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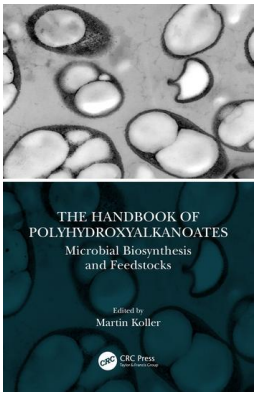

The Handbook of Polyhydroxyalkanoates Microbial Biosynthesis and Feedstocks

Martin Koller

Genetic Engineering as a Tool for Enhanced PHA Biosynthesis from Inexpensive Substrates

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6 Genetic Engineering as a Tool for Enhanced PHA Biosynthesis from Inexpensive Substrates

Lorenzo Favaro, Tiziano Cazzorla, Marina Basaglia, and Sergio Casella

CONTENTS

6.1 Introduction 145
6.2 Engineering Techniques Applied to Obtain Recombinant Strains for PHA Production 146
6.3 The Use of Whey as a Carbon Source 150
6.4 The Use of Molasses as a Carbon Source 154
6.5 The Use of Lipids as a Carbon Source 154
6.6 The Use of Starchy Materials as a Carbon Source 157
6.7 The Use of Lignocellulosic Materials as a Carbon Source 157
6.8 Conclusions and Outlook 158
Acknowledgments 158
References 158

6.1 INTRODUCTION

In order to achieve cost-effective large-scale production of PHA, in-depth research is still required. Besides efficient natural strains converting substrates into PHA, it has become clear during the last few years that the engineering of microorganisms is needed to increase the efficiency of PHA producers and, above all, obtain new proficient bacteria able to use inexpensive, although complex, carbon sources. Indeed, the carbon source required for a hypothetical industrial process could even account for 50% of the total cost [1].

The agricultural production chain, as well as industrial by-products, can be considered interesting sources of a variety of waste and by-product streams potentially suitable as feedstock for microbial production of polyhydroxyalkanoates (PHA), providing, sometimes, the double advantage of saving on disposal costs while producing value-added goods [2,3]. Unfortunately, wild-type strains for the direct and efficient conversion of low-cost waste streams into PHA are not available in nature, and therefore the engineering of strains has become important. Therefore, the economic

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feasibility of efficient PHA biotechnological processes demands inexpensive carbon sources and, possibly, the integration of producing plants where the by-product is generated.

Of course, a similar industrial-scale production is not only dependent on the cost of the substrate, but a number of other critical factors may affect the economic feasibility of the whole process, such as the growth ability of the selected strains, the microbial content of the accumulated PHA and its yield, as well as the downstream process, for example [4].

6.2 ENGINEERING TECHNIQUES APPLIED TO OBTAIN RECOMBINANT STRAINS FOR PHA PRODUCTION

Although many reviews and chapters have dealt with PHA production from organic waste streams by using engineered bacteria, no insight into the engineering techniques applied to obtain such recombinant strains has been proposed. This section is the first survey of the main engineering methods employed so far to develop engineered bacteria strains for PHA production from low-cost substrates. The vectors, their ancestors, as well as the promoter sequences and origin of replication, have been investigated (see Table 6.1 and Table 6.2).

In the case of the PHA-producers platform (see Table 6.1), the most commonly used species is *Cupriavidus necator*, which has been engineered by means of both episomal plasmids and chromosomal integration. Many research projects made use of the plasmid pBBRI MCS or a few derivatives (pBBR1MCS2, pBBR1MCS3, and pBBR1MCS5). Such plasmids have a CmR vector of about 4.7 kb, containing 16 unique cloning sites within the *lacZ*α gene. It is relatively stable both *in vitro* (>10 days) and *in vivo* (>4 weeks) without antibiotic selection [49]. The high plasmid stability is strictly linked to the *oriV*, the origin of replication, with low copy numbers (up to 10) per cell [50]. So far, researchers have used strong promoters, mostly of phagic origin, such as T3 or from *C. necator* itself (*phaABC*) or *Escherichia coli* (*tac*), to allow a constitutive expression of the heterologous gene(s). Nevertheless, the limited number of characterized promoters poses a significant challenge during the engineering of *C. necator* for biotechnological applications, and two recent pioneering papers paved the way for the development of a novel constitutive promoters toolbox that will serve the biotechnology community working on *C. necator* [51,52].

In other cases, chromosomal integration of the gene(s) has been pursued to guarantee the stability of the recombinants under the transcriptional control of strong promoters. However, the copy number of the targeted gene(s) is generally lower than those reported for the episomal vectors.

As reported in Table 6.2, the engineering of non-PHA producers with the operon, *phaABC*, has been achieved by applying a number of different vectors. In contrast, no chromosomal integration has been reported so far. This is mostly because *E. coli* was by far the most commonly used host strain. As such, many stable plasmids have been developed in the last century for the genetic engineering of this microorganism. In most cases, the average copy number was found to be 20, and the *phaABC* operon was cloned under the regulation of the strong phagic promoters, T3 and T7.

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TABLE 6.1
Most-Used Plasmids and Their Major Traits Applied to the Engineering of PHA Producers to Convert Organic Waste Streams into PHA

PHA From	Recipient	GMM[a]	Plasmid	Copy Number[b]	Promoter	Ancestor
Whey permeate [5]	*C. necator* DSM545	mREPT	pSUP102 [6]	chromosomal	*phaABC*	pACYC184
Sucrose [7]	*C. necator* NCIMB11599;437-540	*NCIMB11599* (pKM212-*SacC*) 437-540 (pKM212-*SacC*)	pKM212-SacC [8]	10 (oriV)	tac	pBBR1MCS2
Sucrose [9]	*C. necator* 142SR	142SR (pCUV5-*cscAB*)	pCUV5-cscAB [9]	10 (oriV)	P*lacUV5RBS*	pCUP3
Sucrose [9]	*C. necator* 005dZG	005dZG(pCTRC-*cscAB)*	pCTRC-cscAB [9]	10 (oriV)	P*trcRBS*	pCUP3
Soybean oil [10–13]	*A. eutrophus PHB-4*	PHB^{-}4 (pJRDEE32d13)	pJRDEE32d13 [13]	10 (oriV)	P*phaCAc*	pJRD215
Waste animal fat [14]	*C. necator* Re2058, Re2160	Re2058(pCB113) Re2160(pCB113)	pCB113 [15]	10 (oriV)	T3	pBBR1MCS2
Soybean oil [16]	*C. necator* NSDGΔA	MF03	pK18mobsacB [17]	chromosomal	*phaABC*	pK18
Palm kernel oil [18]	*C. necator* PHB^{-}4	PHB^{-}4 harboring *phaCcs*	pBBR1MCS-C2 [18]	10 (oriV)	T3	pBBR1MCS2
Spent palm oil [19]	*C. necator* PHB^{-}4	PHB^{-}4 harboring $phaC_{USMAA2\text{-}4}$	pMBHC2.5 [20]	10 (oriV)	T3	pBBR1MCS3
Udder, lard, tallow [21]	*D. acidovorans* DSM39	DSM39 *(pBBR1MCS*-5-*lipH-lipC)*	pBBR1MCS-5-*lipH-lipC* [21]	10 (oriV)	T3	pBBR1MCS5
Crude glycerol [22]	*C. necator* H16	H16 (*pBBR-glpFK)*	pBBR1-*glpFK* [22]	10 (oriV)	P*glp*	pBBR1
Crude glycerol [22]	*C. necator* H16	H16_glpFK	pK18ms-glpFK-A2858 [22]	chromosomal	PA2858	pK18
Xylose [23]	*C. necator* NCIMB11599	NCIMB11599 (pKM212- *XylAB*)	pKM212-XylAB [8]	10 (oriV)	tac	pBBR1MCS2

[a] Genetically modified microorganism, [b] Origin of replication is reported within brackets

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TABLE 6.2
Most-Used Plasmids and Their Major Traits Applied in the Engineering of Non-PHA Producers to Convert Organic Waste Streams into PHA

PHA From	Recipient	GMM	Plasmid	Copy Number[a]	Promoter[b]	Ancestor
Whey [24]	*E. coli* SP314	SP314 (pJP24)	pJP24[24]	150;200 (pUC)	T5	pQE32
Bovine whey powder solution [25,26]	*E. coli GCSC 4401*, *GCSC 6576*	*GCSC 4401* (pSYL107) *6576* (pSYL107)	pSYL107[27]	15;20 (ColE1)	T7/T3	pSYL105
Crude glycerol [28]	*E. coli (JM109)*	*HBP01*	*pBAD18-phaAB*	15;20 (pbr322)	P_{BAD}-AraC	pBAD18-kan
			pWQ02		T7	pHP 301
			pWQ04[28]	15;20 (pbr322) 10 (p15A)	T7	pACYCDuet-1
Processed bovine whey powder solution [29–31]	*E. coli* CGSC4401, CGSC3121, CGSC 2507, DSM499, KCTC2223	CGSC4401 (pJC4), CGSC3121 (pJC4), CGSC 2507 (pJC4), DSM499 (pJC4), KCTC2223 (pJC4)	pJC4[32]	15;20 (pbr322)	T7/sp6	pGEM-7ZF
Cheese whey [33]	*E. coli* MG1655	CML3-1 and P8-X8	pMAB26 [34]	15;20 (r6k)	tac	pCNB5
Sucrose [35]	*K. aerogenes* 2688, *E.coli* JMU213	2688 (pJM9131) JMU213 (pJM9131)	pJM9131 [35]	15;20 (PBR322)	T7	p4A
Molasses, sucrose [36]	*E. coli*	*phaC1*	pDRIVE [37]	15;20 (pbr322)	T7	pDRIVE

(*Continued*)

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TABLE 6.2 (CONTINUED)
Most-Used Plasmids and Their Major Traits Applied in the Engineering of Non-PHA Producers to Convert Organic Waste Streams into PHA

PHA From	Recipient	GMM	Plasmid	Copy Number[a]	Promoter[b]	Ancestor
Crude glycerol [38]	*E. coli* HMS174(DE3)	HMS174(DE3)/ pCOLADuet-1::*dhaB1B2::pduP::phaC1*	pCOLADuet-1::*dhaB1B2::pduP::phaC1* 38]	20-40 (ColA)	T7	pCOLAduet-1
Xylose [39]	*E. coli TG1*	*TG1(pSYL107)*	pSYL107[40]	15;20 (ColE1)	T7/T3	pBluescript SK (-)
Cellulose hydrolysate [41]	*E. coli* LS5218	LS5218 (*pGEM-phaABC*)	pGEM-phaABC [41]	15;20 (pbr322)	T7/SP6	pGEM
Xylan, *Eucalyptus* hydrosylate [42,43]	*E. coli* JM109, BW25113	JM109 [pTV118NpctphaC1(ST/QK)] BW2511 [pTV118NpctphaC1(ST/QK)]	pTV118NpctphaC1(ST/QK) [42]	15;20 (pbr322)	lac	pTV118N
Xylan from beechwood [44]	*E. coli* LS5218	LS5218 (pTV118NpctphaC1/ pBBRXBB2)	pBBRXBB2 [44]	10 (oriV)	T7/T3	pBBR1-MCS2
Hydrolyzed corn starch [45]	*E. coli* JM101, DH10B	JM101 (*pBHR68*), DH10B (*pBHR68*)	pBHR68 [46]	15/20 (colE1)	T7/T3	pBluescript SK (-)
Starch [47]	*E. coli* BL21(DE3)	SKB99 (*pLW487* / *pTAmyl*)	pLW487 [47]	15-20 (pbr322)	trc	pEP2
Starch [47]	*E.coli* BL21(DE3)	SKB99 (*pLW487* / *pTAmyl*)	pTAmyl [47]	10 (p15A)	T7	pET24ma
Sucrose [48]	*E. coli* W*ΔcscR*	W*ΔcscR* (pAet41)	pAeT41 [49]	150–200 (pUC)	lac	pUC18

[a] Origin of replication is reported within brackets, [b] Promoter column with "/" reports the availability of two regulatory sequences

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Most of the techniques reported above have been used to verify, at least at a laboratory scale, the possibility of making appropriate microbes to produce PHA from inexpensive carbon sources.

In this section, the most encouraging results from whey, molasses, lipids, and starchy and lignocellulosic materials are reported and discussed. Tables 6.3, 6.4, and 6.5 report the most efficient engineered microbes for PHA production from organic waste streams.

6.3 THE USE OF WHEY AS A CARBON SOURCE

Both the EU (>65%) and North America (24%) strongly contribute to the approximate 120 million tons of whey produced globally per year [5,49] as the principal by-product of the dairy industry (about 90% of the volume of handled milk). Of this, 94% is water, 4.5% lactose, and less than 1% is protein, ash, and fat [53,54]. High BOD (about 60,000 ppm) and COD (up to 80,000 ppm) make whey as a waste to be adequately disposed, giving rise to relevant management problems, even if some part of this by-product could be used for the production of human goods and animal feed [55].

Unfortunately, only a few wild-type microorganisms can directly convert lactose into PHA without its preliminary hydrolysis into glucose and galactose, and at low-efficiency levels [56].

Attempts to increase PHA yields from whey were already made in the early nineties by engineering strains of *C. necator* and *Pseudomonas saccharophila*, since these bacteria, although unable to cleave lactose into glucose and galactose, have some strains recognized as excellent PHA producers [57]. Highly proficient strains of *C. necator* were later genetically modified by introducing the *lacZ*, *lacI*, and *lacO* genes of *E. coli*, encoding for β-galactosidase, *lac* repressor, and *lac* operator, respectively. As reported in Table 6.3, the *lacZ* gene was inserted within a depolymerase (*phaZ1*) sequence, thus obtaining a recombinant strain producing polymer from lactose and whey permeate, while reducing cell depolymerization of the polymer by 30–40% [5].

The opposite strategy would be the translocation of the PHA biosynthesis pathway into suitable hosts efficient in hydrolyzing complex substrates to obtain simple carbon sources but unable to produce and accumulate PHA under natural conditions. This will be possible only if the correct structural genes result in enzymatically active synthases that should be adequately supported by the correct pathways and substrates. Since several PHA synthase pathways have been described [58], a key factor that controls the composition of PHA monomers is known to be the "specificity" of the synthase (PhaC). That is why the earliest and successful PHA genes used have been from *A. eutrophus* (*C. necator*) [59,60]. However, non-producer *E. coli* is the most studied host for PHA gene cloning in view of polymer production (Table 6.4). Indeed, knowledge of related metabolic engineering tools makes heterologous gene expression reasonably simple in this host [61,62], which also lacks an intracellular depolymerization system, and whose culturing, polymer extraction, and purification procedures are well recognized [62]. Most importantly, it is well known that *E. coli* is able to convert lactose directly into galactose and glucose. Therefore, the goal

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TABLE 6.3
PHA Production from Organic Waste Streams by Engineered *C. necator* Strains

Strain	Type of PHA	Operation Mode	Substrate	PHA Concentration [g/L]	PHA Content [wt.-% in CDM]
C. necator mREPT [6]	3HB	Flask	Whey permeate	1.4	22
**C. necator* NCIMB11599 [7]	3HB	Batch reactor	Sucrose	2.0	73
**C. necator* 437-540 [7]	3HB-*co*-LA	Batch reactor	Sucrose	0.1	19
**C. necator* 142SR [9]	3HB-*co*-3HHx	Fed-batch reactor	Sucrose	113	81
**C. necator* EO1 [52]	3HB	Flask	Waste rapeseed oil	7.6	88
**C. necator* EO1 [52]	3HB-*co*-3HV	Batch reactor	Waste rapeseed oil[a]	16.0	86
**C. necator* (*pJRDEE32d13*) [10]	3HB-*co*-3HHx	Fed-batch reactor	Soybean oil	102	74
**C. necator* (*pJRDEE32d13*) [11]	3HB-*co*-3HHx	Flask	Palm kernel oil	3.7	87
**C. necator* (*pJRDEE32d13*) [12]	3HB-*co*-3HV-*co*-3HHx	Flask	Palm kernel oil[b]	5.7	80
**C. necator* Re2058 [15]	3HB-*co*-3HHx	Batch reactor	Palm oil	20.7	71
**C. necator* Re2160 [15]	3HB-*co*-3HHx	Batch reactor	Palm oil	20.5	66
**C. necator* Re2058 (*pCB113*) [14]	3HB-*co*-3HHx	Batch reactor	Waste frying oil, waste animal fat	27.0	60
C. necator MF03 [16]	3HB-*co*-3HHx	Flask	Soybean oil	3.8	79
C. necator PHB^{-}4 harboring $phaC_{Cs}$[18]	3HB-*co*-3HV-*co*-3HHx	Flask	Palm kernel oil[b]	9.6	83
C. necator PHB^{-}4 harboring $phaC_{Cs}$[19]	3HB-*co*-3HHx	Flask	Spent palm oil	6.1	72
**C. necator* H16 $glpK_{Ec}$ [22]	3HB	Flask	Crude glycerol	1.4	64
**C. necator* pKM212-XylAB [23]	3HB	Batch reactor	Sunflower stalk hydrolysate	7.9	73

* Reported as *R. eutropha*; [a] Propionic acid; [b] Sodium valerate was used as 3HV Precursor

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TABLE 6.4
PHA Production from Organic Waste Streams by Engineered *E. coli* Strains

Strain	Composition of PHA	Operation Mode	Substrate	PHA Concentration [g/L]	PHA Content [wt.-% in CDM]
E. coli Δ*arcA* [24]	P(3HB)	Batch reactor	Whey	51.1	73
E. coli GCSC4401 (pSYL107) [25]	P(3HB)	Flask	Bovine whey powder solution	4.5	79
E. coli GCSC6576 (pSYL107) [25]	P(3HB)	Flask	Bovine whey powder solution	5.2	81
E. coli GCSC6576 (pSYL107) [26]	P(3HB)	Fed-batch reactor	Bovine whey powder	69.0	87
E. coli CGSC 4401 (pJC4) [29]	P(3HB)	Fed-batch reactor	Processed bovine whey powder solution	59.6	58
E. coli CGSC 4401 (pJC4) [29]	P(3HB)	Fed-batch reactor with controlled DOC	Processed bovine whey powder solution	96	80
E. coli CGSC 4401 (pJC4) [30]	P(3HB)	Fed-batch reactor with cell recycle membrane	Processed bovine whey powder solution	168	87
E. coli CGSC 4401 (pJC4) [31]	P(3HB)	Fed-batch reactor	Processed bovine whey powder solution	35.7	51
E. coli P8-X8[33]	P(3HB)	Fed-batch reactor	Cheese whey	19	39
E. coli (phaC1) [36]	P(3HB)	Fed-batch reactor	Sugar cane molasses	3.0	75
E. coli (phaC1) [36]	P(3HB)	Fed-batch reactor	Sucrose	2.5	65
E. coli WΔ*cscR* [48]	P(3HB)	Fed-batch reactor	Sucrose	47.7	46
E. coli HMS174(DE3)/ pCOLADuet-1::*dhaB1B2*::*pduP*::*phaC1* [38]	P(3HP-*co*-3HB)	Fed-batch reactor	Crude glycerol	16.2	89
E. coli HBP01 [28]	P(3HP-*co*-3HB)	Fed-batch reactor	Crude glycerol	10.1	46
E. coli TG1 [39]	P(3HB)	Flask	Soybean hydrolysate	4.4	74

(*Continued*)

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TABLE 6.4 (CONTINUED)
PHA Production from Organic Waste Streams by Engineered *E. coli* Strains

Strain	Composition of PHA	Operation Mode	Substrate	PHA Concentration [g/L]	PHA Content [wt.-% in CDM]
E. coli LS5218 [41]	P(3HB)	Flask	Cellulose hydrolysate	3.3	59
E. coli LS5218 (*pTVSTQKAB/pBBRXBB2*) [44]	P(3HB)	Flask	Xylan from beechwood	1.1	33
E. coli [*pTV118NpctC1AB(STQK)*] [42]	P(3HB-*co*-LA)	Flask	Xylan	0.8	29
E. coli [*pTV118NpctC1AB(STQK)*] [43]	P(3HB-*co*-LA)	Flask	*Eucalyptus* hydrolysate	5.4	62
E. coli JM101 and DH10B [45]	P(3HB)	Flask	Hydrolyzed corn starch	1.0	50
E. coli SKB99 [47]	P(3HB)	Flask	Starch	1.2	57

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was firstly reached by cloning PHB-biosynthesis genes [25,26]. Later, a concentrated whey substrate containing high levels of lactose (280 g/L) was used for a fed-batch culture of *E. coli* containing *A. latus* PHB biosynthetic genes [29]. *E. coli* was also used as a host strain for PHA genes coming from *Azotobacter* sp., thus producing the polymer for more than 70% of CDM directly from milk whey, even taking advantage of the absence of the lactose repressor [24]. *C. necator* PHB-synthesis genes fused to a lactose-inducible promoter were also cloned in *E. coli*, thus resulting in remarkable production of the polymer [39].

6.4 THE USE OF MOLASSES AS A CARBON SOURCE

Molasses is a low-value by-product of the sugar industry. Its composition depends on the original source (cane or beet) and refining (sulfured or unsulfured), but 50% of its weight is due to sugars, mainly sucrose and also glucose and fructose [63]. Although pure sucrose is difficult to obtain, store, and transport, molasses is used for a number of industrial fermentations by yeast [64–66], and the problem of the cost of waste disposal persists.

Original attempts to obtain PHA from molasses was made by using *Azotobacter vinelandii* [67], followed by many other efforts with *Alcaligenes latus* (*Azohydromonas lata*), *Pseudomonas corrugata*, and species of *Bacillus*, until recent years [68–74].

However, high PHA production was obtained by genetically engineered sucrose-using *Escherichia coli*, *Klebsiella oxytoca*, and *K. aerogenes*, if properly provided with PHA genes [35]. For instance, in *E. coli*, the *phaC1* from *Pseudomonas* sp. was successfully expressed, thus obtaining 75% polymer concentration [36], as well as PHB biosynthesis genes from *C. necator* giving 80% of CDM and high biomass in a fed-batch culture mode [75]. However, in fed-batch cultivation, the sugar concentration is usually maintained at low levels between feeding pulses, and when sucrose concentration drops below 2 g/L, a *cscR* factor represses the *csc* regulon transcription thus hampering bacterial growth [76]. The obtainment of a *cscR* knock-out *E. coli* mutant [77] overcame this problem, and the *phbABC* operon from *C. necator* allowed more than 1.5-fold PHB production [77].

In contrast, few PHA producers were engineered for sucrose utilization (Table 6.3 and 6.5). For instance, the heterologous *sacC* gene enabled a sucrose utilization pathway in *C. necator* [7]. At the same time, a poly(3-hydroxybutyrate-*co*-lactate) was obtained from sucrose by the same bacterial species expressing genes from *Pseudomonas* sp., *Clostridium propionicum*, *Mannheimia succiniciproducens*, and *E. coli* [8]. Very recently, a recombinant strain of *C. necator* able to produce poly(3-hydroxybutyrate-*co*-3-hydroxyexanoate) [11] has been further provided with *csc* genes from *E. coli* to grow on sucrose; the resulting strain produced vast amounts of the copolymer at high concentrations on CDM [9].

6.5 THE USE OF LIPIDS AS A CARBON SOURCE

A number of industries produce great volumes of lipid-rich waste. Besides slaughterhouses and food processing activities, waste cooking oil and animal fats are

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TABLE 6.5
PHA Production From Organic Waste Streams by Other Engineered Microbes

Strain	Type of PHA	Operation Mode	Substrate	PHA Concentration [g/L]	PHA Content [wt.-% in CDM]
K. aerogenes 2688 (pJM9131) [35]	P(3HB)	Flask	Sucrose	3.0	50
K. aerogenes 2688 (pJM9131) [35]	P(3HB)	Fed-batch reactor	Sucrose	24.0	70
B. licheniformis M2-12 [56]	P(3HB-*co*-3HV-*co*-3HHx)	Batch reactor	*Palm oil mill effluent*	16.2	89
D. acidovorans (pBBR1MCS-5-lipH-lipC) [21]	P(3HB-*co*-3HV-*co*-4HB)	Flask	Udder	N.A.	27
D. acidovorans (pBBR1MCS-5-lipH-lipC) [21]	P(3HB-*co*-3HV-*co*-4HB)	Flask	Lard	N.A.	39
D. acidovorans (pBBR1MCS-5-lipH-lipC) [21]	P(3HB-*co*-3HV-*co*-4HB)	Flask	tallow	N.A.	15

"N.A." stands for not available.

produced in large volumes, often causing water and land pollution together with disposal problems [78,79]. The possibility of using this material as a substrate for PHA production is supported by the above considerations, together with the PHA yield from oil and fatty acid, which is ≥0.65 g PHA/g, while only 0.32–0.48 g PHA/g for glucose [82]. Wild-type *P. aeruginosa* and *C. necator* can accumulate *mcl*-PHA from residual cooking oil and other waste oils [53,80–84], while the same *mcl*-PHA can be produced by *Erwinia* sp. from crude palm oil [85]. Other residual fats from rendering industries can be converted into PHA by *P. resinovorans* and *R. eutropha* (*C. necator*) [83,86].

Once again, the development of more efficient lipid utilizing strains required some genetic modification (Table 6.3 and 6.5). Obruca and colleagues obtained a mutant strain of *C. necator* with an increased NADPH/$NADP^+$ ratio, thus resulting in the accumulation from waste frying rapeseed oil of almost 90% PHB on CDM. Mutants of *Bacillus licheniformis* produced 3-hydroxyvalerate (3HV) [87] and 3-hydroxyhexanoate (3HHx) copolymers from palm oil [53]. Even more interesting, due to thermal and mechanical properties, copolymers with a high content of 4HB were obtained from oily substrates and by-products [88]. A PHA heteropolymer with 4–5 mol-% 3HHx was obtained from olive oil, corn oil, palm oil [89], and later from soybean oil [10], palm kernel oil, and palm acid oil [11,12]

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in a PHA-negative *C. necator* mutant containing a heterologous PHA-synthase gene. In a series of studies [38], high percentages on CDM of several copolymers were obtained by recombinant *C. necator* strains expressing PHA synthases with broad substrate specificity. Both *scl*- and *mcl*-monomers were incorporated into the polymer, and Mifune et al. [16] obtained higher levels of 3HHx by the insertion of *phaJ* from *A. caviae*. The same *phaJ* gene was also isolated from *P. aeruginosa* and cloned into a *C. necator* strain containing a PHA synthase from *Rhodococcus aetherivorans*. The recombinant strains obtained accumulated a copolymer with a 17–30% content of 3HHx from palm oil [15]. Similar 3HHx contents were obtained by a recombinant *C. necator* strain from waste animal fats within a protein hydrolysis plant and residual frying oil [14]. PHA synthase gene (*phaC*) was moved from *Chromobacterium* sp. to *C. necator*, thus allowing the production of the interesting terpolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) with a wide range of 3HV monomer compositions, starting from crude kernel oil and 3HV precursors [18]. Moreover, *mcl*-PHA were obtained from lard by *P. putida* and *P. oleovorans* genetically modified to metabolize triacylglycerols [90], and *Delftia acidovorans* engineered for the co-expression of *lipC* and *lipH* lipase genes from *P. stutzeri* resulted in its ability to grow on fatty substrates from slaughterhouses, thus producing PHA with high molar fractions of 4HB [21].

A key component of lipids is glycerol, and among several bacterial genera, both *Cupriavidus* sp. and *Pseudomonas* sp. can metabolize glycerol and accumulate PHA under aerobic conditions. Currently, glycerol is generated in considerable amounts as a major residue from biodiesel manufacturing, and the recent drop in its price makes this an appealing source for microbial production of PHA. However, the presence of impurities seems to affect both growth and polymer properties [91–93]. For this purpose, many different bacterial strains were found to accumulate the polymer from glycerol [94–98], and 3HV-containing copolymers were also obtained [99–101]. Even in the case of this by-product, some genetic modification was performed on *C. necator* by the introduction of genes involved in aerobic metabolism of *E. coli*. In this case, the overexpression of the glycerol kinase (*glp*) genes enhanced polymer accumulation [22]. More genetic modification was carried out on *P. putida* by the deletion of the transcription repressor gene, *glpR*, to reduce the lag phase and increase *mcl*-PHA accumulation [102].

Homopolymers such as P(4HB), P(3HV), and P(3HP), as well as random copolymers and block copolymers, were obtained by engineering *E. coli* strains from glycerol (see Table 6.4) [73,33,103–106]. In addition, *E. coli* was genetically modified by introducing glycerol dehydratase genes from *C. butyricum*, propionaldehyde dehydrogenase from *Salmonella enterica*, and PHA synthase from *C. necator* [38] for the synthesis of an interesting homopolymer such as poly(3-hydroxypropionate) [P(3HP)]. As these monomers are incorporated into PHA, the resulting copolymer will present lower crystallinity and fragility [35]. The addition of glucose to waste glycerol allowed a higher titer of P(3HP) by another recombinant *E. coli* strain containing the heterologous genes propionaldehyde dehydrogenase (*pduP*), glycerol dehydratase and its factors *dhaB123* and *gdrAB* from *K. pneumoniae*, and *phaC1* from *C. necator* [28].

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6.6 THE USE OF STARCHY MATERIALS AS A CARBON SOURCE

Besides the high amounts of starch and starch derivatives consumed in the human diet as the main carbohydrate [36,37], almost one-third of the rest is used for other applications such as paper and other derived products, pharmaceuticals, textiles, biofuels, and bioplastics [107–112]. The use of hydrolyzed corn starch in place of glucose could reduce the cost of PHB by around 25% [1]. In some cases, some selected bacteria can directly convert raw starch into a polymer and/or a copolymer [111–113].

Once again, strains of *E. coli* have been used as heterologous gene recipients. Poor yields were obtained from hydrolyzed corn starch by cloning *C. necator* PHA synthase genes [44], while in a strain expressing amylase genes from *Paenibacillus* sp. a higher yield was reached [46]. In the efficient amylolytic *Aeromonas* sp., the whole *phaCAB* operon was also inserted to achieve the one-step conversion of starch to PHA, but the results were not particularly encouraging [114]. Since the gram-positive C*orynebacterium glutamicum* is endotoxin-free, it was used in place of *E. coli,* by cloning the operon *phbCAB* of *C. necator* first, and by also inserting alfa amylase from *S. bovis* to use soluble starch [115].

6.7 THE USE OF LIGNOCELLULOSIC MATERIALS AS A CARBON SOURCE

Although lignocellulosic materials are known to be rather recalcitrant to pre-treatments needed to obtain fermentable sugars, lignin, cellulose, and hemicellulose are certainly the most available and cheapest renewable resources in the world [116]. Unfortunately, together with its recalcitrance, this material releases some toxic by-products during hydrolysis, such as 5-hydroxymethylfurfural (5-HMF), thus threatening bacterial growth [117]. However, other than *C. necator* [118,119], a number of bacteria have been isolated as PHA producing from sugarcane bagasse hydrolysate and xylose [120]. While *C. necator* can grow on hydrolysates of different biomass origin, it lacks the enzymes to metabolize pentoses [121, 122], and many efforts are in progress by several research groups to obtain strains able to use xylose and arabinose (Table 6.3 and 6.4). These sugars were metabolized by recombinant strains of *C. necator* expressing *E. coli* genes for arabinose uptake and metabolism [122] and xylose transporters [123], but with a modest polymer production. More recently, high P(3HB) content was obtained from sunflower stalk hydrolysate by the same bacterial species expressing xylose isomerase and xylulokinase genes from *E. coli* [23]. Moreover, the mixture of different sugars, such as glucose and xylose, may induce diauxic growth, resulting in a general slowing down of the process. In this case, although not completely stable yet, a phosphotransferase system (PTS) mutant of *E. coli* was found to use both the above sugars simultaneously [124]. Other attempts at hydrolysate/xylose utilization have been made directly in recombinant *E. coli* strains, one holding *C. necator* PHA biosynthesis genes [39], and another, strain LS5218-STQKABGK [125], showing superior resistance to 5-HMF, one of the major inhibitors released in the pretreated cellulosic material [40]. In order to proceed toward the one-step conversion of cellulosic biomass into PHA, *E. coli* has been further engineered by inserting endoxylanase *xylB* from *S. coelicolor* and *xynB*

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from *B. subtilis* [43], PHA synthase from *Pseudomonas* sp., propionyl-CoA transferase (PCT) from *Megasphaera elsdenii*, and *phaA* and *phaB* from *C. necator* [41]. Efficient production of P(3HB-*co*-LA) was also achieved by the same recombinant strain from *Eucalyptus* hydrolysate [42]. Thus, consolidated bioprocessing of biomasses, typical of biofuel production [126,127], could be expected in the near future for biopolymer production too.

6.8 CONCLUSIONS AND OUTLOOK

The results reported above indicate that many different approaches are in progress globally to obtain new materials at affordable costs. This concept has to necessarily consider the use of cheap feedstocks for chemical, but especially microbiological, conversion. However, residual, low-cost biomasses from a large array of anthropic activities and their compositions are extremely variable. The types of biomass discussed above are probably the most suitable to be processed into PHA because of their availability, carbon content, and low cost.

Moreover, there are other reasonably interesting new feedstocks to be investigated, such as volatile fatty acids (VFA), from the fermentation of agriculture residues or syngas originating from gasification processes. In the latter case, *E. coli* was used again as a gene donor, and a recombinant strain of *Rhodospirillum rubrum* was constructed and found able to produce, besides PHA, the industrially interesting coproduct, H_2 [128–130]. However, there are numerous metabolic pathways developed mainly in recombinant *E. coli* for PHA biosynthesis from unrelated carbon sources [131,132]. In all the contexts, old and new feedstocks, the use of recombinant DNA techniques are becoming more and more important to increase the possibility of obtaining added-value products from waste materials.

Recently, the development of the proficient gene-editing CRISPR/Cas9 technology paved the way for novel and extremely interesting research tools. The genome-editing methods based on CRISPR/Cas9, CRISPR-Cas12a, and/or CRISPRi have been recently reviewed as becoming increasingly crucial for the regulation of metabolic flux to PHA, the development of strong PHA synthetic pathways, and further host strain optimization [133]. The simultaneous integration of genes into multiple loci of the host genome via CRISPR/Cas9 will support researchers in the near future to edit microbial genomes more quickly, targeting both several PHA syntheses and multiple substrate-utilization pathways to be promptly improved.

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