular structural EPS, will have to be identified. In addition, the role of these different EPS molecules will have to be better understood if the aim is to boost their production.

Other approaches than the F/F mode have also emerged in order to obtain targeted microbial communities able to produce PHAs or capsular polysaccharides. These new approaches are based on the fact that, in nature, microorganisms develop simultaneously abilities to scavenge nutrients present at very low concentrations and to store carbon in excess, in the form of either extra- or intra- cellular polymers serving as carbon and energy sources. Therefore, using such approaches, a selection under nutrient limitation will enrich the population with biopolymer producers. More research is needed to confirm this hypothesis and demonstrate its usefulness for producing a large diversity of biopolymers. The effect of an increase of microbial diversity, due to the use of different substrates such as complex feedstocks, will also have to be further studied.

MMC have been suggested for protein production by using wastes and wastewaters as a substrate. An acceptable product quality has been claimed by the researchers. However, the control of the microbial community composition will be of paramount importance in order for the product to be accepted. It will therefore be necessary to understand the factors governing the establishment of targeted microorganisms, but especially understand how to guarantee that other unwanted populations do not develop.

In fine, future studies will have to systematically study the relationships between selective conditions, and the resulting composition of microbial communities as well as the functions they develop.

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