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An insight into enrichment strategies for mixed culture in polyhydroxyalkanoate production: feedstocks, operating conditions and inherent challenges

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ABSTRACT

PHA production using a combination of mixed culture and carbon wastes has been demonstrated as a cost-reducing solution compared to the use of expensive pure culture process. Continuous research has been conducted with the aim to further reduce the production cost by simplifying the production scheme, as well as enhancing the performance of mixed culture in PHA production. Selection of the carbon feedstock and enrichment strategies needs to be essentially considered to obtain a culture enriched with PHA accumulators for a stable PHA production. Most of the studies implementing mixed culture process applied the feast-famine regime with periodical supply of carbon for culture enrichment. Results have revealed that the enriched culture showed a comparable performance with pure culture, in terms of PHA content (30–80%) and yield (0.4–0.8 g PHA/g S). However, a low productivity is the hindering factor to produce PHA at industrial scale by using mixed culture, which could be overcome by improvising the enrichment strategies. This review zooms into the evaluation of two step and three step processes in PHA production by utilising different feedstocks. Critical parameters to be considered for PHA production such as the suitable feedstocks, enrichment conditions, stability of the enriched culture and nutrient supplementation are being highlighted. The possible enrichment strategies that include uncoupled C and N supply and extended cultivation in overcoming the issue of low productivity are presented. The impact of different enrichment strategies on microbial community, characteristics of PHA produced as well as PHA production performance is worth investigating in future.

1. Introduction

Since petrochemical plastics fail to degrade naturally, severity of the plastic disposal is translated into the environmental pollution and an increasing number of landfills [1]. At an alarming level, plastics have been consumed massively to facilitate in the medical sectors and delivery packaging during the ongoing Covid-19 pandemic since 2019. Reduction in the use of the conventional plastics still remain as a challenge as they have been so conveniently made up the countless products that we use every day. Alternative plastic materials that are both user- and eco-friendly are necessary to be adopted to curb plastic pollution problems. Among many, polyhydroxyalkanoates (PHA) is a bio-based plastic that could potentially replace the petroleum-based plastics with similar properties. Since 2008, research related to PHA bioplastics/biopolymers has been increased dramatically in the areas studying PHA characteristics, applications of PHA and developing sustainable production processes [2].

PHA is synthesised via a biological fermentation by PHA producing bacteria [3]. Intracellular storage of PHA bioplastic by these PHA producing bacteria is a way of reserving carbon and energy for survival [4]. In the plastic market, PHA production accounted for only 1.1% of the global bioplastics with production of 6.73 million tonnes in 2018 [5]. Industrial production of PHA bioplastics applies pure cultures such as *Cuprivavidus necator, Alcaligenes* sp. or *Raltonia eutropha* [6]. Various PHA bioplastics namely poly(3-hydroxybutyrate) (P(3HB) or PHB), polyhydroxyvalerate (P(3HV) or PHV), and copolymer P3HB-co-4HB are produced by Danimer Scientific and Tepha Inc. (USA), Kaneka Corporation (Japan), Tianan Biologic (China) and Biomer (Germany) [7]. Production capacity of these PHA producers are in the range of 5,000 up to 10,000 tons per annum. The disadvantages of using pure

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culture process are the requirement of using pure carbon sources (refined sugars or volatile fatty acids) and the aseptic condition to prevent bacterial contamination and to obtain a high cell growth [7–9]. Due to this, the commercial price of PHA is very high from US dollar 4,000 to 15,000 per ton, making it difficult to outcompete with the petrochemical plastics that are much cheaper in the range US dollars 1000–1500 per ton [5]. Some studies in the economic analysis of the PHA production using pure cultures agreed that the pure carbon feedstock is identified as one of the main cost contributors accounting for 30–50% of the overall production cost, followed by the downstream processing cost for extraction and purification [8,10,11].

Research efforts in making PHA more cost-competitive are centralised to two approaches, i.e. engineering bacteria and development of the mixed culture (MC) process. The first approach mainly deals with the PHA producing strains, which are genetically engineered in their basic metabolic pathways (carbohydrate/glycolytic, in situ fatty acid synthesis and β-oxidation). The resultant genetically modified PHA producing bacteria (*E. coli*, *Halomonas* spp. and *Pseudomonas* spp.) are more resistant to contamination especially extremophiles. Hence, the pure culture process is improved with an increasing conversion of the carbon substrate to PHA, and PHA product diversity with a control of the molecular weight and PHA synthases [12,13]. In the second approach, cost reduction in the PHA production is viable by using mixed culture as an alternative to the pure culture. Activated sludge obtained from wastewater treatment facility is a source of mixed cultures which have been reported for its ability to produce PHA biopolymers in a non-septic condition as an advantage [14–16]. In addition to that, the mixed cultures are well-adapted to the use of complex carbon sources from industrial wastes and byproducts [17–20]. Therefore, a significant cost reduction up to 50% for the PHA upstream processing can be realised [6,16,20,21]. Environmental and economic sustainability can be achieved through the development of the mixed culture process that is able to convert the abundantly available low-cost carbon waste sources into value-added biocompatible and biodegradable plastic products.

Most of the complex carbon sources such as waste oils, cheese whey, fruit-canned juices, oil mill effluents and lignocellulose-based hydrolysate need to be pretreated to obtain products such as volatile fatty acids (VFA) with shorter carbon chains before utilising them for PHA production [17–20]. This additional step results in the MC process to be conducted in three steps (pretreatment, culture enrichment and PHA accumulation).

Impurities (salt content, alcohols, minerals and other non-carbon components) contained in the carbon wastes affect the product characteristics (PHB homopolymers or PHA copolymers) [12,22,23]. Since wastewater sludge contains a diversity of different microorganisms/bacteria, enrichment is a high energy demand step to select a PHA producing culture under the anaerobic/aerobic cycle or the feast/famine regime with an intensive aeration [13]. Throughout the bacterial selection, a dynamic change happens in the bacterial community that the PHA producing bacteria are retained and grown. Multiple metabolic pathways of PHA synthesis take place in the mixed culture process, since different carbon components are taken up by various PHA producing bacteria. This results in unstable PHA structures, inconsistent PHA molecular weights in the discontinuous processes conducted in batches [13,24,25]. All of these factors make the mixed culture process more complex and difficult to control the inconsistency of PHA product specification that hinders the scale-up stage for pilot study and large-scale production. The PHA production at a lower cost via the mixed culture process combined with renewable carbon feedstocks has yet to reach commercialisation with some challenges to overcome. More in-depth studies at laboratory and pilot scales are needed to strengthen the research of the MC process.

The experimental studies have proven the feasibility of combining various carbon wastes and activated sludge mixed culture to produce PHA via the anaerobic/aerobic or feast-famine regimes. PHA storage response of the mixed culture is highly comparable with that of the pure culture for yield (0.4–0.8 g PHA/g S) and polymeric content (30–80%). However, the current productivity of mixed culture is ranged from 0.236 to 0.41 g PHA/L.h which hindered the scaling up process in comparison with that of the pure culture at 1.38 g/L.h [9,17,26]. Manipulation of the process parameters leading to an effective selection of the PHA producing culture and PHA accumulation at high yield and content were well reported in most studies. Nevertheless, the key factors to include in devising strategies of increasing PHA productivity are scarce in the current literature. Besides, it is noticed that in the production by using pure culture process, cell growth is the aim to increase the cell density of the PHA producing bacteria, which is then translated into an increased productivity. Conversely, the mixed culture process focuses only in the bacterial selection without given much attention to grow the PHA producing bacteria (PHA producers or PHA accumulators) to achieve higher productivity.

Continuous efforts have been made in lowering the cost of PHA through several means. This review aims to summarise the development of PHA production by using mixed culture and waste carbon sources as an alternative towards low-cost PHA production. The development of two step processes by using suitable feedstocks instead of three steps processes lower the production cost for PHA further. A comprehensive comparison was made between the performance of PHA production from both three steps and two steps processes. Subsequently, the factors to be considered when mixed culture is applied, such as the suitable feedstocks, operating conditions, stability of the mixed culture and nutrient supplementation are further presented. This review emphasised on the enrichment process in mixed culture PHA production, as this is one of the most important steps that governs the PHA yield, content and productivity as well as the quality of PHA produced. In view of the lack of compiled literatures related to improving productivity of PHA production, which is one of the challenges that hinder process up scaling,

Table 1
Comparison between PHA biopolymers and petrochemical polymers [25,27,31,32].

PHA biopolymers	Petrochemical Polymers
Advantages	
• Biocompatible and biodegradable.	• Well-establish production process at low cost.
• Similar thermoplastic properties to polypropylene and polyethylene.	• Many applications in commercial and industrial usage.
• Produced from plant-based materials and carbon waste sources from other agriculture and industry.	• Less water consumption in the production.
• Potential applications in biomedicine, packaging materials to replace non-degradable plastics.	
• In assessment of life cycle, PHA production is more beneficial than polypropylene production in term of depletion of ozone layer, reduction in toxic level.	
Disadvantages	
• High production cost, 15 times higher than petrochemical polymers.	• Non-biodegradable, causing plastic pollution.
• Still under development stage to reduce the production cost, especially the mixed culture process.	• Non-biocompatible, produced from the fossil fuel source.
• Requires high water consumption (65 dm^3/kg polymer).	• Unsustainable and not environmental-friendly.
• Process is carried out in mild conditions (ambient temperature, atmospheric pressure and aqueous medium). Hence, less energy is required.	• Difficult to recycle and reuse.
• Feedstocks originated from carbon wastes contain fertilisers, acids and a significant amount of salts that add to the toxicity level of wastewater and the eutrophication potential.	• Depending on the fossil resources. • Produced under high temperature and pressure conditions and involvement of organic solvents. Hence, more energy required.

this work highlights the possible strategies on productivity enhancement by applying uncoupling carbon and nitrogen supply, as well as extended cultivation. The future prospects of PHA production by using mixed culture are further discussed.

1.1. Polyhydroxyalkanoates (PHA) properties and characteristics

To reduce the dependency on the use of fossil fuel and its related petrochemical industry, PHA biopolymers have been attracting an increase in the research due to its potential to replace the non-biodegradable polymers and to attain sustainable development. A comparison between the conventional petrochemical polymers and the alternative PHA biopolymers is presented in Table 1. Although the production processes of the petrochemical polymers are well established and commercialised with market-competitive prices, they are energy-demanded due to the harsh operating conditions under high pressure and temperature with an involvement of organic solvents [27]. In addition, the petrochemical plastic products are recalcitrant to natural degradation but difficult to recycle and reuse which are found in the characteristics of the PHA biopolymers as big advantages. A study in the life cycle assessment of PHA revealed that it would be more beneficial in the effort of reducing the ozone layer depletion and the toxic level in case of applying the PHA biopolymer production process rather than the petrochemical polymer process [27]. Therefore, continuous research in PHA bioplastic focusing on improving the PHA producing bacterial strain and reducing the production cost for scale-up process shows a hug interest in the PHA production, especially from the waste streams [2].

PHA bioplastics are one group of biopolymers produced by both prokaryotic and eukaryotic microorganisms [28]. The discovery of the PHA bioplastics in the form of P3HB by *Bacillus megaterium* was first reported at the Pasteur Institute in Paris 1926 by Lemoigne - a French scientist [29]. Since then, the PHA-producing microorganisms have been collectively identified in more than 90 genera under both aerobic and anaerobic conditions [28,30].

The degree of polymerisation is one of the indicators for the quality of the polymers produced. A polymeric material with a high degree of polymerisation has more monomer units in their macromolecules, hence increases the molecular weight [33]. PHA bioplastics exhibit a high degree of polymerisation from 105 to 107, which makes its molecular weight in the range of 500,000 to over 1,000,000 Da as high as the conventional polymer plastics [34,35]. Hence, PHA bioplastics are able to undergo further processing by blending with other petroleum-based polymers to overcome their drawbacks such as brittleness, low thermal stability and high fragility [8,36]. Processability of the blends containing PHB and polyethylene glycol (PEG) lowers the processing temperature and brittleness of PHA based plastics [1].

Additionally, biodegradability is a useful property of PHA bioplastics, which the chemical-synthetic plastics do not have. PHA is degraded into carbon dioxide and water by microorganisms living in the soil under aerobic or anaerobic conditions without releasing toxic products [37]. In the marine environment, the mean rate of biodegradation of PHA is reported at 0.04–0.09 mg per day per cm^2, which means it can take between 1.5 and 3.5 years for a water bottle made of PHA plastic to completely biodegrade [38]. Another study in the decomposition of PHA reported that PHB chemically degrades at a temperature just above its melting point at 180℃ into olefinic and carboxylic acid compounds [39]. Other properties of PHA such as elongation at break, tensile strength, and glass transition temperature are 300%, 20 Mpa, 40℃ respectively [25]. With all these properties, PHA becomes a very potential alternative to the non-degradable conventional plastics in many applications such as packaging, additives, plasticisers and lately in the medical and pharmaceutical industries [1,40].

PHA biopolymers are characterised into two groups, namely homopolymers (PHB, PHV, PHMB, and PHMV) and copolymer (PHB-co-HV). Both types of PHA biopolymers are biocompatible and biodegradable; and their physicochemical properties are similar to those of the traditional polymers synthesised from petroleum [16]. The most common product obtained from the PHA production process is the homopolymer PHB [9,40–42]. Crystallinity of the PHB is high in the range of 55–80%. The glass transition temperature and melting point of PHB are 5℃ and 175℃ respectively [16]. Having a low molecular weight of less than 1,000,000 Da, PHB is suitable to apply in making softeners in polymer blends and food packaging [30].

Table 2
Variation of copolymer properties depending on HV fractions [16].

Percentage of HV in the PHB-co-HV copolymer	0% HV	10% HV	25% HV	30% HV
Molecular weight (Da)	$3.5x10^5$	$4.0x10^5$	$17x10^5$	$18x10^5$
Polydispersity	1.2	3.3	2.5	2.1
Glass transition temperature (℃)	−19	1.6	56	42
Melting temperature (℃)	145	168	139	141
Melting enthalpy (J/g)	40.0	44.8	4.7	4.7
Degree of crystallinity (%)	30	34	4	4

Being less commonly produced, the copolymer (PHB-co-HV) has an incorporation of a monomer unit named hydroxyvalarate (HV) in the polymeric chain of hydroxybutyrate (HB). Due to the presence of HV, mechanical properties of the copolymer PHB-co-HV are improved significantly with increasing strength, toughness, and flexibility. Furthermore, an increase in the fraction of HV results in a significant decrease in the melting temperature without affecting the degradation temperature, hence the processing condition becomes easier [9,16,23]. In Table 2, processing properties such as glass transition temperature, melting temperature, melting enthalpy, and degree of crystallinity of the copolymer with 10% HV fraction are higher than that of the homopolymer PHB (0% HV). The glass transition temperature increases dramatically from −19℃ to 1.6℃. The melting temperature increases from 145℃ to 168℃ as both melting enthalpy and crystallinity increase from 40.0 to 44.8 J/g and 30 to 34% respectively. With further increase in the fraction of HV from 10% to 30%, the properties of the copolymer are improved by a drop from 3.3 to 2.1 in the polydispersity but a significant increase in the glass transition temperature 1.6 to 42℃ [16]. Due to the improved physicochemical properties, the copolymer PHBV is more desirable in the production of PHA bioplastics.

1.2. Mechanisms of the metabolic pathways for PHA microbial synthesis

PHA synthesis occurs intracellularly once the carbon substrates enter the bacterial cells via transportation across the cytoplasmic membrane by diffusion. Three basic metabolic pathways take action to produce short-chain length PHA (scl-PHA; 3 to 5 carbon atoms) or medium-chain length PHA (mcl-PHA; 6 to 14 carbon atoms) [12,43]. Genetic engineering mainly deals with these three basic pathways to modify on the PHA biosynthesis mechanisms and the gene of the PHA synthetic enzymes for diversifying PHAs biopolymers [12].

Pathway 1 (acetyl-CoA to 3-hydroxybutyryl-CoA, also known as glycolytic pathway) is taken by the PHA producing strain named *Ralstonia eutropha* (also known as *Cupriavidus necator* or *Alcaligenes eutrophus*). Carbon substrates for pathway 1 are sugars, fatty acids or amino acids. The sugar-based substrates are initially converted to pyruvate via Enter- Doudoroff (ED) for mannose, galactose, and glucose; Pento Phosphasphate Shunt (PPS) for xylose and arabinose [44]. The pyrurate component is further converted to acetyl-CoA as a starting substrate for the scl-PHA synthesis. Acetoacetate substrate is directly converted to acetyl-CoA by the acetoacetyl-CoA synthetase [45]. With the facilitation of PHA storage enzymes (pha A, pha B and pha C), acetyl-CoA is subsequently synthesised to acetoacetyl-CoA, then (R)-3-hydroxybuanoyl-CoA known as 3HB monomer and finally polymerised into poly(3-hydroxybutyrate) or PHB (scl-PHA) as shown in Fig. 1.

The other two pathways are β-oxidation and in situ fatty acid synthesis to produce mcl-PHA as shown in Fig. 2 and Fig. 3 [7,21,48]. Bacterial strains found in the pathway 2 (β-oxidation) are typically

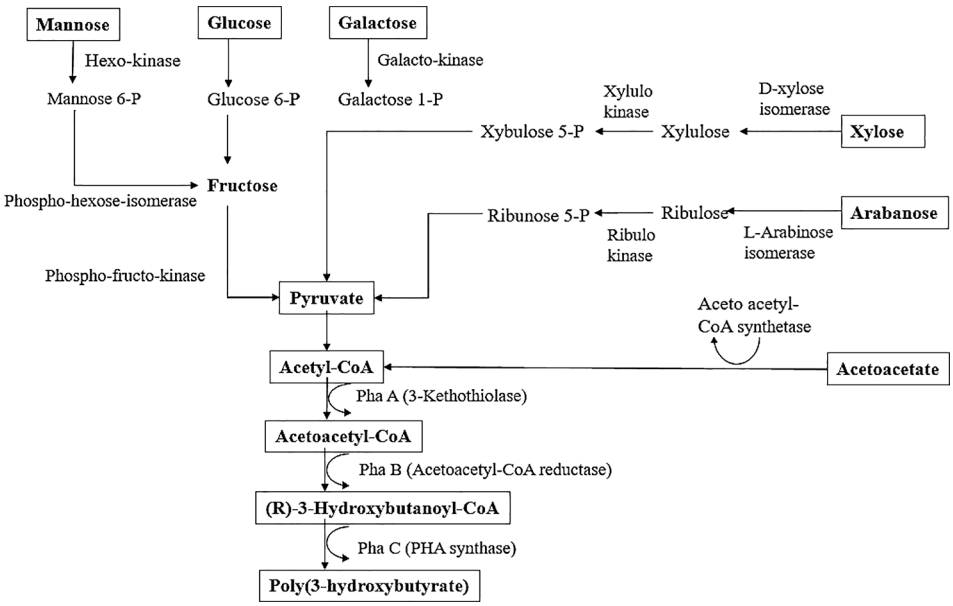

Fig. 1. Pathway 1 – Acetyl CoA to 3-Hydroxybutyryl-CoA to produce PHB (scl-PHA) [7,46,47].

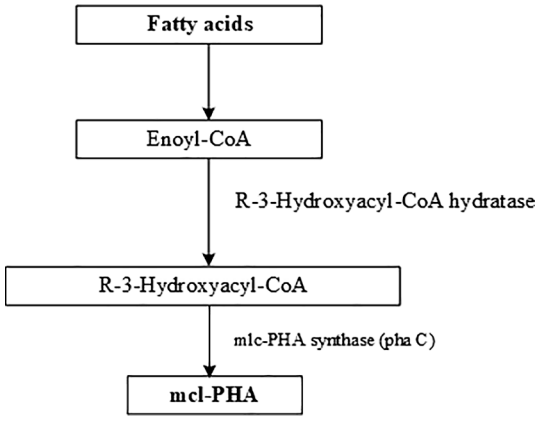

Fig. 2. Pathway 2 – β-Oxidation to produce mcl-PHA [12].

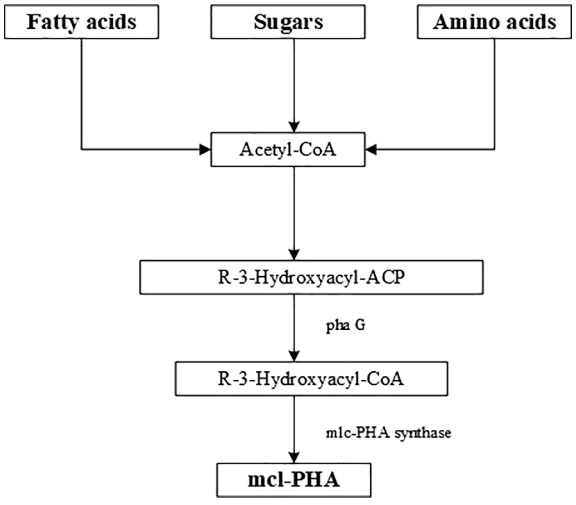

Fig. 3. Pathway 3 – In situ fatty acid synthesis to produce mcl-PHA [12].

Pseudomonas putida, *P. oleovorans*, and *P. aeruginosa* which consume fatty acids to produce enoyl-Coa which subsequently transformed to R-3-hydroxyacyl-CoA (precursor) by R-3-hydroxyacyl-CoA hydratase. This precursor is catalysed by mcl-PHA synthase for polymerisation of mcl-PHA [12].

In the pathway 3 (in situ fatty acid synthesis also known as fatty acid denovo synthesis), uptake of amino acids, sugars and fatty acids by the *Pseudomonas aeruginosa* generates an intermediate acetyl-CoA. This intermediate is converted to R-3-hydroxyacyl-ACP. Pha G, known as 3-hydroxyacyl-acyl carrier protein-CoA transferase is a key catalytic enzyme converting R-3-hydroxyacyl-ACP to R-3-hydroxyacyl-CoA. Polymerisation of R-3-hydroxyacyl-CoA is eventually carried out by mlc-PHA synthase. Hence, depending on the type of carbon substrates being fed to the bacterial cultures, genes involving in different metabolic pathways will be adopted to metabolically synthesise scl- or mcl-PHA biopolymers.

2. PHA production schemes for mixed culture and carbon waste sources

Renewable carbon feedstocks which has zero-cost and generated abundantly are shown to be compatible with the mixed culture [43]. A variety of carbon sources were examined as feedstocks for the mixed culture process to produce PHA as can be seen in Table 3. Generally, the feedstocks are categorised into two types i.e. feedstocks for three-step process and feedstocks for two-step process in the mixed culture PHA production (named as MC-PHA production).

In the three-step process as shown in Fig. 4, feedstocks are usually subjected to pretreatment, culture enrichment and PHA accumulation. Agricultural wastes, wastewater effluents discharged from crude oil, food, pulp and paper industries are particularly subjected to PHA production via three-step processes [43]. In the first step, anaerobic conditions are applied in pretreatment where the acidogenic fermentation is taking place to convert long chain carbon in wastewaters and waste oils into volatile fatty acids (VFAs), which are the precursors for mixed culture enrichment and PHA accumulation. On a side note, agricultural wastes such as sugar bagasse, wheat/rice straw, corn stover, oil palm empty fruit bunches which are rich in cellulosic component would undergo hydrolysis as pretreatment to produce sugars (glucose, fructose, xylose, manose and arabinose). In culture enrichment followed by PHA accumulation, the uptake of the VFA and sugar feedstocks by the PHA

Table 3
Production of PHA from carbon waste sources and mixed cultures.

Carbon sources	Pretreatment	Culture enrichment and PHA accumulation					Results			Ref.
		Carbon substrate	Biomass mixed culture	Nutrients	Operation mode (cycle, HRT, SRT)	Other operating conditions	PHA storage	Type of PHA	Biomass growth and others	
Olive oil mill effluent	Pretreated with bentonite, Anaerobic fermentation, Centrifuge	VFAs (acetic, lactic & propionic acids) OLR = 8.5 g COD/L.d	Activated sludge MC C_X = 300 mg VSS/L		SBR (F/F regime)	pH 7.5 25℃	q_{PHA} = 420 mg COD/g COD.h C_{PHA} = 465 g COD/L after 350 h $Y_{PHA/S}$ = 1 mg PHA/mgVFA Final PHA content = 0.54 g PHA/g VSS or 54%	Copolymer PHB-HV HV content 11% (on molar basis) due to the presence of propionic acid		[57]
VFAs (acetic, lactic & propionic acids)		Cs = 8.5 g COD/L OLR = 8.5 g COD/L.d	Activated sludge MC		SBR (F/F regime)		q_{PHA} = 649 mg PHA/g VFA.h $Y_{PHA/S}$ = 0.45 mg PHA/ mg VFA PHA content 50%	Copolymer 31%		[54]
Paper mill wastewater	Acidogenic fermentation 100 rpm pH 6 room temperature	VFAs OLR = 4.5 g COD/L.d	Activated sludge MC	NH_4Cl and other nutrients supplied to influent to obtain C limiting condition	SBR (F/F regime) SRT = HRT = 2 d No settling Cycle 24 h	Aeration 30℃ pH 7.0	$Y_{PHA/S}$ = 0.80 gCOD PHA/ gCOD S PHA content 76.8% Productivity 2 g/L.d	PHA producing bacteria: *Plasticicumulans acidivoran*	Biomass growth: C_X = 1.79 gVSS/L C_X = 1.29 gTSS/L	[43]
Sugarcane wastewater	Anaerobic fermentation pH 4.8 35℃	VFAs (acetate, propionate, butyrate, valerate 72%, saccharide 20.6%, undefined inert substances 9.2%) Cs = 252 gCOD/L	Activated sludge MC C_X = 3.65 g/L		SBR (F/F regime) SRT 5 d HRT 1 d Cycle 12 h	pH 7 20℃	PHA content 61.26% $Y_{PHA/S}$ = 0.68 mg COD/mg COD q_{PHA} = 0.31 mg COD/mgX.h		F/F ratio 0.04 $Y_{X/S}$ = 0.18 mgCOD/mgCOD	[53]
Sugar molasses	Acidogenic fermentation pH 6 30℃	VFAs Cs = 45 Cmmol VFA/L	Activated sludge MC	Amonia phosphate C/N/P = 100/8/1 (molar basis)	SBR (F/F regime) SRT 10 d HRT 1 d Cycle 12 h	pH 8 500 rpm 23-25℃	PHA content 74.6% q_{PHA} = 0.43 Cmol PHA/ Cmol X.h $Y_{PHA/S}$ = 0.81 Cmol PHA/ Cmol VFA		F/F ratio 0.21–1.1 good PHA storage	[58]
Palm oil mill effluent	Anaerobic fermentation 30℃, pH 4.5	VFAs Cs = 750–950 mg VFA/L OLR = 360 mg VFA/L.d	Activated sludge MC	Nutrients supplied for bacterial growth C/N/P = 10/2/1 (molar basis)	SBR (F/F regime) HRT = SRT 2d No settling	pH 7 28-30℃	PHA content 64% q_{PHA} = 0.24 mgPHA/mgX.h	HB:HV = 77:23 (% mol)	$Y_{X/S}$ = 0.03 mgX/ mgVFA	[52]

(*continued on next page*)

Table 3 (*continued*)

Carbon sources	Pretreatment	Culture enrichment and PHA accumulation					Results			Ref.
		Carbon substrate	Biomass mixed culture	Nutrients	Operation mode (cycle, HRT, SRT)	Other operating conditions	PHA storage	Type of PHA	Biomass growth and others	
					Cycle 24 h	Aeration 1vvm	$Y_{PHA/S}$ = 0.59 mg PHA/mg S			
Crude glycerol (glycerol 89%, 6.98% moisture, 1.7% salts)	Without pretreatment	OLR = 360–1000 mgC/L.d	Activated sludge MC	Basic nutrient for bacterial growth	SBR (F/F regime) HRT = SRT 2d No settling Cycle 24 h	pH 7 Aeration 1/3 vvm 28-30℃	PHA content 80% $Y_{PHA/S}$ = 0.7 mgC PHA/mgC S q_{PHA} = 0.16 mgC PHA/mgC X.h Productivity 236 mg PHA/L.h	Copolymer PHB-HV (60:40)	F/F ratio 0.26–0.4 C_X = 1.7–2 g/L $Y_{X/S}$ = 0.01 mgC X/mgC S	[17]
Crude glycerol (70% glycerol, 30% methanol & other free fatty acids)	Without pretreatment	OLR = 50 CmM/d = 4.6 g/L.d	Activated sludge MC	Ammonia chloride and phosphorus C/N/P = 100/6/1 (molar basis) N sufficient for biomass growth	SBR (F/F regime) SRT 5d HRT 2d Settling Cycle 24 h	Aeration 1 L/min pH 8.0–8.4 400 rpm 20-22℃	PHA content 59% $Y_{PHA/S}$ = 0.44 g/g q_{PHA} = 0.046 Cmmol HB/Cmmol X.h q_{GB} = 0.018 Cmmol GB/Cmmol X.h	PHB and glucose biopolymer (GB)	F/F ratio less than 0.2 favouring PHA storage and selection C_X = 9.983 Cmol X/L $Y_{X/S}$ = 0.310 Cmmol X/Cmmol S	[42]
Crude glycerol (72% glycerol, 25.7% methanol, 2.58% FFA and FAME)	Without pretreatment	Cs = 30 CmM OLR = 2.8 g/L.d	MC culture acclimatised to bio-oil	C/N/P = 100/8/1 (molar basis) N sufficient for biomass growth	SRT 5d HRT 2d Settling Cycle 24 h	Aeration 1 L/min 400 rpm pH 7.2–8.2 20-23℃	PHA content 47% $Y_{PHA/S}$ = 0.44 g COD/g COD Productivity 0.27 g PHA/L.d	HB and GB	F/F ratio 0.04–0.12 for PHA storage response $Y_{X/S}$ = 0.11 Cmol X/Cmol S	[59]
Crude glycerol	Without pretreatment		MC enriched in yeast and bacteria				PHA content 7.4%	Multiple biopolymer products TGA 4.6% in yeast cell PG 28% PHB, PHV and PHBV	Methanol for cell growth	[22]
Crude glycerol	Anaerobic fermentation	VFAs, 1,3-PDO Cs = 90 CmM OLR = 3.7 g COD/L.d	Activated sludge MC		SRT = HRT 1d Cycle 12 h	Aeration 1 L/min 500 rpm pH 8	q_{PHA} = 1.13 Cmol PHA/Cmol X.h $Y_{PHA/S}$ = 0.84 gCOD PHA/gCOD S PHA content 76%	Copolymer PHB-co-HV		[26]

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Table 3 (*continued*)

Carbon sources	Pretreatment	Culture enrichment and PHA accumulation					Results			Ref.
		Carbon substrate	Biomass mixed culture	Nutrients	Operation mode (cycle, HRT, SRT)	Other operating conditions	PHA storage	Type of PHA	Biomass growth and others	
						30℃	C_{PHA} = 1.48 g/L Productivity 0.41 g PHA/L.h			
Cheese whey (lactose 79.3%, protein 9.1%, fat 0.7%, ashes less than 8.5%)	Acidogenic fermentation	VFAs (acetate butyrate, propionate, lactate, valerate, ethanol)	Activated sludge MC	C/N/P = 100/4/0.6	HRT 1d	Aeration 1vvm	$Y_{PHA/S}$ = 0.96 Cmol PHA/Cmol S	Copolymer with 13.2% HV (%mol)	Uncouple C&N feeding strategy has higher PHA production than the conventional ADF strategy.	[18]
	300 rpm	OLR = 100 Cmmol/L.d = 8.5 g/L.d		Uncouple C and N feeding strategy	SRT 4d	300 rpm	q_{PHA} = 0.4 Cmol PHA/Cmol X.h			
	30℃ pH 6				Settling Cycle 12 h	pH 8.1–8.9 23-25℃	Productivity 6.02 gPHA/gX.d			
Sodium acetate		Sodium acetate	Activated sludge MC	N and P supply	HRT 8 h	Aeration 1.2–1.8 L/min	PHA content 70% (wt.%)	PHB	C/N ratio 6–13.2 for C limited (acetate uptake rate)	[60]
		Cs = 30 and 165 mM			SRT 1d	pH 7	$Y_{PHA/S}$ = 0.06 Cmol PHA/Cmol S		C/N ratio 15–24 for N limited (ammonia uptake rate)	
					Settling Cycle 4 h	20℃			$Y_{X/S}$ = 0.29 Cmol X/Cmol S	
VFAs		VFAs	Activated sludge	N and P supply for Nitrogen limited condition	Cycle 23 h	250 rpm	PHA content 29.98%		$Y_{X/S}$ = 0.591 gVSS/gVFA	[34]
					Settling	28℃	$Y_{PHA/S}$ = 0.334 g PHA/g VFA q_{PHA} = 11.64 mg/L.h		q_X = 20.59 mg/L.h	
Acetate sodium		Acetate sodium	MC	N and P supply	HRT 1d	Aeration	$Y_{PHA/S}$ = 0.6 g PHA/g S		$Y_{X/S}$ = 0.24 g X/g S	[61]
		Cs = 1400 mg/L			SRT 5d	30℃	q_{PHA} = 0.292 mg PHA/mg VSS.h			
					Settling Cycle 12 h		PHA content 64.7%			
VFAs		VFAs	Enriched MC for extended cultivation for 10 days, on basis of feast famine	C/N/P = 100/6/1.5 (mass ratio)	SRT 10 days	pH 7	PHA content 71.4%		Final cell density 17.22 g/L	[56]
		Cs = 4.8 g COD/L			Cycle 12 h	21℃	$Y_{PHA/S}$ = 0.49 gCOD PHA/gCOD VFA		Biomass magnification 43 and 52 with and without sludge discharge	
		OLR = 1.2 g/L.d					Productivity 1.21 g PHA/L.d			
Synthetic wastewater		Sodium acetate	Activated sludge	C/N/P = 100/12/2 nitrogen sufficient	HRT 12 h	No pH control	**Under nitrogen deficient:**	PHB		[62]
		Cs = 300 mg COD/L		100/2/2 nitrogen deficient	SRT 8d	20℃	$Y_{PHA/S}$ = 0.61 Cmmol PHA/Cmmol S	PHV		

(*continued on next page*)

Table 3 (*continued*)

Carbon sources	Pretreatment	Culture enrichment and PHA accumulation					Results			Ref.
		Carbon substrate	Biomass mixed culture	Nutrients	Operation mode (cycle, HRT, SRT)	Other operating conditions	PHA storage	Type of PHA	Biomass growth and others	
					Settling Cycle 6 h		PHA content 20.71% q_{PHA} = 0.276 Cmmol PHA/ Cmmol X.h q_S = 0.490 Cmmol S/ Cmmol X.h **Under nitrogen sufficient:** $Y_{PHA/S}$ = 0.43 Cmmol PHA/ Cmmol S PHA content 14.24% q_{PHA} = 0.180 Cmmol PHa/ Cmmol X.h) q_S = 0.433 Cmmol S/ Cmmol X.h	P3H2MV		
Synthetic wastewater		Sodium acetate Cs = 300 mg COD/L	Activated sludge	C/N/P = 100/ 12/2 nitrogen sufficient C/N/P = 100/ 2/2 nitrogen deficient	HRT 12 h SRT 8d Settling	No pH control 20℃	PHA content 43.3% $Y_{PHA/S}$ = 0.69 gCOD PHA/ gCOD S		Biomass stable after 40d C_X = 2310 mg/L	[63]
VFAs (acetate and propionic acid mixture)		VASs (acetic and propionic acids) Cs = 8.5 g/L OLR = 8.5 gCOD/L.d	Activated sludge	C/N = 14.3 Cmol/Nmol for uncouple feeding strategy	SRT = HRT 1d No settling Cycle 6 h	Aeration greater than 2 mg O_2/L pH 7.6 25℃	$Y_{PHA/S}$ = 0.4 g Cod PHA/g COD S C_{PHA} = 1300 mg COD/L	Copolymer HV content 20%		[55]
Acetate and methanol		$C_{acetate}$ = 13.5 mM $C_{methanol}$ = 27 mM	Activated sludge	C/N = 8 Cmol/Nmol	SRT 1d Settling at the end of feast to remove supernatant Cycle 12 h 30℃	Aeration 750 rpm pH 7	PHA content 60% $Y_{PHA/S}$ = 0.62 Cmol PHA/ Cmol S	PHB	$Y_{X/S}$ = 0.38 Cmol X/Cmol S	[64]
Acetate		Acetate C_S = 1.2 g COD/L OLR = 1.2 g COD/L.d	Activated sludge	C/N/P = 100/ 10.3/6.1	HRT 1d SRT 2d Settling	Aeration 22-24℃	**Under no pH control 8.8–9.2, and N limited conditions:** PHA content 51% g PHA/g VSS			[65]

(*continued on next page*)

Table 3 (*continued*)

Carbon sources	Pretreatment	Culture enrichment and PHA accumulation					Results			Ref.
		Carbon substrate	Biomass mixed culture	Nutrients	Operation mode (cycle, HRT, SRT)	Other operating conditions	PHA storage	Type of PHA	Biomass growth and others	
						no pH control	$q_{PHA} = 0.16$ Cmol PHA/ Cmol X.h			
					Cycle 12 h	8.8–9.2	$Y_{PHA/S} = 0.33$ Cmol PHA/ Cmol S			

Note: VFA: volatile fatty acids; SRT: solid retention time; HRT: hydraulic retention time; S: substrate; X: biomass.
q_{PHA}: PHA production rate.
q_X: biomass or cell growth rate.
$Y_{PHA/S}$: yield of PHA on carbon substrate.
$Y_{X/S}$: yield of biomass on carbon substrate.
C_{PHA}: PHA concentration.
C_X: biomass concentration.
C_S: substrate concentration.
OLR: organic loading rate.

producing bacteria in the mixed culture will take place via the three metabolic pathways (glycolytic, β-oxidation and in situ fatty acid synthesis) as mentioned in the previous section.

In the two-step process (Fig. 5), the pretreatment step is omitted when using glycerol-based feedstock or synthetic VFA mixtures, which can be directly be fed to the enrichment and PHA accumulation steps. A study in examining crude glycerol (a byproduct from biodiesel processing) as feedstock for the mixed culture process has found that glycerol entering the bacterial cells via the Embden-Meyerhof-Parnas (EMP) pathway to produce pyruvate [30]. The intermediate pyruvate formed in the cells is further converted and polymerised into scl-PHA (PHB) via the glycolytic pathway (acety-CoA to 3-hydroxybutyryl-CoA) and/or to produce mcl-PHA via the in situ fatty acid synthesis pathway as illustrated in Fig. 6 [30,49]. Therefore, the glycerol-based feedstock can be as effective as other common carbon feedstocks like fatty acids and sugars entering the pathways 1 and 2 to produce both scl- and mcl-PHA biopolymers. This is because in term of oxidation level, the three carbon atoms in the glycerol molecules are chemically in a higher reduced state than that of glucose or lactose. When uptaking glycerol, a more reduced physiological state in the bacterial cells is achieved, thus favours the synthesis of intracellular PHA [30].

3. Enrichment of mixed culture by imposing feast/famine regime

Back in 1996, studies done by Majone *et al.* and Satoh *et al.* have reported that activated sludge was able to accumulate PHA due to the presence of some bacteria with PHA storing ability under the aerobic conditions, besides the presence of non-PHA storing bacteria [50,51]. Enrichment is an essential step in the PHA production as proven by the experimental result of Majone *et al.*, which has shown that the performance of the enriched MC obtained under the unbalanced growth conditions was 405 mg for PHA storage rate and PHA content of 44%. These results were significantly higher than the MC without enrichment (21 mg and 10% respectively). Due to such big differences, the enriched MC was evaluated to contain more bacteria capable of accumulating PHA in comparison with the initial activated sludge which contained less PHA accumulating bacteria [51]. Therefore, the enrichment strategies for the culture selection play an important role in obtaining a MC with a high PHA storing capacity.

In a mixed culture, when the external carbon source is available, the PHA producing bacteria consume carbon for both cell growth and intracellular accumulation of the PHA as carbon or energy storage. When there is an environmental stress such as carbon depletion, the PHA-producing bacteria can survive by changing its metabolism to consume the accumulated PHA in their cells. In contrast, bacteria without the ability to store PHA (non-PHA accumulating bacteria) will be hard to survive during the starvation period [51]. Based on this finding, a mechanism of the enrichment strategy is developed with alternative periods between carbon availability (Feast) and carbon starvation (Famine) applied to the MC. This strategy creates a selective pressure on the mixed culture, which gradually enriches with PHA producing bacteria. which are *Alphaproteobacteria* and *Betaproteobacteria* as the most dominant species [17,52].

In many studies, this carbon feeding strategy is recognised as an effective way for the culture selection and become the fundamental carbon feeding strategy known as aerobic dynamic feeding (ADF) or feast/famine (F/F) regime [17,20,53]. The imposition of the F/F regime creates an internal growth limitation on the MC. To explain for this point, the absence of external carbon supply in the famine phase causes a decrease in the intracellular components (RNA and enzymes) required for cell growth of PHA storing bacteria. When an excessive carbon supply is resumed in the feast phase, the amount of growth enzyme becomes insufficient, and instead the storage enzyme is triggered the storage of PHA [7,47]. Operation of the F/F regime in the enrichment is commonly conducted in a sequencing batch reactor (SBR) which can be

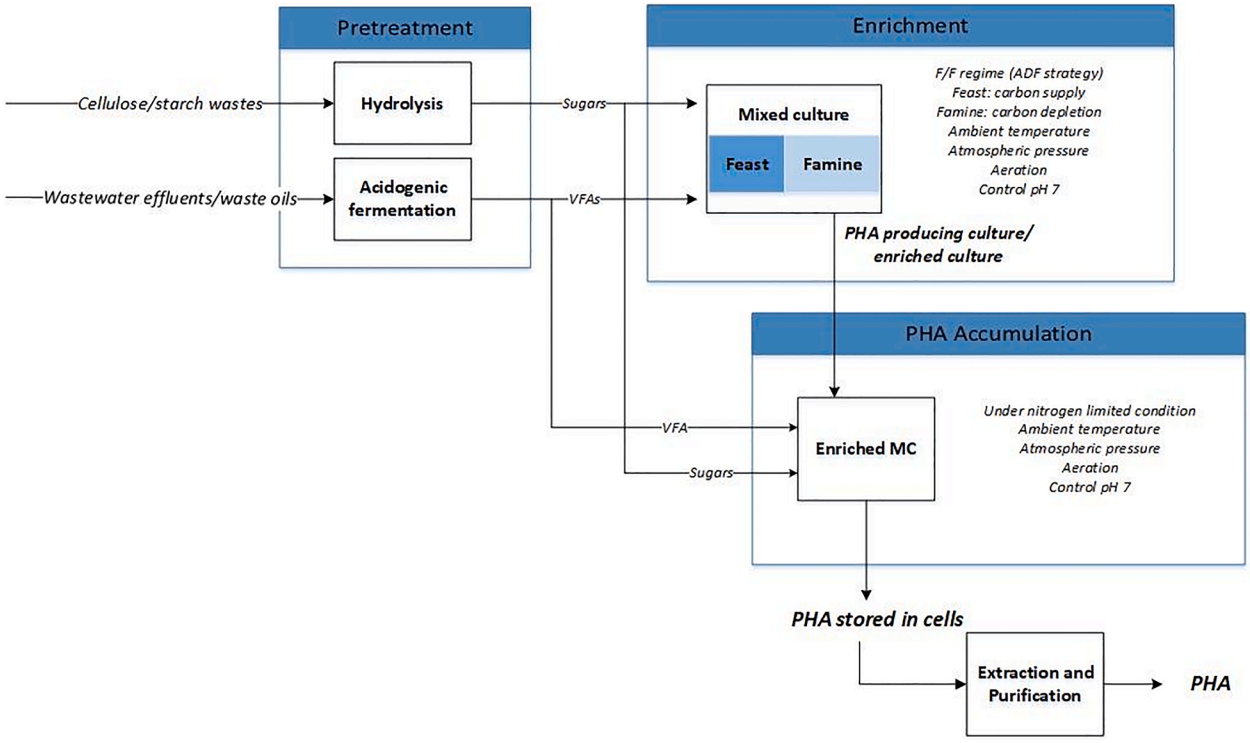

Fig. 4. MC-PHA production process in three steps.

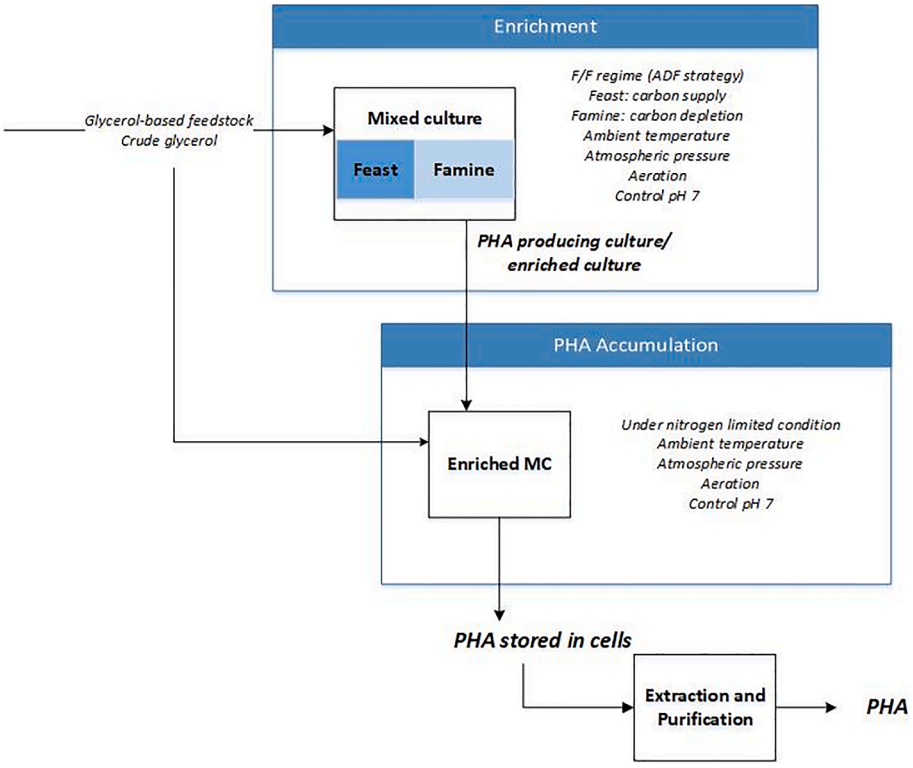

Fig. 5. MC-PHA production process in two-steps.

set up for the intermittent carbon supply to the MC. SBRs operate in cycles consisting of feeding, reaction (feast and famine), settling, and discharge. After several cycles (when F/F ratio being stabilised at approximately 1:4) in the enrichment stage, a stable MC enriched with PHA storing bacteria is obtained and proceeded to the PHA accumulation stage under the nitrogen-limiting condition [17,21,54].

In the enrichment, an effective selection of PHA storing bacteria depends on the response of activated sludge MC to the feast-famine regime [47]. The enriched MC will be able to show a stable performance in producing PHA in the accumulation stage in terms of stable

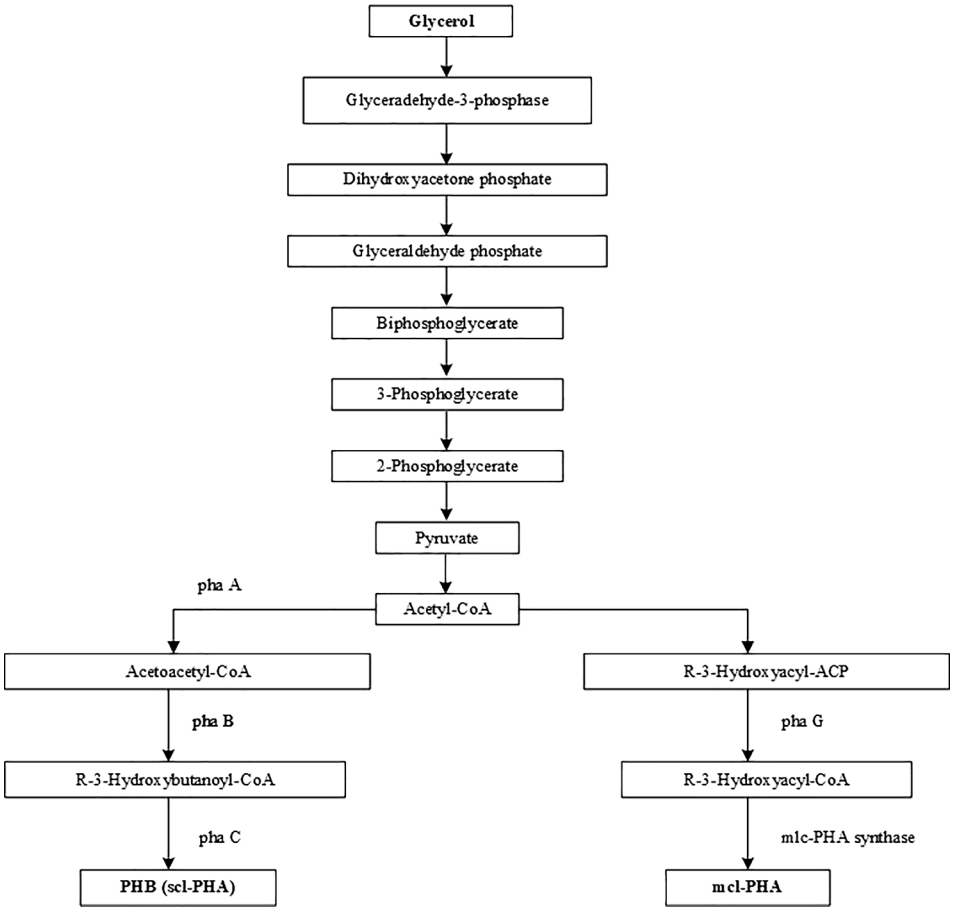

Fig. 6. Metabolic pathways from glycerol to PHA.

PHA content, yield of PHA to carbon substrate, volumetric productivity, polymer composition, and culture stability. These aspects should be included in evaluating the performance of PHA production by the MC.

A majority of studies focused on the F/F regime for the enrichment with varying operating conditions of the SBRs and different carbon waste as feedstocks as shown in Table 3. Results of these studies often showed a high PHA storage in terms of PHA yield (0.4–0.8 g PHA/g S) and accumulated content (30–80%). However, cell growth (biomass growth) of the enriched culture is another aspect to consider in the MC-PHA production besides the PHA storage, as this could be one of the factor that causes the lower productivity compared to PHA production by using pure culture. Some recent research has been conducted to gain more understanding in the cell growth and its effect on improving the PHA productivity of the enriched culture by devising new strategies based on the F/F regime [18,55,56]. Hence, both aspects (PHA storage and cell growth) can be ensured for an efficient MC-PHA production.

4. Aspects to be considered in MC-PHA production

4.1. Type of feedstocks

4.1.1. Feedstocks for the three-step process

Wastewater effluents in the waste streams of other industry are complex carbons that need to be converted to a simpler carbon (VFAs) prior to enrichment and PHA accumulation steps as shown in Fig. 4. In Table 3, the acidogenic fermentation conducted under the anaerobic conditions is a common pretreatment method applied to the feedstocks which are from wastewater effluent streams, lignocellulosic hydrolysate and molasses [43,57,58].

In the studies applying the anaerobic fermentation, the use of VFA mixtures usually resulted in the production of copolymer P(HB-co-HV) at high yield and PHA content with HV in the range 11%-31% [54,57]. An example is shown in the olive oil mill extraction where the bentonite pretreated wastewater effluent was fermented anaerobically at pH 6.5 and fluxed with CO_2 and N_2 at 25℃. The obtained supernatant was a VFA mixture containing acetic acid (40%), lactic acid (40%) and propionic acid (20%) which was subsequently fed to an activated sludge mixed culture. In the last PHA accumulation step, copolymer P(HB-HV) was obtained with 11% HV content on a molar basis, yield of 1 mg PHA/mg VFAs and PHA content of 54% [57]. In another study by using anaerobic fermented palm oil mill effluent as feedstock, the mixed culture has accumulated copolymer P(HB-co-HV) with 23% HV content, 59% PHA yield and 64% PHA content [52]. Furthermore, wastewater collected from the paper mill and sugarcane processes was also examined in the three-step process and shown to result in a good PHA storage with high yield from 0.68 to 0.80 g COD PHA/g COD VFA and high PHA content in the range 61–77% [43,53].

In the cheese-making process, cheese whey - a byproduct rich in carbon compounds such as lactose, protein and fat was fermented to obtain a mixture of VFAs at various concentrations of acetate, butyrate, propionate, lactate, valerate and ethanol [18]. The activated sludge mixed culture being fed with the fermented cheese whey showed a good performance in producing PHA copolymer with HV content of 13.2%, high yield of PHA at 0.96 Cmol PHA/Cmol S, and the production rate at 6.02 g PHA/g X.d. Instead of applying anaerobic fermentation, less extensive pretreatment methods were applied to cheese whey via the enzymatic hydrolysis followed by permeation. The pretreated cheese whey hydrolysate (glucose) was used to grow a culture consortium of

Haloferax mediterranei and *Cupriavidus necator* mRePT in a fed-back bioreactor to produce copolymer PHB-co-HV and mlc-PHA at contents of 53% and 35.6% respectively [66,67].

Sugar components in the molasses was also applied to produce VFA feedstock for MC-PHA production via anaerobic fermentation [58]. As a result, PHA storage response of the enriched culture fed with VFAs was able to accumulate PHA at a content of 74.6%, at a high yield of 0.81 Cmol PHA/Cmol VFA.

On the other hand, pretreatment of lignocellulosic material was carried out via hydrolysis followed by detoxification to obtain glucose [68]. In two studies, wheat straw and sugarcane crop residues were first pretreated via ammonia fibre expansion (AFEX) and alkaline pretreatment respectively to make lignocellulosic material less recalcitrant. Subsequently, enzymatic hydrolysis was performed to convert the cellulosic and hemicellulosic components into glucose and xylose, which were then used for growing biomass in the pure culture of *Halomonas boliviensis* LC1 and *Bacillus firmus* N II respectively in the fermenters. The enriched cultures were then used for accumulation of PHB at content of 89% (1.9 g/L CDW) and 72% (146 g/L CDW) in batch [69,70]. Till date, mixed culture originated from activated sludge was not being tested for PHA production by using lignocellulosic substrate.

For carbon waste feedstocks used in the three-step process, nutrient supplement such as nitrogen and phosphorus is necessary for the cell growth in the MC-PHA production since the feedstocks are poor in nutrients as suggested in some studies [18,43,58]. Therefore, dosage of the nutrient supplement and its ratio to the amount of carbon supply were examined further to determine the status of enrichment reactor under nitrogen sufficiency or deficiency that is further discussed section 4.4.

4.1.2. Feedstocks for the two-step process

In recent years, crude glycerol, a byproduct of biodiesel production has been examined in the research as a potential feedstock for the mixed culture [17,30,71,72]. Originally, vegetable oils extracted from palm fruit, grape seed, soybean, used cooking oil, and animal fats are substances containing triglycerides. The triglycerides in these fatty substances undergo a *trans*-esterification process which reacts with an alcohol (methanol or ethanol) in the presence of catalysts to form fatty acid methyl esters (FAME) known as biodiesel, and another alcohol called glycerol or glycerine together with other inorganic impurities [73]. While biodiesel is the main and desired product as an alternative to petroleum-based diesel fuel, crude glycerol is the byproduct with a low commercial value in the biodiesel production industry. Since crude glycerol is in the simplest three carbon form, it was used in the two-step process for PHA production as illustrated in Fig. 5 [17,22].

Conversion of crude glycerol into PHA bioplastic was successfully carried out by using pure cultures as well as bacterial consortium. *Cupriavidus necator* or *Rostoria entropha* is the most common bacterial strain being used in pure cultures, besides *Thermophilic Caldimonas manganoxidans*, *B. megaterium k and C. hydrocarbooxydans* [9,11,40,41,48,74–76]. Results from these recent studies showed that the PHA content in dry cell weight ranges from 40 to 80%, at PHA yield in the range 0.4–0.8 g PHA/g carbon substrate. In a study using thermophilic *Caldimonas Mangaoxidans* bacteria, the content of PHA was 71% at a concentration of 8.4 g/L [40]. With bacteria extracted from marine environment namely *Bacillus cereus MCCB 281* using sea water as a culture medium, PHA content of 68.27% at concentration of 2.54 g/L was obtained [11]. The waste stream of the biodiesel production containing residual glycerol and carboxylic acids was also being utilised as feedstock in a separate study [41]. The waste stream was first neutralised before feeding it to the pure culture for PHA production. The pure culture used in this study was a consortium of two different bacteria *B. megaterium k* and *C. hydrocarbooxydans*. The obtained PHA concentration was 8.01 g/L with 44.9% PHA content [41].

Crude glycerol was also used as a carbon feedstock for the mixed culture to produce PHA in some studies focusing on the two-step process as shown Table 3. In a stable enriched MC, the accumulation of PHA could reach up to 80% with a high yield of 0.8 mgC PHA/mgC substrate [17]. In a similar study, the PHA content and yield were 76% and 0.99 Cmol PHA/Cmol substrate respectively, resulted from the enriched culture under the feast/famine regime fed with crude glycerol [26]. These results have suggested that the combination of mixed culture and crude glycerol has resulted in comparable or even higher yield as compared to its pure culture counterpart. The PHA yield and content obtained from the MC-PHA production from crude glycerol were evaluated to be as high as those studies using wastewater and cheese whey. However, PHA productivities of the mixed culture fed by crude glycerol were very low, ranging from 263 mg PHA/L.h to 0.41 PHA/L.d as compared with the productivity of the pure culture [17,26,59].

Table 4
Variation of the operating parameters in the SBRs.

Operating parameters for SBRs	Three-step process	Two-step process
OLR	0.36–8.5 g/L.d	0.36–4.6 g/L.d
SRT	2 to 4 days	up to 5 day
HRT	1 day	1–2 days
Cycle length	12 h	between 12 h to 24 h
Ref.	[18,52,54,56,57]	[17,26,42,59]

Interestingly, crude glycerol has shown to be not only feasible to be used as feedstock for MC-PHA production in the two-step process, but also able to produce copolymer PHB-co-HV as a new finding reported in few studies recently [17,22,26]. This is because crude glycerol contains a mixture of mainly free glycerol and glycerides which is accounted for 70% wt. alongside with methanol used in the biodiesel process, and a small fraction of free fatty acids (FFAs), fatty acid methyl esters (FAMEs) and ash [17,42,59]. Characterisation of crude glycerol collected from different biodiesel factories reported that compositions were varied significantly from 22.9% to 63.0% for glycerol and from 6.2% to 12.6% for methanol [77]. Therefore, crude glycerol is considered as a mixed carbon substrate, which can be possibly applied to produce PHA copolymer. Ratios of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3 HV) monomers in the copolymer were 60:40 and 80.9:19.1 [17,26]. In the work of Mohandas *et al.*, the percentage of HV in the copolymer was reported at 13 mol % in production of PHA by using MC enriched by crude glycerol [11]. In one study conducted by Fra-Vazquez's group, crude glycerol as carbon substrate used for PHA production process resulted in not only the formation of PHV with 7.4% accumulated in the cell, but also 27.6% of polyglucose (glucogen) and 46.3% of triacylglycerides (TAGs) [22]. As compared with homopolymer PHB, copolymer PHBV is more desired due to its better mechanical properties such as higher strength, toughness and flexibility [9,16,23]. Therefore, crude glycerol is a very promising feedstock for MC-PHA process producing the copolymer at a lower cost.

Among the carbon sources originated from wastes and industrial byproducts, crude glycerol is found to be the only carbon byproduct which has been investigated so far for the two-step process using MC for PHA production. Besides crude glycerol, other glycerol-based material such as glycerine pitch (unrefined glycerine – a byproduct form crude glycerol refinery in the biodiesel production) can be a very potential carbon feedstock to examine further. This is because glycerine pitch comprises glycerol (55–65%), diglycerol (15–25%), less than 10% of other fatty acids, methanol and inorganic salts which are varied depending on the efficiency of the refining system [78]. A study in examining unrefined glycerine pitch as feedstock for the pure culture process carried out in two steps reported the biosynthesis of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) P(3HB-co-4HB) copolymer using *Cupriavidus* sp. USMAHM13 [79]. Therefore, future research in utilising glycerine pitch feedstock for the two-step MC process would become a new study area of the PHA production.

For triacylglycerides (TAGs) present in fats, vegetable oils and waste cooking oils, a majority of studies using lipid wastes as a carbon source conducted in two steps focused on using pure culture such as *Cupriavidus*

necator, *Pseudomonas oleovorans* to produce medium to long chain length PHA and copolymer P (3HB-co-3HV) [36,37,74]. A big challenge for the oil-based feedstock is its hydrophobic nature resulting in a difficulty in microbial PHA production. Instead of direct feeding to the bacterial cultures, a pretreatment step "saponification" is required to convert TAGs into fatty acids to be used as feedstocks for PHA production [37]. For glucose and starch/cellulose/lignocellulose hydrolysate, the uptake of sugars by the mixed culture results in biosynthesis of glycogen or polyglucose rather than PHA [47]. Therefore, to utilise sugars for PHA production, an acidogenic fermentation of sugars to obtain VFAs by applying the three-step process is more advisable.

4.2. Operating conditions of enrichment reactors

Operating conditions of a sequential batch reactor (SBR) for enrichment such as organic loading rate (OLR), hydraulic retention time (HRT), solid retention time (SRT), cycle length, temperature, pH, dissolved oxygen level by aeration affect the performance of PHA production. At lab scale, bacterial fermentation taking place in the enrichment SBR (working volume from 1 to 5 L) is normally conducted at room temperature 20-30℃, pH 7–9, stirring 20–750 rpm and aeration at air flow rate of 1–2 L/min or 1/3–1 vvm in majority of the studies as mentioned in Table 3. However, other operating parameters of the SBRs such as OLR or carbon concentration, HRT, SRT and cycle length can be varied with the choice to the carbon feedstocks for two-step or three-step process as summarised in Table 4.

The operating conditions presented Table 3, that are a 12 h cycle and high OLRs ranges from 0.36 to 8.5 g/L.d are commonly applied in the culture selection of the three-step process using the SBR systems with SRT of 2–4 days and HRT of 1 day [18,52,54,56,57]. These operating conditions resulted in an effective selection of the PHA accumulating bacteria in enrichment process. In these studies, PHA storage response of the enriched MC showed a high PHA content from 50 to 75%, corresponding to high yield from 0.5 to 0.8 g PHA/g S in the batch test. For example, in the PHA production from fermented cheese whey, the MC enrichment carried out in an SBR operating at 12 h cycle, SRT of 4 day, HRT 1 day and OLR at 8.5 g/L.d resulted in an enriched culture with PHA polymer storage capacity at a yield of 0.96 Cmol PHA/Cmol S [18]. The 12 h cycle was also applied in the three step process fed with VFAs obtained from pretreatment of sugarcane wastewater and sugar molasses in two other studies have also reported high PHA yields of 0.68 mg COD PHA/mg COD VFA and 0.81 Cmol PHA/Cmol VFA respectively [53,58]. As for the two-step process using crude glycerol without pretreatment, it seems a longer cycle of 24 h but lower OLRs from 0.36 to 4.6 g/L.d favoured the culture enrichment conducted in the SBR operating with SRT up to 5 days and HRT of 1–2 days [17,26,42,59]. The PHA production by using the enriched culture obtained from the two-step process has a PHA content up to 80% and PHA yield at 0.84 g PHA/g S which are as high as the results obtained from those SBR systems by using three-step processes[17,26].

The differences in the cycle lengths and OLR ranges applied for the two-step and three-step processes could be attributed to the inclusion of the pretreatment step. Because the complex carbon sources like wastewater effluent and cheese whey are converted to a simpler carbon feedstock such as VFAs via the anaerobic fermentation, the three-step process can thus be operated at a shorter cycle of 12 h combining with a higher OLR. Whereas, using glycerol-based feedstock without pretreatment requires a longer time for the substrate uptake compared to when VFAs was fed, and thus a longer cycle of 24 h combined with a lower OLR are more suitable for the two-step process. Furthermore, for the three-step process a change in the cycle length from 12 h to 24 h could help to improve the productivity of the enriched MC which was found in the investigation of paper mill wastewater pretreated in anaerobic conditions [60]. The enriched MC showed not only a high polymer storage capacity (yield at 0.80 gCOD PHA/gCOD S, PHA content of 76.8%) but also a high productivity of 2 g/L.d, being reported as one of the highest among the studies using carbon waste source [43]. In the case of using crude glycerol in two-step process, the usual cycle length of 24 h could be reduced to 12 h, unless the anaerobic fermentation applied in pretreatment step was added to convert crude glycerol to VFAs and 1,3-propanediol (1,3-PDO) [26]. This study has reported a high production of PHB-co-HV copolymer with yield of 0.84 gCOD PHA/gCOD S, 76% PHA content and productivity of 0.41 g PHA/L.h. Therefore, the cycle lengths between 12 and 24 h combined with the OLRs for the two-step or three-step process is essential to govern the PHA content, yield and productivity.

In addition, it was found that as the cycle length decreased from 24 h to 12 h, the bacterial culture shifted its biosynthetic behaviour in which glucose biopolymer (GB) accumulation favoured over hydroxybutyrate (HB) accumulation [59]. The HB production rate at the SBR operating at 24 h cycle was at 0.046 Cmmol HB/Cmmol X.h, which reduced almost half compared to SBR operating at 12 h cycle. On the other hand, as the cycle length reduced from 24 h to 12 h, the GB production rate increased two times from 0.018 to 0.037 Cmmol GB/Cmmol X.h. Thus, when using crude glycerol as a carbon feedstock for the MC enrichment, SBR operation at the 24 h cycle would result in an enriched MC favouring the PHA accumulation rather than the 12 h cycle. The cycle length should be carefully selected by taking into consideration the suitable OLR range or carbon concentration, the associated pretreatment, and the favour for PHA biosynthetic behaviour of the enriched culture.

OLR was shown to have an effect not only on the PHA production, but for biomass growth of MC as well [17,54]. In the study by using crude glycerol, increasing OLR from 360 to 1000 mg C/L.d resulted in a significant increase in the biomass concentration from 0.7 to 1.7 g/L, which promoted the PHA storage response to accumulate PHA at a high content of 78%, PHA yield 0.7 mgC PHA/ mgC S. Besides, PHA productivity has also increased from 193 to 210 mgC/L.h with an increase in OLR. However, a further increase in OLR to 1250 mg C/L.d resulted in a sudden decrease in the PHA production with PHA content, yield and productivity dropped significantly to 27%, 0.3 mgC PHA/ mg C S and 65 mgC/L.h respectively. This increase in the OLR has resulted in a failure of the MC in responding to the PHA culture selection as F/F ratio increased from 0.26 to 2 [17]. A similar observation was also reported in the study using the VFA mixture [54]. In this study, as the OLR increased from 8.5 to 31.25 g COD/L.d., the biomass concentration increased from 3.9 to 8.7 g COD/L but the PHA production rate decreased sharply from about 500 to 20 mg COD/ g COD.h. The highest performance in PHA production was achieved at the intermediate OLR of 20 g COD/L.d where the PHA productivity and polymer storage rate were at 0.25 g PHA/L.d and 649 mg COD/ g COD.h. In addition, both studies also reported a change in the F/F ratios exceeding 2, which was unfavoured for the selection of PHA accumulating bacteria in the mixed culture at the highest OLR applied.

The effect of carbon concentration on the PHA production in the enrichment was similar to that of the OLR as being investigated in other work [53,58]. For the fermented sugar molass fed to the MC enrichment with the carbon concentration in the range 30–60 Cmmol VFA/L, the selected culture had a PHA storing capacity with the highest accumulated PHA content of 74.6% at the intermediate carbon concentration of 45 Cmmol/L [58]. In a separate study by using fermented sugarcane wastewater, two levels of the carbon concentration at 1400 and 2500 mg COD/L were fed to two different SBRs operating under same conditions (SRT 5 days, HRT 1 day in a 12 h cycle). The results showed the enriched culture at the lower carbon centration level (1400 mg COD/L) produced a higher PHA content at 51.2%, with yield of PHA to carbon substrate at 0.5 mg COD/mg COD and the polymer production rate at 0.33 mg COD/mg X.h. On the other hand, operation of the SBR at the high carbon concentration (2500 mg COD/L) showed a deterioration in the stability which made it unfeasible to be used for the PHA accumulation step [53]. Therefore, from the studies focusing on the OLR and carbon concentration, feeding of the carbon substrate should be applied at the intermediate level to obtain an enriched culture with high PHA storage

Table 5
Comparison between N deficient and N sufficient conditions in the MC enrichment.

	SBR operating conditions					
SRT (days)	8		8		1	
HRT (h)	12		12			
Cycle (h)	6		6		8	
Volume (L)	3		3		2	
Feedstock	Synthetic wastewater		Synthetic wastewater		Sodium acetate	
N supplement	N sufficient	N deficient	N sufficient100/12	N deficient100/2	N deficient17	N sufficient
C/N ratio	100/12	100/2				7.4
NH_4Cl concentration	36 mg/L	6 mg/L	24 mg/L	6 mg/L	8.5 mM	8.5 mM
PHA content	12.14%	18.63%	38.3%	43.3%	Fraction of growth in feast 70%	Fraction of growth in feast 20%
PHA yield	0.43 Cmmol PHA/ Cmmol S	0.61 Cmmol PHA/ Cmmol S	0.51 gCOD PHA/g COD S	0.69 g COD PHA/g COD S	0.06 Cmol PHB/Cmol S	0.02 Cmol PHA/Cmol S
References	[62]		[63]		[60]	

capacity and consistent performance in PHA production.

SRT of enrichment reactor is another important parameter affecting PHA production. SRT is the duration which the bacteria are maintained in the mixed culture SBR system, and it controls the biomass concentration throughout the process [80]. An investigation in the effect of the SRT on the PHA production using MC showed that at 5 day SRT, the enrichment of the PHA accumulating culture was more effective (F/F ratio at 0.06) and achieved a higher performance (PHA content 29.03%, PHA yield to carbon substrate 0.76 mg COD/mg COD, PHA production rate 0.51 mg COD/mg X.h). Whereas for the SBR operating with a longer SRT for 10 days, the enriched MC has shown to accumulate a lesser amount of PHA in the cells (PHA content, yield and production rate at 24.52%, 0.68 mg COD/mg COD, and 0.45 mg COD/mg X.h respectively) [53]. Besides that, the effect of varying the SRT from 3 to 10 days was examined in the mixed culture enrichment in the SBRs fed with synthetic wastewater containing acetate as the sole carbon source [81]. In this study, the PHA production capacities of MC obtained at the short SRT of 3 days had a higher PHA content of 31% compared to the longer SRT of 10 days, which has recorded a lower PHA content at 21%. This was because at shorter SRT, the selective pressure on the MC was stronger to favour the PHA producing bacteria as demonstrated in the study applying 2 day SRT [82]. Another reason was the weeding out effect, which gradually eliminated the non-PHA producing bacteria during the enrichment process. At a shorter SRT, the PHA producing bacteria were enriched and outgrew in the enriched culture. Whereas a longer SRT would allow more non-PHA producing bacteria to grow and dominate the culture [53]. Therefore, the PHA production of the MC was shown to be affected by the SRT, which should be applied at a lower range from 3 to 5 days.

From these reported studies examining the impact of operating conditions on PHA production, it reveals an interdependent relationship between the OLR, carbon concentration, cycle length and the F/F ratio. In the feast/famine regime, the F/F ratio should be lower than the range of 0.3–0.4 as this is generally indicating successful enrichment of the MC with high PHA storage capacity [18,47,83]. It is because the carbon depletion period (famine phase) imposes an internal growth limitation, thus induces the PHA storage within PHA producing bacteria. When feast phase is in place, the PHA producing bacteria tend to consume the available carbon substrate for PHA storage instead of for cell growth. The mechanism of inducing PHA storage in the MC can only come into effect when the famine phase is long enough to suppress the cell growth. In case of increasing the OLR or carbon concentration but without prolonging the cycle length, bacteria would take a longer time to consume the carbon substrate in the feast phase, resulting in a shorter famine phase and a higher F/F ratio. Consequently, the PHA-producing bacteria will probably incline towards cell growth and therefore accumulate less PHA.

4.3. Stability of the mixed culture for PHA production

Beside the establishment of the F/F regime and the associated SBR operating conditions favouring the PHA storage, stability of the mixed culture throughout the enrichment needs to be considered in a long-term operation. The parameters to measure the stability include F/F ratio, biomass concentrations (TSS/MLSS – total suspended solid/mixed liquor suspended solid and/or VSS/MLVSS – volatile suspended solid/mixed liquor volatile suspended solid), and sludge volume index (SVI). A long-term enrichment normally lasted from 3 up to 12 months, which consisted of a period of acclimatisation, followed by a steady state. The F/F ratio and biomass concentrations are stabilised subsequently [17,53]. In the study using unpretreated crude glycerol fed to the SBR operating at 24 h cycle with SRT and HRT of 2 days, a stable MC was obtained after 8 months when the biomass concentration and F/F ratio were remained on average at 700 mg MLSS/L and 0.2 respectively [17]. Before reaching the steady state, it took two months for the MC to get acclimatised, in which the biomass concentration dropped suddenly from 1200 to 170 mg MLSS/L [84]. It was observed that in the OLR range 360–1000 mgC/L.d corresponding to the F/F ratio range 0.2–0.26, the total accumulated PHA obtained in the batch test was well maintained at approximately 1200 mg/L with the PHA content from 77% to 78%. Hence, it could be said the enriched MC was stable in producing PHA. However, increasing the OLR to 1250 mgC/L.d immediately affected the F/F ratio and caused a significant reduction in the PHA production. Therefore, the stability of the MC should be maintained at the range of OLRs corresponding to the F/F ratio below 0.28 for a good PHA storage during the enrichment [17,85,86].

The physical states of MC, which was reflected by MLSS and MLVSS concentrations and the sludge volume index (SVI) were also used to evaluate the stability of the MC in the enrichment process [53]. Operation of two SBR systems were carried out under similar conditions (initial biomass concentration at 3.6 g MLSS/L, carbon concentration at 2.5 g COD/L, and 12 h cycle length). For the SBR-1 operating at the SRT of 5 days, there were a remarkable decrease in the biomass concentration from 3500 to 2000 mg MLSS/L and a steady increase in the SVI from 1250 to 1750, which were noticed in the first 20 days during acclimatisation period. For the rest of 70 days, the biomass concentration increased again and maintained at around 3500 mg MLSS/L with the SVI stabilised at 1500. Such a good recovery in the physical states of the MC resulted in a stable PHA accumulation in the batch test (PHA content 60%, PHA yield 0.65 mg COD/mg COD). On the other hand, a doubling of SRT to 10 days in SBR-2 with the same operating conditions had a severe effect on the stability of the MC enrichment. After 40 days, the biomass concentration decreased steeply from 4500 to 1500 g MLSS/L, while the SVI suddenly decreased and maintained relatively at approximately 600 without recovery. Therefore, the MC obtained from the SBR-2 in such unstable physical state could not be used in the batch test. A short SRT of 5 days was therefore more favoured to achieve a good

stability for the culture selection in the enrichment and the resultant PHA content and storage yield were at 61.26% and 0.68 mg COD PHA/mg COD VFA in the PHA accumulation conducted in batch [53].

4.4. Nutrient supplementation

Cell cultivation whether on the pure or mixed microbial cultures generally requires an adequate supply of energy for cell maintenance, growth, reproduction and product biosynthesis via the supply of carbon substrates, supplement of other nutrients (nitrogen, phosphate and sulphur) and traces of elements such Mg, Mn, Fe and K which are all present in the culture media [87,88]. Besides carbon source, nitrogen in the form of organic (yeast extract, peptone and whey) or inorganic (ammonia NH_3 and ammonia salts NH_4^+, NO_3^-, NO_2^-) compounds need to be supplied. Microorganisms that grow under aerobic condition prefer nitrogen to be supplied in the form of ammonia salts such as NH_4^+, whereas those living in the anaerobic condition prefer the nitrates and nitrites [87].

The transition between carbon availability (feast) and starvation (famine) imposed on the mixed microbial culture activated sludge triggers the selective pressure to enrich the culture with PHA storing bacteria. Besides the dynamic carbon feeding, the dosage or level of nutrient supplement to the MC is essentially being controlled to sustain the survival of these PHA storing bacteria not only during the culture selection but also for the subsequent PHA accumulation. Since the entire process is carried out in the aerobic condition provided by continuous aeration, ammonia salts (NH_4^+) such as ammonia chloride (NH_4Cl) or ammonia sulphate ($(NH_4)_2SO_4$) are the inorganic source of nitrogen nutrient which is usually supplemented in the culture media as reported in many studies [52,54,59,64]. Although the addition of these ammonia salts can cause the system condition to be acidic which is unfavourable for the PHA storage, this issue can be settled as the pH of enrichment reactor is always controlled at 7.0 in most cases [43,52,53,56,60,64].

In the mixed culture with the availability of both carbon and nutrients, the PHA producing bacteria function favours both cell growth (growth of cell biomass) and PHA storage, whereas the non-PHA producing bacteria will only be focusing on cell growth, which is well-known in the MC studies [42,55,89]. When the environmental stress under available carbon with limited nitrogen condition, the response of PHA producing bacteria to this stress is to suppress the cell growth, but promote the PHA storage instead. Due to this fact, a nitrogen-limited condition is established by the absence of the nitrogen in the media. This is usually applied to the enriched culture in the accumulation step in order to maximise the PHA storage in the cells [17,42,47,65].

However, the role of nitrogen supplement in the enrichment (culture selection and biomass growth) is contradicted among the studies that examined the effects of nitrogen-deficient and nitrogen-sufficient conditions with varying carbon to nitrogen ratios (C/N ratios on molar basis). The implementation of the F/F regime on the activated sludge mixed culture is usually associated with the nitrogen supplement to allow biomass/cell growth during the culture selection process. In those studies establishing a nitrogen-sufficient condition for biomass growth in enrichment, the C/N ratios were varied from 100/2 to 100/8 (molar basis) as shown in Table 3. In the research of examining the feasibility of using crude glycerol for MC-PHA production in the two-step process, enrichment of PHA accumulating culture was carried out in the nitrogen-sufficient condition (C/N: 100/6) [42]. The SBR was fed with glycerol at OLR 4.6 g/L.d and NH_4Cl in a 1.5 L working volume with SRT 5 days and HRT 2 days for 24 h cycle. An enriched MC was obtained (F/F ratio less than 0.2) for a good PHA with 59% content of PHA and yield of 0.44 g PHA/g S [42]. At a higher C/N ratio of 100/8 to support a sufficient biomass growth during the enrichment, the enrichment resulted in MCs with good PHA storage at F/F ratios in the range 0.04–0.21, high PHA content (47% and 74.6%) and high yield of PHA (0.44 g PHA/g COD S and 0.81 Cmol PHA/Cmol S) using crude glycerol and fermented sugar molass (carbon concentration 30 CmM and 45 Cmmol/L respectively) [58,59]. As carbon waste or industrial byproduct sources such as cheese whey, crude glycerol, and wastewater are usually poor in nutrients, supplement of nitrogen nutrient in the enrichment stage is essential for the growth of the enriched culture [18,55,56,60].

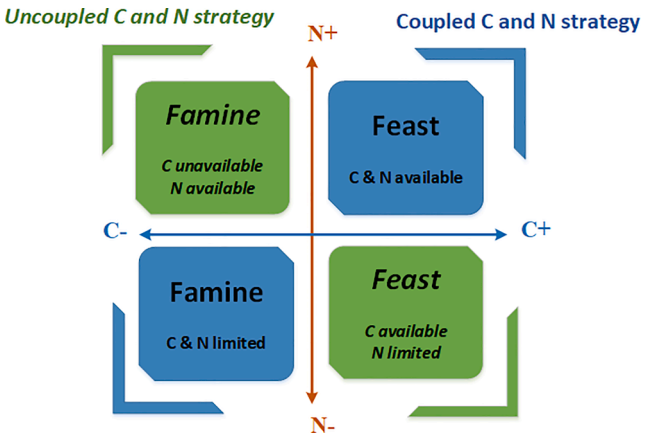

Fig. 7. Mechanisms of the coupled and uncoupled carbon (C) and nitrogen (N) supply strategies.

In contrast to those studies examining the MC enrichment under nitrogen sufficient condition, the nitrogen-deficient condition was reported to favour the culture selection in the MC which resulted in a higher PHA storage capacity [62,63]. In these two studies, synthetic wastewater containing sodium acetate as a sole carbon source was fed to two SBRs operating under the same conditions with SRT 8 days, HRT 12 h, cycle 6 h in 3 L working volume. The only difference was in the nitrogen supply conditions, i.e. one under nitrogen sufficiency (C/N: 100/12) and another under nitrogen deficiency (C/N: 100/2) in the enrichment. In batch test for PHA accumulation, PHA storage capacity of the enriched MC obtained from nitrogen deficient enrichment was higher in term of PHA content and yield than that under nitrogen sufficient enrichment as can be seen in Table 5.

Furthermore, the effect of nitrogen deficiency on the selection and storage of the PHA in enriched culture was also investigated in a study using sodium acetate as carbon substrate [60]. Operating conditions in the SBR under nitrogen deficiency (C/N: 17 Cmol C per Cmol N) were set with HRT of 8 h, SRT of 1 day, and 4 h cycle length. The performance of the enriched MC showed a high accumulation of PHA at 70% with a yield of 0.06 Cmol PHB/Cmol S and percentage of growth fraction (increase in biomass growth by percentage) at 85% under nitrogen-deficient condition (C/N: 17 Cmol C per Cmol N) which was 3 times higher than that obtained under only carbon limited condition (C/N: 7.4 Cmol C per Cmol N). Due to the contradiction in the necessity of the nitrogen supplement to the MC in the enrichment, future studies on the relationship between nitrogen deficient and sufficient conditions with the biomass growth and PHA production need to be established.

4.5. PHA productivity - a challenge in the MC-PHA production

Productivity or volumetric productivity is defined as the rate of producing PHA per unit reactor volume per unit time by the enriched culture [17,85]. It is one of the key parameters used to evaluate the performance of PHA production process, besides PHA storage response (PHA content and yield) [31,56]. In most studies examining the mixed culture process so far, the reported PHA content (up to 89%) and yield (up to 0.8 g PHA/g carbon substrate) have achieved the desired target to be as high as that of the commercialised pure culture process, but not the volumetric productivities [43].

The current productivities reported in those mixed culture PHA production (Table 3) are less than 0.5 PHA/L.h which is approximately 10 times lower compared to the pure culture PHA production from sugar

Table 6
Bacterial activities in the MC enrichment by the uncoupled C and N supply strategy.

	Feast	Famine
Condition	C available, N limited	C unavailable, N available
PHA producing bacteria	Consume carbon from the external supply to store PHA in the bacterial cells.	Utilise the stored PHA in the bacterial cells and available N to grow. Eventually become dominated in the MC.
Non-PHA producing bacteria	Hard to consume the external C source for cell growth under N limited condition.	Struggle to survive, even harder to grow without the external C supply and no stored PHA. Eventually outcompeted.
Favouring modes	PHA storage	Cell growth

feedstock having a higher productivity of 5 g PHA/L.h with PHA content ranging from 80 to 90% [47]. The PHA production rates are usually lower than 1 mg COD/mg COD X.h in a mixed culture systems in most studies [57,58,64,65]. In two-step processes, the highest productivity of 0.41 g PHA/L.h was reported in a study using crude glycerol, highlighted its strength as a potential feedstock for PHA production [26]. Productivity could also be impacted by influent carbon concentration as evident in a study using fermented wastewater in the three-step process with low productivity of 0.08 g/L.h due to the very low influent carbon concentration at just 200 mL/L [43]. Such low productivities in the MC-PHA production would render a considerable increase in the capital investment in building large bioreactors with higher capacities. An increase in the productivity of the MC-PHA production process can contribute to the reduction in the production cost of PHA, especially equipment related cost [31,90]. Thus, for an efficient MC-PHA production suitable for scaling up, its productivity needs to be comparable to that of the PC-PHA production [6].

Based on the previous research in the effect of nitrogen and carbon feeding, biomass growth is promoted by nutrient supplementation such as nitrogen and/or phosphorus [30,47]. However, if the MC is supplemented by the nitrogen nutrient for bacterial growth to increase its productivity, this becomes contradicted with the imposition of the F/F regime. This is because the internal growth limitation and nitrogen deficiency should be imposed for having an effective selection of PHA accumulating bacteria in the enrichment and maximising PHA storage in the accumulation step [18,56]. The F/F regime generally provides an intermittent supply of carbon substrate to the MC for the bacterial selection, but insufficient nitrogen supplementation for the bacterial growth [56]. To sum up, ADF strategy which only promotes the selection of PHA producers, but limits its cell growth, which might contribute to the low productivity [47]. Thus, the current productivities of the MC were inherently low in those studies based on the conventional F/F regime. An enhancement in the growth of PHA accumulators in these systems could increase the productivity of PHA production. Therefore, it is necessary to combine nitrogen supplementation with F/F regime in the MC-PHA production process in a more effective way to obtain a MC with both high PHA accumulator growth rate and PHA storing capacity.

5. Strategies to increase productivity in the MC-PHA production

5.1. Uncoupling carbon and nitrogen supply

In implementing the F/F regime for enrichment, it is common to feed carbon (C) together with nutrients containing nitrogen (N) to the MC in the beginning of the feast phase. Uptake of both C and N components is generally for the bacterial growth in the MC. Beside the bacterial growth, the PHA producing bacteria consume the external carbon source for PHA storage. When the external carbon sources are depleted, the MC enters the famine phase in which the nitrogen nutrient is either continued or stopped to be supplied [17,48,58]. As mentioned, the long famine suppresses the bacterial growth due to the internal growth limitation caused by the carbon depletion. In the absence of the external

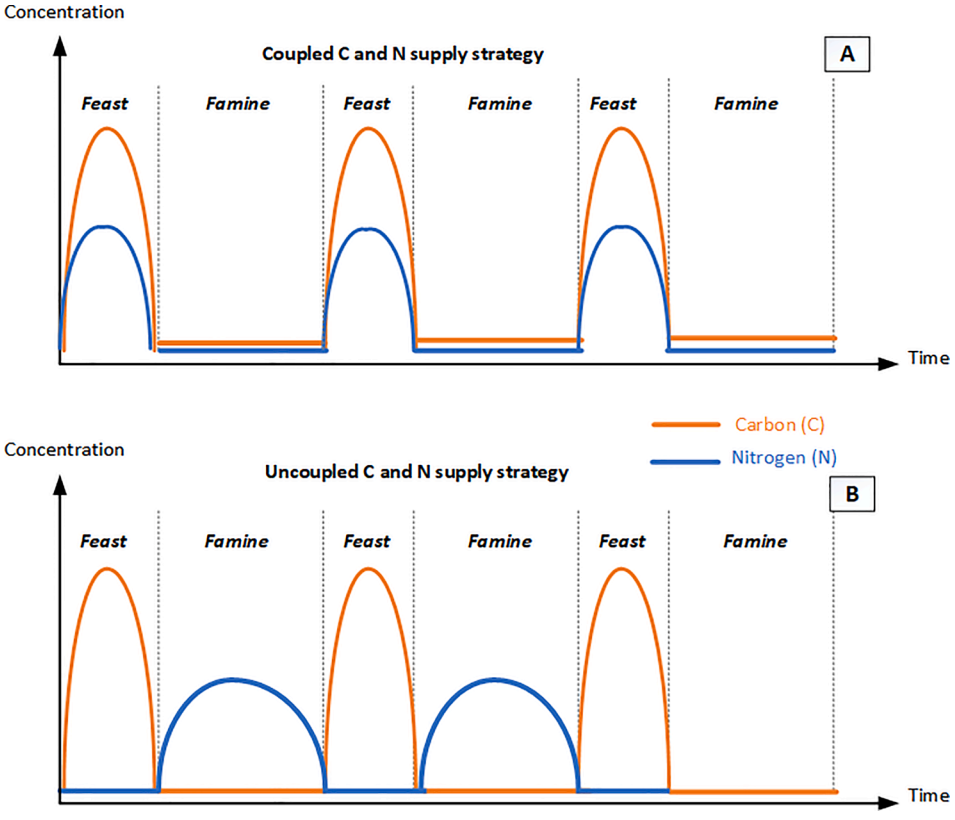

Fig. 8. An illustration of the concentration profiles for the coupled (A) and uncoupled (B) carbon and nitrogen supply strategies.

Table 7
Performance of the enriched MC by uncoupling C and N supply [18,55] and extended cultivation [56].

Using VFAs tested for enrichment only	Uncoupled C and N supply	Coupled C and N supply (Conventional F/F regime)
PHA yield (g COD PHA/g COD S)	0.4	0.2
HV content in PHA polymer	20%	10%
PHA concentration (mg COD/L)	1200	600
PHA content in biomass	28%	15%
Biomass concentration (g VSS/L)	2.2	1.8
Using fermented cheese whey tested for enrichment and accumulation		
PHA yield (Cmol PHA/Cmol S)	0.96	0.86
PHA production rate (Cmol PHA/Cmol X.h)	0.40	0.25
Carbon uptake rate (Cmol S/Cmol X.h)	0.42	0.29
HV content in PHA polymer (Cmol %)	13.0%	13.2%
Specific PHA productivity (g PHA/g X.d)	6.02	4.43
Using a mixture of VFAs tested for enrichment and accumulation	**Extended cultivation with sludge discharge**	
Productivity (g PHA/L.h)	1.22	
Cell density (including PHA) (g/L)	17.22	
Biomass magnification/increase in biomass after extension	43 after 10 days of the extended cultivation	
PHA yield (g COD PHA/g COD S)	0.84	
PHA content	71.4%	

carbon, it is very hard for the non-PHA producing bacteria to survive, and eventually being outcompeted, whereas the PHA producing bacteria are able to survive by utilizing the stored PHA for cell maintenance. The supply of carbon together with nitrogen is named as "coupled C and N supply" which bacterial growth and PHA storage occur simultaneously in the feast phase of the F/F regime (Fig. 7). In the coupled strategy, the advantage is evident in effective selection of a culture enriched with PHA producers, but the low cell growth resulting in low productivity is the drawback of this strategy.

With the aim of promoting cell growth while ensuring an effective selection of PHA producing bacteria in MC, the "uncoupled C and N supply" strategy was first proposed in the enrichment step by Silva *et al.* further investigated by Oliveira *et al.* using VFAs and fermented cheese whey respectively [18,55]. In these two studies, the uncoupled strategy was implemented in the MC enrichment by feeding just carbon substrates in the start of the feast while supplementing only the nitrogen nutrient at the start of famine when carbon source was completely depleted (Fig. 7). Under N limited condition, the carbon uptake from the external supply by the PHA producing bacteria is for PHA storage. Once the external carbon was completely depleted, the stored PHA was used as the carbon source for the PHA producing bacteria to grow when nitrogen was supplemented in the famine phase. Since the external supply of carbon and nitrogen is not being supplied at the same time throughout the process, it creates an even harsher environment for the non-PHA bacteria to sustain the feast and famine. Bacterial response to the uncoupling strategy is described more in Table 6. Fig. 8 illustrates the differences in the concentration profiles of C and N supply in the enrichment applied the coupled and uncoupled strategies.

The performances of the enriched cultures obtained from the coupled and uncoupled strategies are compared in Table 7. The uncoupling strategy resulted in an enriched MC with a higher performance in PHA production compared to the coupling strategy (conventional F/F regime) not only in term of PHA storage capacity (PHA yield, PHA content), but also in term of biomass concentration, carbon uptake rate, PHA production rate and productivity. It was evident that the uncoupled C and N supply strategy was effective to enrich MC with selective growth of PHA storing bacteria, hence leading to its higher biomass concentration at 2.2 g VSS/L and consequently double in PHA yield at 0.4 g COD PHA/ g COD S and PHA concentration at 1200 mg COD/L [55]. The enriched MC was further examined for its PHA storage capacity in the accumulation stage and shown an increase in the productivity at 6.02 g PHA/g X.d [18]. Compared to the conventional F/F strategy (coupled C and N supply), the OLR applied in the enrichment was usually set in the low range 0.36–4.6 g/L.d to ensure the stability of MC and a fast PHA storage response [17,65,91]. The uncoupling strategy applied in the enrichment was able to operate at a higher OLR of 8.5 g COD/L.d in the culture enrichment [18].

Based on the experimental results, the uncoupled C and N supply strategy seems more superior than the coupled C and N supply which is usually applied in many other studies. In term of the operating conditions, applying the uncoupling strategy allowed the enrichment reactor to operate at higher OLR (8.5 g COD/L.d) without affecting the stability and efficiency of the culture selection as well as maintaining stable F/F ratio [18]. For most of the enrichment reactors applying the coupling

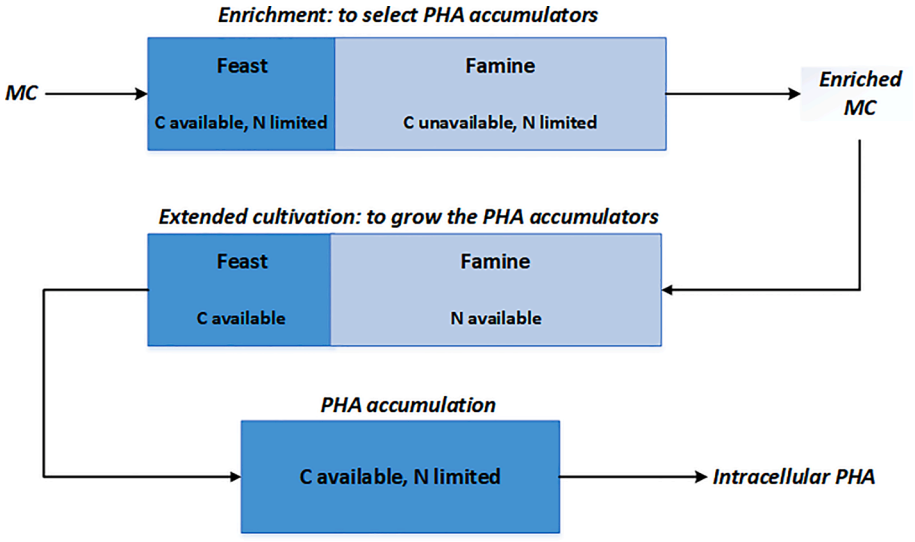

Fig. 9. Mechanism of the extended cultivation strategy for the MC-PHA production.

strategy, operation at relatively low OLR (2 g COD/L.d or even lower) was recognised as the limitation in order to maintain a stable culture at low F/F ratio. In terms of culture performance, the higher PHA yield and productivity resulted from the uncoupling strategy showed not only its higher efficiency in selection of a PHA producing culture but also a higher cell growth as compared to that of the coupling strategy.

5.2. Extended cultivation

With the aim for selective growth of PHA storing bacteria in the enriched MC, subsequently increasing the PHA productivity, the "extended cultivation" was another strategy proposed by Huang et al. (2017). This strategy was conducted in a way that a period of bacterial growth was embedded between the enrichment step and the PHA accumulation step as shown in Fig. 9. The MC was first submitted to the F/F regime for enriching the culture with PHA accumulators. In the work of Huang et al., a mixture of VFAs was fed to an SBR operating at OLR 1.2 g/L.d, SRT for 10 days in 12 h cycle, under nitrogen-limited condition. After the enrichment, the cultivation was extended under the same SBR operating condition to further grow the PHA accumulators contained in the MC. This extended cultivation was lasted for 10 days by separately supplying carbon feedstock in the feast and nitrogen nutrient in the famine phase. With such an embedded period for bacterial growth, performance in producing PHA by the enriched MC was shown to improve significantly as shown in Table 7 [56].

The attempts in both strategies (the extended cultivation and the uncoupled carbon and nitrogen availability) were to separate the bacterial growth (nitrogen supplement) from PHA storage (carbon supply). The difference between them is that for the extended cultivation strategy, the MC enriched with PHA storing bacteria was extended in the cultivation for another 10 day period after the F/F enrichment, while for the uncoupling strategy, the bacterial growth took place in the famine phase after the feast phase. In the batch test conducted under the nitrogen-limited condition for the PHA accumulation, the performance of the MC obtained from the extended cultivation was recorded at PHA content of 71.4% and yield of 0.84 g COD PHA/g COD VFA. These results are comparable to that of the uncoupling strategy as can be seen in Table 7. Notably besides the high PHA storing capacity, productivity of the enriched MC was also improved to 1.22 g PHA/L.h with the final cell density of 17.22 g/L through the application of extended cultivation strategy.

6. Future prospects

As an effort to replace the petroleum-based plastics, ongoing research has been aiming to improve the cost competitiveness of the PHA production schemes. With the findings in the critical parameters affecting the culture selection by manipulation of the operating conditions, they are interdependent and eventually affect F/F ratio, stability, PHA storage capacity and PHA productivity. Therefore, synergistic interactions of all the critical parameters affecting culture enrichment and PHA accumulation are worth exploring in the process optimisation study to maximise the PHA storage in the cells and for further development of kinetic models.

With a limited number of studies, the new strategies such as uncoupled carbon and nitrogen supply and extended cultivation were shown to be very promising in increasing the PHA productivity, since the aspect of the bacterial growth was addressed effectively. Relationship between microbial community and characteristics of PHA produced by implementing these new strategies could be further established. Besides, dosages of nutrient supplement and carbon supply that lead to the highest PHA storage capacity could be included in the process optimisation.

Another prospect of PHA production at low cost can be seen in valorisation of the complex wastes/byproducts. Although crude glycerol has shown to be suitable for the two-step process to produce PHA using mixed culture, other feedstocks such as glycerine pitch should be considered in future investigations. The metabolic pathways of intracellular PHA synthesis based on the carbon substrates (fatty acids, sugars and amino acids) can be used to predict the polymeric products (scl- or mcl-PHAs) and the presence of the PHA producing bacterial strains. However, the mechanism of synthesising PHA copolymers in the mixed culture is not well understood. Therefore, an in-depth study in finding the groups of PHA bacterial strains and their multiple pathways adopted in the mixed culture would provide a better understanding in synthesis of PHA copolymers. Scope of future study is advised to include characterisation of PHA produced from mixed culture and carbon wastes to comprehend the impact of PHA production schemes on the resultant PHA properties.

7. Conclusions

One of the major factors hindering the commercial production of biodegradable PHA polymers is identified to be the high production cost. In the continuous effort to make PHA production commercially feasible, the application of mixed culture and carbon waste feedstocks has taken a step further in improving the cost effectiveness. Two aspects are crucial to consider in the MC-PHA production i.e. selection of feedstock suitable with the production scheme adopted and its operating conditions applied in enrichment. Recent research has shown that it is feasible to simplify the production scheme from three to two steps when using crude glycerol. Beside feedstock, critical parameters such as OLR, cycle length and SRT act synergistically under the imposed feast-famine regime, affecting the stability of enrichment reactor as well as PHA production in term of yield, content and productivity. To address the issue of low productivity inherent from the conventional ADF strategy, separation of PHA storage in the feast phase from bacterial growth in the famine phase that were being applied in the uncoupled carbon and nitrogen supply and the extended cultivation strategies have showed significant results in improving the PHA productivity. Future studies in MC-PHA production have prospects in investigating the microbial community and characteristics of PHA produced in relation to the implementation of these new enrichment strategies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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