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Synthetic biology of microbes synthesizing polyhydroxyalkanoates (PHA)

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ABSTRACT

Microbial polyhydroxyalkanoates (PHA) have been produced as bioplastics for various purposes. Under the support of China National Basic Research 973 Project, we developed synthetic biology methods to diversify the PHA structures into homo-, random, block polymers with improved properties to better meet various application requirements. At the same time, various pathways were assembled to produce various PHA from glucose as a simple carbon source. At the end, *Halomonas* bacteria were reconstructed to produce PHA in changing morphology for low cost production under unsterile and continuous conditions. The synthetic biology will advance the PHA into a bio- and material industry.
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Contents

1. Introduction

Polyhydroxyalkanoates (PHA), polylactic acid (PLA), poly(-butylene succinate) (PBS), polyethylene (PE), poly(trimethylene terephthalate) (PTT), polypropylene (PPP), polyethylene terephthalate (PET) and poly(propylene carbonate) (PPC) are biodegradable or biobased polymers with at least one monomers produced by microbial conversions or microbial industrial biotechnology [1]. The weaknesses of microbial or enzymatic processes compared with the chemical processing make industrial biotech products less competitive with the chemical ones. However, taking advantages of the molecular biology and synthetic biology methods as well as changing process patterns, bioprocesses could be developed as competitive as chemical ones, these

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including the minimized cells, open and continuous fermentation processes et al. [2] This review aims to report progresses made by the China National Basic Science Research Project 973 during 2012–2016 on synthetic biology of PHA.

Many bacteria have been found to produce various polyhydroxyalkanoates (PHA) biopolyesters. In many cases, it is not easy to control the structures of PHA including homopolymers, random copolymers and block copolymers as well as ratios of monomers in the copolymers. However, the weakening of beta-oxidation cycle in *Pseudomonas putida* and *Pseudomonas entomophila* led to controllable synthesis of all kinds of PHA structures including monomer ratios in random and/or block copolymers when fatty acids are used as PHA precursors. Introduction of functional groups into PHA polymer chains in predefined proportions has become a reality provided fatty acids containing the functional groups are taken up by the bacteria for PHA synthesis. This allows the formation of functional PHA for further grafting (Fig. 1). The PHA diversity is further widened by the endless possibility of controllable homopolymerization, random copolymerization, block copolymerization and grafting on functional PHA site chains(Fig. 1) [3].

PHA diversity is generated by engineering the three basic synthesis pathways including the acetoacetyl-CoA pathway (pathway I), in situ fatty acid synthesis (pathway II), and/or beta-oxidation cycles (pathway III), as well as PHA synthase specificity and process control. It is now possible to tailor the PHA structures via genome editing or process engineering. The increasing PHA diversity and maturing PHA production technology should lead to more focused research into their low-cost and/or high-value applications [4] (see Fig. 2).

Similarly to the genome, transcriptome, and proteome, the PHA spectrum exhibits diverse and dynamic modifications – the term 'PHAome' has been created to reflect not only the diversity of monomers, homopolymers, random and block copolymers, functional and graft polymers, molecular weights, and combinations of the above, but also the ranges of PHAs with various molecular weights and monomer ratios that are present at a particular time point in a bacterial cell. It has become very important to understand the PHAome and ensuring an ample supply of PHAs to promote the discovery of new properties and applications of this family of advanced materials [5].

2. The development of new technology for pathway cloning and assembly

PHA synthesis involves a lot of pathways. However, construction of large gene clusters containing DNA fragments is still a difficult and expensive task. To tackle this problem for complicate PHA synthesis pathway assembly, we developed a gene cluster extraction method based on *in vitro* single-strand overlapping annealing (SSOA). It starts with digesting the target gene cluster in an existing genome, followed by recovering digested chromosome fragments. Subsequently, the single-strand DNA overhangs formed from the digestion process would be specifically annealed and covalently joined together with a circular and a linear vector, respectively. The method could clone a 18 kb DNA fragment encoding NADH: ubiquinone oxidoreductase. Combined with genetic information from KEGG and the KEIO strain collection, this method will be useful to clone any specific region of an *E. coli* genome at sizes less than similar to 28 kb. The method provides a cost-effective way for genome assembly, alternative to expensive chemically synthesized gene clusters [6].

Anaerobic metabolic pathways dedicated to co-production of hydrogen and PHB were established in *E. coli* due to the advantages of directing fluxes away from toxic compounds such as formate and acetate to useful products. *E. coli* over-expressing very large hydrogenase 3 cloned using the above SSOA method and/or acetyl-CoA synthase showed improved poly-3-hydroxybutyrate (PHB) and hydrogen production when grown with or without acetate as a carbon source [7].

A method was developed to generate single-stranded DNA

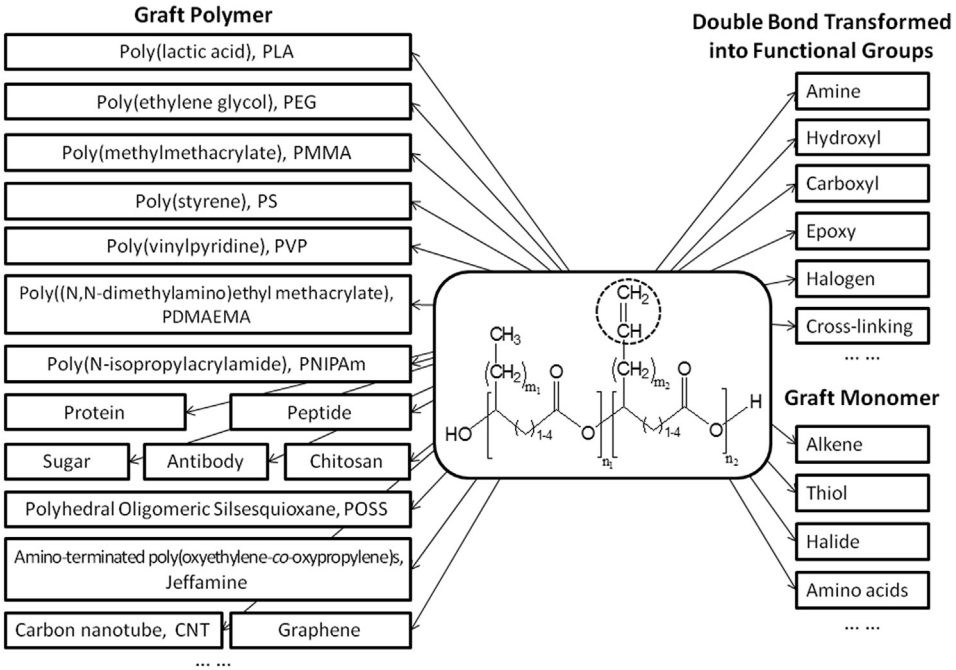

Fig. 1. Introduction of functional groups into PHA polymer chains in predefined proportions has become a reality provided fatty acids containing the functional groups are taken up by the bacteria for PHA synthesis. The PHA diversity is further widened by the endless possibility of controllable homopolymerization, random copolymerization, block copolymerization and grafting on functional PHA site chains [3].

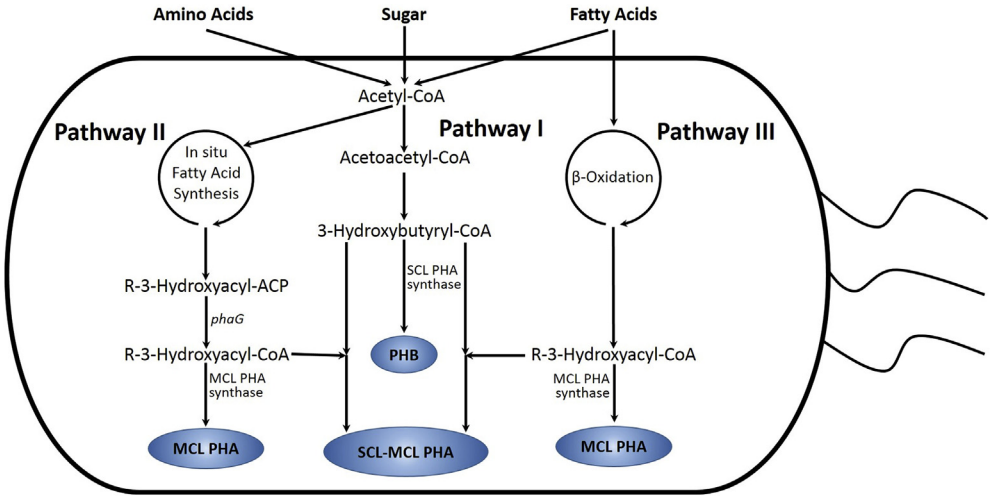

Fig. 2. PHA diversity is generated by engineering the three basic synthesis pathways including the acetoacetyl-CoA pathway (pathway I), in situ fatty acid synthesis (pathway II), and/or beta-oxidation cycles (pathway III), as well as PHA synthase specificity [4,5].

overlaps based on Nicking Endonucleases (NEases) for DNA ligation-independent cloning LIC, the method was termed NE-LIC. This NE-LIC allows generating 3'-end or 5'-end ss DNA overlaps of various lengths for fragments assembly. The mechanism can be applied to many other LIC strategies. Finally, the NE-LIC was successfully applied to assemble a pathway of six gene fragments responsible for synthesizing microbial PHB [8].

It is very important to assemble multiple DNA (genes) fragments into a large circular DNA structure in one step in synthetic biology. We successfully used linear plasmid and Cyclization After Transformation (CAT) strategy to conduct DNA linear assembly combined with CAT method to demonstrate an increased overall construction efficiency for assembling multiple DNA (genes) fragments. It has been used in construction of a VB12 metabolic pathway consisting of multiple genes [9].

Bacterial hemoglobin promoters P_{vgb} were cascaded assembled for enhanced expression under micro-aerobic growth. The highest PHB production was obtained when a promoter containing eight cascaded P_{vgb} repeats P_{8vgb} was used. This study revealed that *vgb* promoter containing cascaded repeats P_{8vgb} was useful for product formation under low aeration intensity [10].

3. Designing new pathways for new PHA synthesis by *E. coli or Pseudomonas*

Block PHA were reported to be resistant against polymer aging that negatively affects polymer properties. Diblock copolymers PHB-*b*-PHHx consisting of poly-3-hydroxybutyrate (PHB) block covalently bonded with poly-3-hydroxyhexanoate (PHHx) block were therefore produced by a recombinant *Pseudomonas putida* KT2442 with its beta-oxidation cycle deleted to its maximum. It is possible to produce PHA block copolymers of various kinds using the recombinant *Pseudomonas putida* KT2442 with its beta-oxidation cycle deleted to its maximum [11].

Poly(4-hydroxybutyrate) (P4HB) is a highly elastic polymer, whereas poly(3-hydroxypropionate) (P3HP) is a polymer with enormous tensile strength. A recombinant *Escherichia coli* that produces homopolymers of P3HP and P4HB was constructed for the synthesis of the block copolymers [12]. When the strain was grown in the presence of 1,4-butanediol (BDO) as a 4HB precursor, P4HB block was formed. Sequential supplementation of 1,3-propanediol (PDO) as a 3HP precursor allowed formation of a block copolymer of p3HP-*b*-P4HB. Block copolymerization of P3HP and P4HB adds a new vision on PHA polymerization by generation of new polymers with superior properties [13].

At the same time, copolyesters of 3-hydroxypropionate (3HP) and 4-hydroxybutyrate (4HB), abbreviated as P(3HP-co-4HB), was synthesized by the same *Escherichia coli* harboring a synthetic pathway consisting of five heterologous genes including *orfZ* encoding 4-hydroxybutyrate-coenzyme A transferase from *Clostridium kluyveri*, pcs' encoding the ACS domain of tri-functional propionyl-CoA ligase (PCS) from *Chloroflexus aurantiacus*, *dhaT* and *aldD* encoding dehydratase and aldehyde dehydrogenase from *Pseudomonas putida* KT2442, and *phaC1* encoding PHA synthase from *Ralstonia eutropha* [14]. A pathway was constructed in *Escherichia coli* for the production of P(3HB-co-4HB) from glucose. Native gene *sad* encoding *E. coli* succinate semi-aldehyde dehydrogenase was expressed under the control of CRISPRi, using five specially designed single guide RNAs (sgRNAs) for regulating carbon flux to 4-hydroxybutyrate (4HB) biosynthesis. The system allowed formation of P(3HB-co-4HB) consisting of 1–9mol% 4HB. It was found that CRISPRi was a feasible method to simultaneously manipulate several genes in *E. coli* [15].

Meng et al. in this lab further constructed a pathway in *E. coli* for production of poly(3-hydroxypropionate) and p(3-hydroxybutyrate-co-3-hydroxypropionate) from glucose, demonstrating the feasibility of using various pathways in *E. coli* for production of diverse PHA [16].

The beta-oxidation weakened *Pseudomonas putida* was established as a platform for the production of polyhydroxyalkanoates (PHA) with adjustable monomer compositions and microstructures. Its produced a novel diblock copolymer P3HHx-*b*-P(3HD-co-3HDD) consisting of 49 mol% P3HHx and 51 mol% P(3HD-co-3HDD) [35.25 mol% 3HDD (3-hydroxydodecanoate)] [17].

This lab succeeded to synthesize controllable composition of poly(3-hydroxyalkanoate) (PHA) consisting of 3-hydroxydodecanoate (3HDD) and phenyl group on the side-chain when chromosome of *Pseudomonas entomophila* was edited to weaken its β-oxidation. Cultured in the presence of 5-phenylvaleric acid (PhV), the edited *Pseudomonas entomophila* produced only homopolymer poly(3-hydroxy-5-phenylvalerate) or P(3HPhV). While copolyesters P(3HPhV-co-3HDD) of 3-hydroxy-5-phenylvalerate (3HPhV) and 3-hydroxydodecanoate (3HDD) were

synthesized when the strain was grown on mixtures of phenylvaleric acid and dodecanoic acid. It was possible to tailor-made novel functional PHA using the chromosome edited *Pseudomonas entomophila* [18,19].

A semi-rational approach for high-efficient PHB pathway optimization in *E. coli* based on PHB synthesis *phbCAB* operon cloned from native producer *Ralstonia entropha* (*R. entropha*) was developed (Fig. 3). Rational designed Ribosomal Binding Sites (RBS) libraries with defined strengths for each of the three genes, were constructed based on high or low copy number plasmids in a one-pot reaction by an Oligo-Linker Mediated Assembly (OLMA) method. Applying this approach, strains accumulating 0%–92% PHB contents in Cell Dry Weight (CDW) were achieved. The semi-rational approach combining library design, construction, and proper screening is an efficient way for PHB and other multi-enzyme pathways optimization [20].

PHBHHx copolymer poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) production suffers from high cost due to expensive C12 fatty acid. Wang et al. [21] designed metabolic pathways to synthesize P(HB-co-HHx) directly from glucose using recombinant *Escherichia coli.* By combining the BktB-dependent condensation pathway with the inverted beta-oxidation cycle pathway, they were able to synthesize a PHBHHx with a 10 mol% HHx fraction in the *E. coli*.

By engineering the reversed fatty acid beta-oxidation cycle, Zhuang et al. [22] were able to synthesize medium-chain-length (mcl) PHA in *Escherichia coli* directly from glucose. Furthermore, when a low-substrate-specificity PHA synthase from *Pseudomonas stutzeri* 1317 was employed, recombinant *E. coli* synthesized 12 wt% of cell dry weight short-chain-length (scl) and medium-chain-length (mcl) PHA or scl-mcl PHA copolymers, of which 21mol% was 3-hydroxybutyrate and 79mol% was medium-chain-length monomers. The reversed fatty acid beta-oxidation cycle offered an efficient metabolic pathway for mcl-PHA biosynthesis in *E. coli* and can be further optimized.

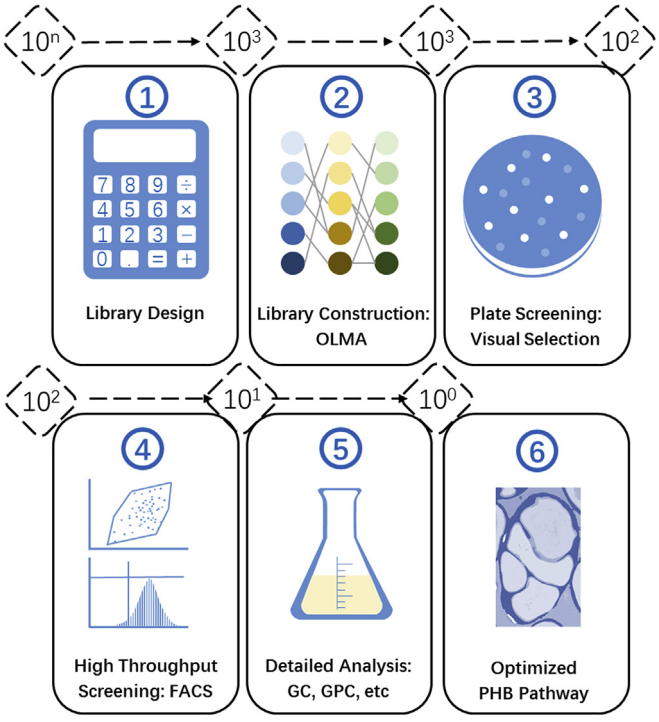

Fig. 3. Scheme for PHB pathway optimization. Applying proper rational design approaches like RBS library design, the capacity of possible variations in a library can be reduced from random combinations to a smaller number of combinations (10^3), then the rational designed library was successfully constructed through one-step OLMA method. On-plate visual selection was involved to identify pathways accumulating PHB and reduce the library capacity to 10^2. High throughput screening via FACS to screen PHB producers can further reduce the interested pathways for further detailed analysis to about 10^1. Detailed analysis can be carried out according to different requirements, the desired optimized pathway can thus be selected from the library [20].

Polylactide (PLA) is another representative of bio-based biodegradable polyester and has been used in areas of biomedical implants, food packaging and drug delivery because of its lower price. However, a large scale application of PLA has been hampered by a disadvantage of poor thermal and mechanical properties. Therefore, copolymerization of LA with other hydroxyalkanoate (HA) monomers is a better choice to improve the physical properties of PLA or PHA. Recently, several polyhydroxyalkanoate (PHA) synthases were engineered to utilize lactyl-CoA (LA-CoA) and 3-hydroxybutyryl-CoA (3HB-CoA) as substrates [23,24]. Taguchi reported the one-step biosynthesis of the LA-incorporated PHA copolyester, P(6 mol% LA-*co*-94 mol% 3HB), with a number-average molecular weight of 1.9×10^5. However, the property of copolymer does not have too much change because mechanical properties of PHB are similar to PLA [23]. Therefore, Shozui et al. introduced other monomers such as 3-hydroxyvalerates (3HV), 3-hydroxyhexanoate (3HHx) into the LA copolymers for better and more diverse properties [25–27]. Li et al. constructed an *E. coli* strain to produce a novel glycolate-containing biopolymers poly(-glycolate-co-lactate-*co*-3-hydroxybutyrate) from glucose. The recombinant *E. coli* was able to accumulate the novel terpolymer with a titer of 3.90 g/L in shake flask cultures [28]. Assessment of thermal and mechanical properties demonstrated that the resulted terpolymer possessed decreased crystallinity and improved toughness, in comparison to poly(3-hydroxybutyrate) homopolymer.

4. Bacterial morphology engineering

Many bacteria can accumulate inclusion bodies such as sulfur, polyphosphate, glycogen, proteins or PHA. A larger intracellular space is needed for more inclusion body storages. Various approaches were taken to increase the bacterial cell sizes including deletion on actin-like protein gene *mreB*, weak expression of *mreB* in *mreB* deleted mutant, and weak expression of *mreB* in *mreB* deleted mutant under inducible expression of division ring FtsZ inhibiting protein SulA, all resulted in increasing bacterial sizes and PHB granules accumulation (Fig. 4). It was found that over 100% PHB accumulation increase was observed in *E. coli* overexpressing of *mreB* in *mreB* deleted mutant under inducible expression of FtsZ inhibiting protein SulA [29–31].

5. *Halomonas* based biotechnology and synthetic biology

Disadvantages of bioprocessing are obvious: most fermentation (bioprocessing) processes are discontinuous ones lasting one to several weeks. Bioprocessing consumes a lot of precious fresh water, the sterilization and aeration processes also demand a lot of energy. In addition, final products in fermentation broths are generally very low, ranging from mg to 200 g/L. The lower yield leads to a high downstream purification cost. In contrast, chemical industry can reach at least 500 g/L at the end of the process. Most seriously, the substrate to product conversion efficiency is very low in fermentation, in PHA industry, the most common substrate to PHA conversion stands around 1/3 or 33% (g/g), while the chemical synthesis of plastics such as PE, PET or PS et al. can be as high as over 90% or even close to 100% (Table 1) [32]. We must learn from the advantages of chemical industries while maintaining the advantages of bioprocessing. New technology should be developed to

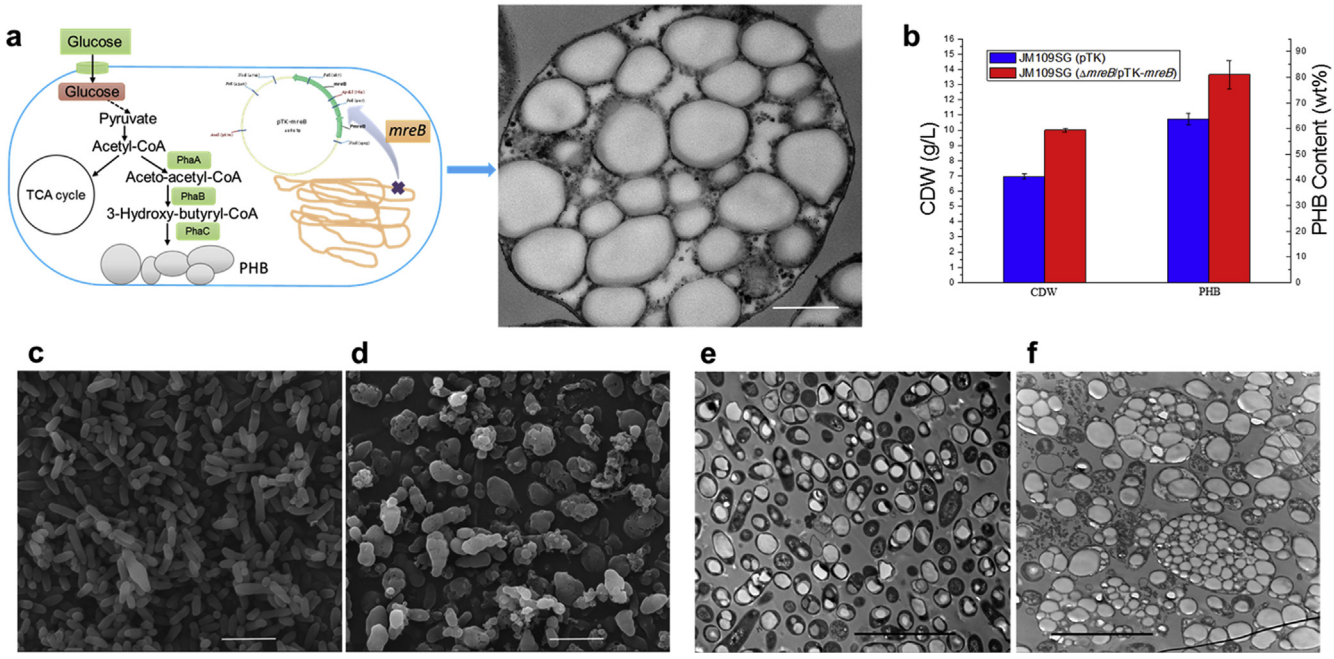

Fig. 4. Electron microscopy studies on morphology and PHB production by *E. coli* JM109SG (Δ*mreB*) overexpressing *mreB* [30].

Table 1
Comparisons between bio- and chemical processing [32].

Comparison parameter	Biotechnology	Chemical technology
Raw materials	Sustainable agriculture resources including CO_2	Petroleum
Reaction conditions	Ambient temperature and atmosphere pressure, aqueous medium	Mostly under high temperature, high pressure and organic solvents
Process	Mostly discontinuous batch processes	Mostly continuous processes
Process duration	From inoculation to fermentations to downstream take one to two weeks	Mostly completed within days
Energy consumption	High due to sterilization and continuous aeration	Depending on products
Water consumption	Heavy water consumption	Less water consumption
Final product conc.	Mostly low, from mg to 200 g/L	Mostly over 500 g/L
Cost of product recovery	Very high	Low
Substrate to product conversion efficiency	Low	Mostly very high
Risk	Low level	High due to flammable, explosive, toxic gas or product leakages
Waste water	Mostly non-toxic and easily treated	Mostly toxic, acidic or alkali, difficult to treat

address the setbacks on bioprocessing. *Halomonas* based biotechnology we developed supported by the National Basic Research 973 project represents the Advanced Bioprocessing to learn from Chemical Industries [33,34].

Halophilic bacteria offer such possibilities as they grow in medium to high salt concentrations under high pH in a wide temperature window, such growth conditions.

can effectively prevent contamination by other microorganisms [33]. In this lab halophilic bacteria were grown in unsterile and continuous processes in seawater like medium for at least one month without any microbial contamination [35], technology has also been developed for genetic manipulation of halophilic bacteria, allowing the synthetic biology construction of above mentioned super PHA production strains [36] for production of PHBV copolymers consisting of 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV) with high substrate to PHA conversion efficiency [37]. In addition, New *Haloarchaea* has also been found to produce PHBV [38].

It is commonly known that chromosomal expression is more stable yet weaker than plasmid one is. To overcome this challenge, a novel chromosomal expression method was developed for halophile *Halomonas* TD01 and its derivatives based on a strongly expressed *porin* gene as a site for external gene integration. The gene of interest was inserted downstream the *porin* gene, forming an artificial operon *porin*-inserted gene. This chromosome expression system was proven functional by chromosomal expression of heterologous PHB synthase gene to completely restore the PHB accumulation in endogenous *phaC* knockout *Halomonas* TD01. The integrated heterologous PHB synthase gene was expressed at the highest level when inserted at the locus of *porin* compared with insertions in other chromosome locations. An inducible system allowed on-off switch of gene expression in *Halomonas* TD strains was also developed based on *porin*. The stable and strong chromosomal expression method in *Halomonas* TD spp was established [39].

Systems and synthetic biology approaches have been studied to design and construction a PHA super production strain able to grow fast to high cell density (>200 g/L) utilizing low cost substrates including cellulose, starch or even kitchen wastes under a very high carbon source to PHA conversion efficiency of at least 50% (g/g)

[32]. The synthetic cell will be able to achieve oxygen limitation induced >90% PHA accumulation in cell dry weights. After completing the PHA production, the very large cells will be induced for flocculation precipitation followed by induced cell lysis to release large PHA granules. Based on these design, the cost of PHA will be reduced to 50% of the original.

The above super PHA production strain (Fig. 5) will be constructed to allow precisely control their PHA structures to form homopolymers, random- and block copolymers as well as functional polymers with precise monomer structures and ratios for consistent properties. High value added applications based on unique PHA polymer properties and chiral hydroxyalkanoic acids will be developed for the high end markets.

6. PHA production from waste material

Waste materials or waste water can be used to produce PHA, which provides a cost reduction. Pittmann and Steinmetz reported the production of polyhydroxyalkanoates (PHAs) as a side stream process on a municipal waste water treatment plant (WWTP) at different operation conditions. It was demonstrated a strong influence of the operating conditions on the PHA production. Lower substrate concentration, 20 °C, neutral pH-value and a 24 h cycle time are preferable for high PHA production up to 28.4% of cell dry weight (CDW) [40]. PHA composition was influenced by cycle time only and a stable PHA composition was reached.

Yue et al. isolated a halophile *Halomonas campaniensis* strain LS21 which could use kitchen wastes like mixed substrates as nutrients for production of bioplastic PHB. The recombinant *H. campaniensis* LS21 containing a PHB synthesis genes *phbCAB* was grown in artificial seawater containing mixed substrates similar to kitchen wastes (soluble and insoluble cellulose, proteins, fats, fatty acids and starch) for 65 days without interruption. In the presence of 27 g/L NaCl under a pH around 10 at 37 °C, the recombinant produced approximately 70% PHB during the 65 days fermentation process. This study demonstrates the advantages of this strain in terms of low cost substrates (kitchen wastes like mixed substrates) [41]. The strain is currently being used as a chassis for many synthetic biology study.

7. Future prospects

The large scale application of PHA as a low biodegradable plastic has been hampered by its higher production cost. The recent advances on systems and synthetic biology provides feasible technologies for developing a super microorganism capable of producing diverse PHA with a low cost. Among various host strains, halophilic bacteria are becoming more and more attractive due to their unique properties including strong adaptation to high osmotic pressure and high pH thus agaist contaminations. All of these unique properties make halophiles as candidates of a super chassis for a lot of engineerings. With the application of synthetic technologies, the halophiles should be constructed to have the capacity of growing fast to high cell density and produce PHA with diverse properties, a high conversion efficiency from carbon source to PHA. Also, easy downstream processing technologies should be developed for halophiles so that the product PHA can be competitive in cost with petroleum plastics.

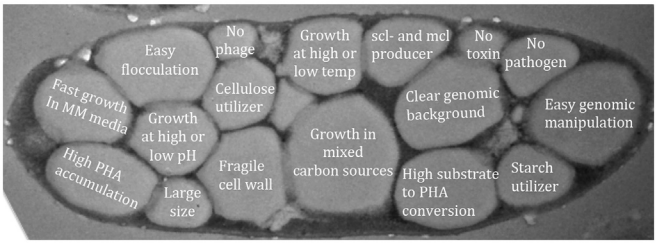

Fig. 5. Desirable properties for a PHA industrial production strain [32].

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

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