Regulating the monomer of polyhydroxyalkanoate from mixed microbial culture: with particular emphasis on substrate composition: A review

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ABSTRACT

Polyhydroxyalkanoate (PHA), produced by mixed microbial cultures (MMCs), is a biodegradable biopolyester that alleviates the global plastic crisis in the nearest future. Even PHA has become a research hotspot, reviews on PHA composition determining thermodynamic and processing properties of PHA products are rare. The primary focus of the evaluation relies on the factors affecting the PHA monomer composition of MMC-PHA production. Besides, since the volatile fatty acids (VFAs) composition of the substrates strongly influences the PHA monomer composition, regulating the ratio of even-carbon to odd-carbon VFAs, PHA, vary properties could be obtained predictably. So oriented acid production process, the first stage process in three-stage MMC-PHA production that can regulate the VFAs composition, is also comprehensively introduced to help understand PHA monomer regulation.

Keywords: Mixed microbial cultures, Monomer composition, Oriented acid production, Polyhydroxyalkanoate (PHA), Volatile fatty acids (VFAs)

1. Introduction

Polyhydroxyalkanoates (PHA), a kind of intracellular polyester that is environmental-friendly and can be degraded naturally, is produced by microorganisms under unbalanced external nutritional conditions (excess carbon source, lack of nutrient elements such as nitrogen and phosphorus) [1]. In a naturalistic setting, PHA is mainly a storage material for carbon sources and energy in organisms, and it is considered to be effective energy storage means for microorganisms to resist the environmental impact. After extraction and industrial processing, PHA products have similar material properties to traditional petrochemical plastics and can be used as biodegradable packaging materials, tissue engineering materials, medical materials, etc. [2]. Many microorganisms in nature contain PHA hydrolases, which can hydrolyze PHA into oligomers and monomers and use these products as the basis for bacterial growth. In recent years, with environmental concerns, PHA has attracted significant attention due to its complete biodegradability. At the same time, PHA is also widely considered a potential replacement for traditional petrochemical plastics, and it is hoped to alle-

viate the current pollution crisis of plastics [3].

Currently, there are two main methods to produce PHA from bacteria. One is the pure culture mode, the main mode of commercial and industrial-scale production. Single strain or engineering bacteria with good PHA synthesis ability is used to produce PHA. However, the pure culture mode needs strict aseptic environment conditions and high-purity carbon source substrate, making the production cost of PHA products still at a high level. The high production cost has dramatically reduced the economic competitiveness of PHA compared with traditional petrochemical plastics and limits the wide application of PHA products [1]. The other method is using mixed microbial communities (MMCs) as the biological carrier for PHA synthesis. In the pure culture mode, fermentation facilities, operating environment and substrates need to be sterilized strictly to prevent other hybrid bacteria, which would destroy the PHA production capacity, from adulterating into pure cultured microorganisms. While compared with the pure culture mode, the MMC-PHA production mode has richer microbial diversity to resist the environmental impact, so PHA synthesis can be carried out in a more open environment. Detailly, the MMC-PHA

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production process does not require strict substrate sterilization or an aseptic operation environment, which avoids the high energy cost [1]. Besides, low value and easily available organic wastes, such as volatile fatty acids (VFAs), crude glycerol, etc., were usually used as carbon source substrates in MMC-PHA production. Hence MMC-PHA production mode is cheaper, more environmentally-friendly, and more sustainable and it is a hot research topic for biopolymer scientists at present and even in the future.

In addition to the similar Physico-chemical performances, such as corrosion resistance, to those of traditional petrochemical plastics, the bioplastic products made from PHA also have complete biodegradability, excellent biocompatibility, and so on. The biopolymer researchers have found that the PHA's thermal and mechanical properties can be regulated by controlling its monomer composition [4]. Furthermore, the PHA's monomer composition has a great relationship with the degradation rate under various environmental conditions [5]. In the past 20 years, many reviews have summarized and analyzed how to improve PHA content in MMCs, but the articles reviewing PHA composition in MMCs are rare. Therefore, this work attempts to provide an outline of the current research relative to PHA's monomer composition adjustment from MMC-PHA production and their processing conditions for the regulation of PHA performance in MMC-PHA production.

2. The Structure and Properties of PHA

The first kind of PHA, polyhydroxybutyrate (PHB), was first identified and discovered by Lemoigne from *Bacillus Megaterium* in the early 20th century [6]. During the 1960s and 1990s, researchers gradually found more types of PHA [7-9]. Compared with traditional fossil plastics, different kinds of PHA have great differences in structure and properties, which provides unlimited possibilities for the processing properties of bioplastics. According to the report, PHA has unique biodegradability, biocompatibility, controllable thermal processing properties, and other mechanical properties similar to traditional petrochemical plastics [10]. The diversity of its properties is mainly affected by the monomer type, monomer proportion, and molecular weight of PHA [11].

Structurally, PHA is a linear polyester composed of ester linkage linking hydroxyalkanoates (HA) monomers. The general formula of PHA is shown in Fig. 1. In the formula, n represents the amount of HA in the polymer chain, which varies from 100 to 30,000. X is the number of methylene groups in the main chain of HA, with a value between 1-4. R represents side-chain alkyl groups, which range from methyl (C_1) to tridecyl (C_{13}). In addition, the R groups can also be other branched-chain groups, such as halogenated, epoxide, aromatic, etc. [12].

Fig. 1. The generic structural formula of PHA [12].

PHA can be divided into three types according to the number of carbon atoms in the monomer. Monomer with 2-5 carbon atoms is short-chain PHA(SCL-PHA), commonly includes PHB, polyhydroxyvalerate(PHV), and poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV). PHBV is the copolymer of PHB and PHV. The carbon chain length with 6-14 carbon atoms is medium-chain PHA(MCL-PHA), such as P(HHx). And the short-chain and medium-chain PHA combine to form short-medium chain PHA(SCL-MCL-PHA), such as P(HB-co-HHX) [13]. Generally, SCL-PHA has high crystallinity and poor tensile strength, while MCL-PHA exhibits soft, elastic, and low crystallinity properties. Their combination, SCL-MCL-PHA, shows different elasticity and tensile strength according to the proportion of two monomer types in the copolymers [14]. Generally speaking, the larger amount monomers that make up PHA, the higher the molecular weight of PHA. The molecular weight of PHA typically ranges from 50,000 to 1 million Dalton, and it is widely thought that the higher the molecular weight, the tensile properties of the PHA expression come out [15].

The mechanical properties and processing properties of PHA are mainly affected by the components of the PHA monomer and the proportion of each component [16]. There are two most common HA monomer types in MMC-PHA production: hydroxybutyrate (HB) and hydroxyvalerate (HV) [17]. The PHA composed of only one kind of constituent is named homopolymer, such as PHB and PHV. While the PHA consisting of two or more constituents is a kind of copolymer, like PHBV. These three polymers are the most common and easy to produce PHA types in the MMC-PHA process. Homopolymer, like PHB, has very strong hardness and water resistance due to its high crystallinity and high brittleness, but its low thermal stability is not conducive to ductile processing [18]. When a certain proportion of HV monomer is added to PHB, its ductility can be significantly enhanced. The higher the HV content, the stronger the toughness of its copolymer PHBV is, and the more effective it can improve the processing property of the polymer. PHBV showed a lower melting point, higher hardness, and better tensile property overall [19]. In addition, PHA with high HV content shows better water sensitivity and oxygen permeability [10, 20]. These studies conducted on the mechanical properties of PHA show that the proportion of HB and HV would affect the PHA-polymer final processing and mechanical properties, so we can synthesize PHA according to different processing requirements flexibly by regulating the monomer composition of PHA.

3. The Conventional Process of MMC-PHA Production

So far, the research reports on PHA synthesis by MMCs mostly focus on the research that used VFAs as carbon sources. Some studies used low-cost agricultural and industrial surplus raw materials or organic waste streams, such as fermented molasses, crude glycerol, etc., as carbon sources for MMC-PHA production [21]. But VFAs are the most preferred substrate in MMC-PHA production because VFAs are the precursors for PHA biosynthesis. The other substrates, like crude glycerol, tend to form glycogen instead of

Fig. 2. Basic scheme with three steps of MMC-PHA production.

PHA [22]. So, the typical MMC-PHA production process obtains three steps (Fig. 2): (1) Anaerobic fermentation, in which the complex bio-waste resources are converted into VFAs through anaerobic fermentation; (2) MMCs enrichment, the dominant cultures with a good ability of PHA synthesis from activated sludge or other sources would be enriched in the condition of unbalanced nutritional condition. The main purpose of this stage is to screen out MMCs with PHA synthesis ability and then maximize enriching them. Therefore, this stage usually consists of two steps. Firstly, the bacteria with PHA synthesis ability will be screened out under the case of excessive carbon sources and the restriction of nitrogen and phosphorus. Then, under unrestricted conditions, the screened MMCs rapidly proliferate to a considerable concentration [23]. Generally speaking, PHA output will not be obtained at this stage. Because MMCs with PHA synthesis ability are screened and enriched through the cyclic process of "PHA synthesis, accumulation, and consumption" [24]. To obtain PHA output continuously, most of the biomass is extracted for PHA synthesis in the next stage, and the rest is left in the enrichment reactor to continue the enrichment cycle. (3) PHA accumulation, add VFAs produced from the first step into MMCs which have been enriched in the second step to accumulate the maximum amount of PHA and then recover it [1]. The main purpose of this stage is to obtain the maximum amount of PHA accumulation. In this stage, MMCs will have a small amount of proliferation (due to the limitation of nitrogen and phosphorus, the cytoplasmic synthesis of MMCs is limited). The main source of MMCs depends on the supply of Stage 2 [4].

4. The Effect Factors on PHA Monomer Composition in MMC-PHA Production

In pure culture, pure substrate and specific PHA-producing bacteria or transgenic engineering bacteria can be used to produce particular monomers. While the MMC-PHA production is a biological process that enriches the corresponding microbial population basing on natural ecological selection [25]. Therefore, based on the process development basis of ecological selection, it is a more practical method to produce specific PHA monomers by manipulating the

process conditions in the MMC-PHA production process at the moment.

According to the previous studies, although MMCs contain different PHA-producing species, and each species of PHA-producing bacteria accumulates PHA with different molar mass and monomer composition distribution, some rules still can be found in the MMC-PHA production process. It can be observed from research that the types of microorganisms in MMCs and the composition of VFAs used as carbon source substrates greatly affect the PHA's monomer [1]. Most notably, one point should be emphasized that the VFAs composition plays a more vital role in regulating PHA monomer biosynthesis rather than the biodiversity of the MMC [21, 26]. These two aspects will be reviewed and analyzed in the following part.

4.1. Microbial Communities in MMC-PHA Production

In MMC-PHA production, the most common biological carrier used for PHA production is MMCs in activated sludge. Since Wallen and Rohwedder discovered the stored PHA particles in the mixed bacterial community cells of activated sludge in 1974 [27], the metabolism of PHA in activated sludge has aroused the interest of wastewater treatment researchers, and the synthesis of PHA by mixed culture method was first applied to the phosphorus removal process in wastewater treatment [28]. To enhance the effect of biological phosphorus removal, researchers acclimated and enhanced the PHA synthesis and utilization capacity of phosphorus removal bacteria by different proportions of short-chain fatty acids [29-31]. With the progress of research, researchers have found that bacteria with PHA accumulation capacity in activated sludge can be divided into aerobic bacteria, phosphate accumulating organisms (PAOs), glycogen-accumulating organisms (GAOs) and filamentous bacteria [32]. *Acinetobacter*, *Aeromonas*, *Pseudomonas* in PAOs and *Alcaligenes*, *Spirillum*, *Azotobacter* and *Pseudomonas* in GAOs had strong PHB accumulation capacity [33]. The aerobic bacteria named plasticicumulans enriched from activated sludge by Johnson et al. can obtain PHA more than 80% of the cell dry weight in lab-scale [34], close to the production yield of pure culture. Some of these bacteria have the function of producing specific PHA. For example, *Alcaligenes* and *Aeromonas* are strains that can specifically produce SCL-PHA and PHHx in pure culture, respectively [21, 35, 36]. The reason for this phenomenon may be that there are specific PHA synthetases in the cells of the related bacterium. However, in the MMC-PHA production process, the completely open operating conditions easily make the synthetic PHA products unstable in nature and uneven in quality. Isolation and enrichment of specific bacteria from MMCs may be contrary to the original intention of the MMC-PHA production process. As a result, researchers prefer to enrich specific types of microbial communities by feeding specific types of VFAs substrates to MMCs to regulate the population metabolism and PHA synthesis quality of MMCs [37, 38]. Therefore, in recent five years, a concept called 'Directed acid production (or oriented acid production)' has been developed, which is proposed in the background of regulating the production of specific PHA monomers [39-42]. More specifically, in the first stage of MMC-PHA synthesis, a particular proportion of VFAs was obtained employing operating parameters, operating conditions, screening of fermentation substrates and so on, then this kind

of VFAs would be used as substrates for MMC-PHA synthesis, thus to obtain PHA with different monomer composition.

4.2. The Association between MMC-PHA Production and Oriented Acid Production

VFAs, the important intermediate product after the end of acid production and fermentation stage of organic matter anaerobic three-stage process, are considered as the preferred substrate for MMC-PHA production [43]. VFAs mainly refer to the short-chain fatty acids with less than six carbon atoms, and the common VFAs conclude acetic acid, propionic acid, valeric acid, and butyric acid. VFAs have a wide range of production sources, and they can be produced by anaerobic fermentation from the biowaste resources, for example, high-carbon wastewater (such as food industry wastewater, municipal landfill leachate, etc.), carbohydrate waste (i.e., biowaste resources rich in simple carbohydrates, such as glucose and sucrose) and so on [44]. According to the number of carbon atoms in the main chain, VFAs can be divided into odd carbon VFAs(OCFA) and even carbon VFAs(ECFA). OCFA generally refers to propionic acid, n-valeric acid and isovaleric acid, while ECFA refers to acetic acid, n-butyric acid and isobutyric acid.

In MMC-PHA fully open system, the VFAs-rich stream usually contains some sugar and protein, but according to the experimental report, the presence of sugar and protein does not affect the monomer composition of PHA [39]. The proportion of ECFA and OCFA in VFAs flow directly affects the monomer composition of PHA. Generally, the usage of ECFA in the MMC-PHA production process tends to generate HB monomer, whereas OCFA tends to yield HV and other longer-chain monomers, and researchers have been clearly identified this rule by employing sole VFA [4, 22, 42, 45].

The role of VFAs as substrates can be divided into two parts [39, 46]. One is for the growth of MMCs, and the other is for

the synthesis and storage of PHA. After VFA enters the cells through active transport under the consumption of adenosine triphosphate (ATP), and then be activated into acyl-CoA molecules(i.e. acetyl-CoA and propionyl-CoA, Fig. 3) through the β -oxidation pathway [47]. When the nitrogen and phosphorus in the substrate are limited, the tricarboxylic acid cycle (TCA) process would be delayed, limiting the biological process of bacterial synthesis of cytoplasm and genetic material, and leading to the slow growth rate of bacteria. As a result, the remaining acyl-CoA molecules that cannot be completely used in the TCA progress enter the PHA synthesis pathway [48]. In the PHA synthesis pathway, acetic acid and propionic acid can be directly activated to acetyl-CoA and propionyl-CoA, respectively. The rest of ECFA was converted to acetyl-CoA by the β -oxidative way, while OCFA was converted to crotonyl-CoA. The synthesis of PHB is the most widely studied and classic synthesis pathway of the MMC-PHA process. Firstly, 3-ketothiolase(PhaA) condenses two molecules of acetyl-CoA to form a molecule of acetoacetyl-CoA, and then acetoacetyl-CoA reductase(PhaB) reduces it to 3-hydroxybutyrate-CoA, the precursor of PHB, through H provided by NADPH. Finally, after the formation of the HB monomer, PHB synthase (PhaC) polymerizes the HB monomers, connects the bonds between monomers, and extends to form a biopolymer PHB. The synthesis process of PHV is similar to that of PHB, but the corresponding synthetases are slightly different [48]. In the PHV synthesis process, the HV monomer needs one molecule of acetyl-CoA and one molecule of crotonyl-CoA to be converted together [46].

In the first stage of the MMC-PHA process, controlling the composition of VFAs to obtain the particular PHA monomer synthesized is a macroscopic but effective method. It should be noted that oriented acid production does not refer to a specific type of acid but regulates the ratio of ECFA to OCFA in acid production to adapt to the percentage of PHB to PHV in PHA production[49].

Fig. 3. Pathway of different VFAs in PHA synthesis [21, 46].

5.2. Impact Factors for Oriented Acid Production

The concentration and component spectrum of VFAs results from different acidogenic metabolic pathways in acidogenic bacteria [71]. However, substrate characteristics, acidogenic bacteria involved in the reaction, and other operating conditions, such as pH and temperature, will greatly impact the acidogenic metabolic pathways, thus affecting the production and composition of VFAs [75]. In this paper, the influencing factors of VFAs components are sorted out from the following three aspects: substrate composition, acidogenic bacteria types and operating characteristics of acid fermentation ecological conditions.

5.2.1. Types and composition of substrates

Biowaste resources used for acid fermentation can be divided into agricultural and forestry waste, food waste and waste from food processing plants, high concentration wastewater and municipal sludge [50]. The composition can be divided into three kinds of biowaste resources rich in carbohydrates, protein, and triglycerides. Although it was mentioned above that ECFA is easier to be produced than OCFA, a regular pattern can be found from existing studies (Table 1 [75-79]: when the substrate is rich in lipids and proteins, OCFA (propionic acid and valeric acid) is more likely to obtain more output proportion of the total VFAs production [76, 77].

The effect of substrate types and composition on VFAs composition reflects the role of substrate specificity [80]. The above studies indicate that it is possible to produce OCFA by anaerobic fermentation with biowaste resources rich in lipid or glycerin. The chemical composition of proteins and lipids is likely to be the main reason to promote the output of OCFA. The free ammonium ion produced after proteins hydrolysis is conducive to promoting the content of COD in the substrate [81]. And the biowaste resources rich in fat contain a lot of oil and grease, and it can produce a lot of glycerine and high fatty acids after hydrolysis, and glycerol is an important precursor for the synthesis of propionic acid [82]. At the same time, oil can release a lot of heat energy during anaerobic decomposition to meet the Gibbs free energy required for propionic acid production by microorganisms. Meanwhile, a high concentration of higher fatty acids can significantly inhibit methanogens and acetic acid-producing bacteria [83], making the fermentation system to be more inclined to propionic acid fermentation. In addition, some scholars believe that glycerol is beneficial to propionic acid fermentation because of its high reducibility and can be oxidized to propionate ketone, thus producing more propionic acid to maintain the redox balance [84].

In other studies, by adding different kinds of waste to a single

type of waste, changing the composition of the substrate can also achieve the purpose of changing the variety of fermentation products. Huang et al. [85] mixed peanut meal rich in protein with rice washing water rich in carbon to achieve propionic acid fermentation when C/N≈56. Ma et al. [86] found that the OCFA production could be improved by adding protein and lipids-rich waste, such as food waste to waste-activated sludge. Xiong et al. [87] added crude glycerol to the sludge for co-fermentation, which greatly increased the proportion of OCFA to total VFAs compared with the single sludge fermentation. This may be due to the high nitrogen content of sludge itself and the addition of crude glycerol is a carbon-rich substrate; by mixing the two in proportion, the C/N ratio of the single substrate can be improved, which provides an appropriate nutritional balance for acidogenic bacteria, thus promoting the metabolic activities of acidogenic bacteria and enhancing the activities of key enzymes.

However, although some researchers believe that the composition of VFAs mainly depends on the design of substrates [68, 88], the composition and types of substrates are not decisive factors to determine the type of acid production. Substrate types may significantly affect the VFAs composition by affecting the metabolism of microorganisms and the evolution of the community structure of dominant bacteria. Therefore, from this point of view, the types of acidogenic bacteria and the physiological conditions of their growth may have a greater influence. Other studies have shown that the same substrate can have various fermentation types through pretreatment, fermentation conditions and other regulation. When exploring the impact of substrate components in oriented acid production progress on VFAs composition, it is necessary to systematically study and evaluate the coupling interactions among the substrate types, acidogenic bacteria and operating conditions in the acidogenic system.

5.2.2. Types of acidogenic bacteria

In general, biowaste resources can be hydrolyzed and produce acid by microorganisms existing in their system in the state of natural fermentation. These microorganisms can co-evolve into a relatively stable community under specific ecological conditions and produce corresponding VFAs. Most acidogenic microorganisms are anaerobes and some facultative anaerobes, and most of these bacteria belong to phylum *Firmicutes*. Previous studies showed that phylum *Firmicutes* can secrete extracellular enzymes related to the VFAs product such as lipid, protein, and polysaccharide hydrolysis [89]. This characteristic enables biowaste resources to be fully hydrolyzed and acidified.

As mentioned above, in the acid production mechanism, the

Table 1. Literature on the OCFA Production from Substrates Rich in Lipids and Proteins

Substrates types	Operating conditions	Total VFA yield	Main OCFA compositions of total VFAs yield	Reference
Meat and bone meal	pH = 10, T = 55°C	2,643 mgCOD/L	Isovaleric acid 27%	[76]
Crude glycerol	pH = 4.4, T = 35°C	2,271 mgCOD/L	propionic acid 74%	[76]
Grease, kitchen wastes	pH = 6, T = 35°C	23.22 g/L	propionic acid 32.2%	[78]
Hogwash waste grease (drowning oil, waste meat and frying oil)	Natural fermentation in 35°C	6,430.22 mg/L	propionic acid 54.73%	[79]

activities of key enzymes in different bacterial communities are various. That is, different bacterial communities have their metabolic characteristics and substrate preference, thus showing different metabolic pathways, namely under the different strains of type, pyruvate is converted into acetic acid, propionic acid, butyric acid, pentanoic acid opportunities is not the same [68]. In the acid-producing system, the common dominant flora is *Proteobacteria*, *Scleroderma*, and *Bacteroidetes*, in which genera were used as the dominant taxa, mainly including *Bacteroides*, *Clostridium*, *Micrococcus*, *Streptococcus*, *Radiomycetes* and *Granulocytes* [90]. As the executive body of VFAs production, exploring the evolution process of the community structure of acidogenic bacteria and classifying the dominant bacteria into ECFA producers and OCFA producers can reveal the interspecific relationship between different microbial communities and the correlation between microorganisms and acid production system, which will greatly benefit the future research on oriented acid production progress.

Huang et al. [41] found that the functional bacteria which can improve propionic acid yield include *Proteococcus*, *Petrimonas*, *Clostridium*, *Lactococcus* and *Bacteroides*. Among them, *Proteococcus*, *Petrimonas* and *Clostridium* are the producers that can directly use the substrate to generate propionic acid, and the rest are collaborators of auxiliary propionic acid production. Another researcher, Huang zhouyue [40], found that at the class level, γ -*Proteobacteria* dominated the output of OCFA, while α -*Proteobacteria* and β -*Proteobacteria* were closely related to the production of ECFA, and at the genus level, *Veillonella*, *Acinetobacter* and *Pseudomonas* have positive effects on OCFA production. Cibis et al. [90] isolated acetic acid, propionic acid and butyric acid-producing bacteria from biogas plants, and explored their growth characteristics (such as substrate utilization rate, substrate conversion, etc.), and obtained valuable information about the formation of acetic acid, propionic acid or butyric acid by conversion of important polymers and metabolites during organic matter degradation.

In addition, oriented acid production progress can be promoted by adding obligate strains when controlling the VFAs composition. After the initial fermentation of activated sludge and kitchen waste, Chen et al. [91] sterilized the original acid solution and inoculated *Propionibacterium* in the fermentation broth. As a result, the propionic acid content up to 7.13 g COD/L, accounting for 68.4% of the total VFAs. It is worth noting that although inoculation with obligate strain can achieve the goal of oriented acid production, it is still necessary to evaluate further whether the use of pure bacteria inoculation of large-scale output to produce specific acid will offset the low-cost dividend brought by biowaste resources fermentation.

5.2.3. Ecological conditions of the acid production process

The ecological environment conditions (such as pH, temperature, residence time, etc.) in the acid production system have an important impact on the composition of VFAs products (Table 2 [92-101]). In the acid production process, the ecological factors mainly affect the cell proliferation, growth, and metabolism of acidogenic bacteria by regulating the activity of key enzymes, which determines the VFAs composition [91].

Table 2. Literature on Obtaining Specific VFAs Components from Kinds of Biological Waste

Main VFA components	substrate	Operating conditions				Total VFAs yield	Main VFA proportion of total VFAs yield	Ref.
		pH	T	ORP	HRT/SRT	OLR		
Acetic acid	lime-treated sugarcane bagasse	6.95-7.05	55°C	-	19.1 d	2.07 g/(L·d)	0.55 g/gVSS	[92]
	alkaline, thermal pretreated WAS	-	35°C	-	10 d	-	5,602 ± 73 mgCOD/L	[93]
	municipal solid waste	10	35°C	-	10 d	-	8,320 mgCOD/L	[76]
	food waste	6	30°C	-	20 d	-	0.918 gVFA/gVSS	[94]
	PS & WAS	-	55°C	-	10 d	2.5-3.5 kg VS/d	3.7 gCOD/L	[95]
Propionic acid	dairy whey effluent	6	37°C	-	95 h	-	0.97 gCOD/gCOD	[96]
	paper & chicken manure	5.3-6.6	40°C	-	32.6 d	-	18.3 ± 0.4 g/L	[97]
	WAS/rice	6.0-9.0	21°C	-	8 d	-	520.1 mgCOD/gVSS	[61]
	WAS	-	35°C	-	4 d	-	3,840 mgCOD/L	[98]
Propionic acid	grease & kitchen wastes	6	35°C	-	-	-	2,322 mg/L	[78]
	crude glycerol	4.4	35°C	-	-	-	2,271 mgCOD/L	[76]
	hogwash waste grease	-	35°C	-	-	-	6,430.22 mg/L	[79]
Butyric acid	slaughterhouse by-products	6.8	38°C	-	-	-	0.38 g/g dry matter	[99]
	chicken manure	7	55°C	-	-	-	10.99 ± 0.21 g COD/L	[92]
	food waste	6	30°C	-	17 d	-	0.79 gCOD/gVS	[100]
Valeric acid	sucrose and piggery waste	8.9	35°C	-	-	-	11.89 gCOD/L	[101]
	meat and bone meal	10	55°C	-	-	-	2,643 mgCOD/L	[76]
	chicken manure	7	35°C	-	-	-	10.65 ± 0.05 g COD/L	[92]

5.2.3.1. pH

pH has an important impact on the key enzymes involved in acid production reactions in microorganisms. The too acidic or alkaline environment may lead to denaturation of enzyme proteins or affect their activity [102-104]. By affecting the activity of intracellular enzymes [105] or the metabolic balance of acidogenic bacteria [106], pH impacts the life and metabolism process of acidogenic bacteria. A suitable pH microenvironment can promote the growth of required acidogenic bacteria and meanwhile suppress the non-essential bacteria [107]. Therefore, each microorganism has its growth pH range. When the pH in the acid-producing reactor is close to the ecological requirements of some kind of acid-producing population, this kind of microorganism would become a dominant population through ecological competition, then affecting the types of VFAs produced. As a result, the VFAs composition may be changed when the range of pH in the acid production system changes. However, there are inconsistencies about the optimal range of pH values in the literature. Many researchers believed that a weakly acidic environment (pH range 4-5) would be the best pH range for acetic acid production and that of butyric acid is 6-7 [108], while Farouk et al. [109] considered that both acetic acid and butyric acid can be effectively promoted under the pH range of 5-7. In contradiction, in other studies, alkaline conditions lead to an increase in the yield of acetic acid or butyric acid. For example, the acetic acid production in the acid-producing reactor of food waste reaches 91% at pH = 9 [81]. In another study, in the anaerobic conversion of sucrose and piggery waste, butyric acid was the main VFAs product at pH = 8.9 [101]. This indicates that the attainment of the desired VFAs component is not solely dependent on pH, other parameters should also be considered when adjusted pH during acidogenic fermentation.

5.2.3.2. Temperature

Like pH value, temperature plays an important role in affecting the growth of acidogenic bacteria, the activities of key enzymes and the hydrolysis of particulate organic matter into soluble substances [110]. The metabolic rate of acid production process usually increases with the increase of temperature. From the thermodynamic perspective, in the suitable temperature range, the temperature rise is conducive to promoting the biowaste resources to release more carbohydrate and protein, and other nutrients, enhancing the enzyme activity of acidogenic bacteria to encourage the generation of specific VFA [111]. The growth rate and live metabolism of dominant bacteria are different under different temperature changes. It can be observed from the literature listed in Table 2 that medium and high temperature (30–55°C) is the universal temperature range for VFAs production. However, the effect of temperature on obtaining the specific VFAs components during fermentation seems limited. Yuan et al. [111] found that when the fermentation temperature of waste-activated sludge increased from 4.0°C to 14.0°C, the percentage of propionic acid in the total VFAs output increased only slightly from 20% to 29%. In previously published studies, the contribution of temperature to the fermentation acid production process is more reflected in enhancing the total VFAs yield. But from another angle, adjusting the temperature combining with other fermentation factors can also be improved the output of required VFAs components in the acid production

system. But remarkably, high-temperature treatment in large-scale production needs a lot of energy consumption, which may offset the benefits of the MMC-PHA production chain to a large extent.

5.2.3.3. Oxidation-reduction potential (ORP)

Due to the lack of an electron transport system in the anaerobic fermentation acid production system, a large amount of NAD^+ is usually produced during substrate oxidative dehydrogenation. Therefore, NAD^+/NADH has become a common redox pair in the intracellular metabolism of acid-producing bacteria [102]. ORP is the main index reflecting the electron transfer and redox balance of acidogenic bacteria in the reaction process [112]. However, information on the effect of ORP on the VFAs composition is still limited. Cohen et al. [113] have concluded that high ORP can lead to propionic acid fermentation. Ren et al. [102] proposed that when the ORP was low (ranges from -400 to -200 mV), the ethanol type and butyric acid type fermentation often appeared, while it is easy to occur the propionic acid fermentation type when ORP ranges from -250 to +100 mV. Zhao et al. [114] used waste molasses as fermentation substrate; by adjusting fermentation conditions pH and ORP, a similar conclusion was also obtained. Yin et al. [100] studied the effects of ORP and inoculation on VFAs production and found that ORP reached the maximum yield in the range of -100 to -200 mV. Information about how ORP affects specific VFA production still needs more researches.

5.2.3.4. Substrate residence time and organic loading rate

It is all known that acidogenic fermentation is a process based on the hydrolysis and acidogenic stages of anaerobic digestion. In acidogenic fermentation of complex biowaste resources, hydrolysis usually was identified as a rate-limiting step [115]. Ferrer et al. [95] studied the influence of SRT on the acid production of mixed sludge of primary sludge and residual sludge and found that propionic acid was the main VFAs component when SRT = 9.4 days. As mentioned before, the substrate has an important influence on the composition of VFAs, the hydrolysis rate of substrates with different components is different, and at the same time, the growth rate of dominant flora that can produce specific types of VFAs is different. Therefore, the VFAs product spectrum is related to the residence time (containing sludge retention time (SRT) and hydraulic retention time (HRT)) to some extent. The studies of residence time will help to promote the development of oriented acid production.

The organic load rate (OLR) is a correlated parameter of HRT or SRT. It is reported that low OLRs can lead to the starvation of the cultures and thus stimulate the activity of microorganisms and accelerate the acid production process [116]. Li et al. [117] pointed out that when the OLR was at a high level, propionic acid was more easily obtained from the lactate-propionic acid pathway. This may be because when OLR is high, or HRT is low, more oxidizing compounds are converted to reducing compounds. Yu et al. [118] observed similar results at moderate temperatures: as OLR increased, the yield of propionic acid increased while the yield of acetic acid decreased. However, Jiang et al. [69] reported that under low OLR conditions, propionic and butyric acid production was facilitated, while the percentages of acetic and valerate in total VFAs increased with the increase of OLR. The underlying

mechanism behind these observed phenomena remains to be further explored.

5.2.3.5. Other factors

In addition to the above factors, the researchers also studied other factors.

Kim et al. [119] found that the addition of trace elements positively affected VFAs composition. They added trace elements such as calcium, iron, nickel and cobalt in the thermophilic anaerobic sludge digestion system. The results showed that the addition of these trace elements contributed to the high concentration production of propionic acid.

As for additives, Yang et al. [120] pointed out that the addition of β -cyclodextrin (β -CD) had no significant effect on the composition of VFA. However, Huang et al. [41] reported that when glycerol (C/N) was 15-20, pH was 8.0-9.5, and β -CD was 0.2 g/gTss, they obtained more than 60% propionic acid.

Some researchers believe that the accumulation of VFAs in the acidogenic system would inhibit VFAs producing bacteria [69]. This principle is similar to that of reversible reaction: when a kind of VFA in the system accumulates to a certain extent, the production would be inhibited so that the formation of specific acid would be limited. Arslan et al. [121] selectively removed propionic acid from the acid production system by electrodialysis, thus doubling its productivity.

5.2.4. Brief summary

From the perspective of microbial community ecology, the interaction between the acidogenic bacterial community and its growth environment is an important factor affecting the VFAs composition, and the type of substrate also plays an important role. From the research mentioned above, we can find that due to the differences in working conditions and community structure in different acid production systems, the interaction of various influencing factors in the acid production system will obviously affect the product spectrum of VFAs. If only from a single influencing factor, the

research on the same influencing factor may lead to inconsistent conclusions, proving the importance of synergistic effect between microorganisms and growth environment on VFAs components control. However, in the existing studies, more attention has been paid to the impact of operating parameters on VFAs product spectrum in the acid production system, which is only a surface phenomenon, and there is still a large blank space in the principle and mechanism behind these phenomena. In future research, revealing the mechanism of substrate, microorganism, and operating factors of the acid production system to control the formation of VFAs components will help provide a good theoretical basis for optimizing oriented acid production process.

5.3. Research Status of MMC-PHA Production Combined with Oriented Acid Production

So far, the experience of MMC-PHA production combined with oriented acid production is still limited. In the current process report on the MMC-PHA production combined with oriented acid production, waste sludge is a common substrate for acid production (Table 3). The common feature of these processes is that the concentration of total organic matter and VFAs in the actual hydrolysate is very high. To improve the adaptability and stability of the MMC-PHA synthesis system, the hydrolysate needs to be pretreated (such as diluting [122] or removing the unnecessary nutrients [41] before adding to PHA biosynthetic reactor as substrate. Another method is to acclimate MMCs directly with hydrolysates with high VFAs concentration in the MMC enrichment stage. This way can help the MMCs fully adapt to the concentration of hydrolysate stock solution. Then in the subsequent phase, the PHA accumulation stage, the hydrolysate can be directly used as the substrate to maximize the accumulation of PHA without pretreatment [42].

In the future, developing efficient and low-cost VFAs pretreatment technology can be a good point. Meanwhile, the researches about economic analysis and life cycle assessment (LCA) on the

Table 3. Production Status of MMC-PHA Production Combined with Oriented Acid Production

Feedstock for VFAs production	Operating conditions in VFAs production	Total VFAs yield	Main VFAs components and proportion	PHA yield (%DCW)	PHA monomer content(%)		Ref.
					PHB	PHV	
Papermaking wastewater	pH = 6, T = 30°C, HRT = 16 h	≈6,000 mg COD/L	propionic acid (40%)	48	47.00	53.00	[122]
Co-ferment kitchen waste and sludge	pH = 6.5, T = 35°C, OLR = 4 gVSS/(L·d), SRT-8 d	18,062.66 mg COD/L	ECFA/OCFA = 2.53	47.65	79.00	21.00	[63]
excess sludge	pH = 6.8, T = 55°C, SRT = 5 d	5,947.50 mg COD/L	valerate acid (52.05%)	42.31	68.40	23.70	[42]
WAS	pH = 8, T = 35°C, SRT = 15 d, C/N = 16, β -CD = 0.1 g/gTSS	4,986 ± 88 mg COD/L	OCFA = 59.6 ± 2.8%	56.4±3.9	27.6 ±0.8	72.4 ±5.2	[41]
WAS	pH = 9, T = 36°C, HRT = 24 h	1,019 ± 51.0 mg COD/L	Acetic acid (45.3%) Propionic acid (31.5%)	60.3	91.80	8.20	[4]

MMC-PHA production combined with oriented acid production should also be encouraged.

6. Conclusions

In the process of MMC-PHA synthesis, it is one of the current research hotspots to obtain PHA products with different processing properties and mechanical properties by controlling the synthesized PHA monomer. In the laboratory scale, the control of VFAs as substrates can realize PHA monomers' specific production in MMCs. However, the oriented acid production process mechanism to achieve this goal has not been clear, and it still needs to be further explored. These research experiences will help to combine the three-stage process of MMC-PHA production more closely. Furthermore, the development of the oriented acid production process can undoubtedly promote the wide commercialization of MMC-PHA production.

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Author Contributions

T.W. (Master student) did writing and original draft preparation. Q.F. (Professor) did supervision, writing, reviewing and editing.

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