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Potential of Diverse Prokaryotic Organisms for Glycerol-based Polyhydroxyalkanoate Production

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Abstract

The potential and performance of various Gram-negative, Gram-positive and archaeal wild type microorganisms, and bacterial mixed cultures, as well as the application of genetically engineered strains as whole-cell biocatalysts for glycerol-based polyhydroxyalkanoate production are analyzed and assessed. This encompasses the comparison of growth and polyhydroxyalkanoate accumulation kinetics, thermo-mechanical properties of isolated glycerol-based polyhydroxyalkanoate of different composition on the monomeric level, and the presentation of mathematical models developed to describe glycerol-based polyhydroxyalkanoate production processes. For all these aspects, the article provides a detailed compilation of the contemporary state of knowledge, and gives an outlook to expected future developments.

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1. Introduction

1.1. Gram-Negative Eubacterial Production Strains

The use of glycerol for microbial polyhydroxy-alkanoate (PHA) synthesis has been analyzed using pure cultures of various natural species, predominate-ly belonging to Gram-negative eubacteria. The subsequent paragraphs summarize the respective research endeavors, includeing the production of *scl*-PHA homo- co- and terpolyesters, and elastomeric *mcl*-PHA.

Poly3-R-hydroxybutyrate-co-3-R-hydroxyvalerate (PHBHV) copolyesters with an exceptional high content of 3HV of about 85% molmol^{-1} were produced on 10 liter bioreactor scale by Haage et al. by cofeeding glyceroland the 3HV-related substratesodium pentanoate using *Azohydromonas australica* DSM 1124 (formerly *Alcaligenes latus*) as production strain. This high content of 3HV in PHBHV constitutes the highest value ever reported in the literature for PHBHV production on two substrates in parallel. In contrast to cultivation of this strain on sucrose, for instance, where considerable amounts of PHA are already accumulated during the balanced growth ("growth associated PHA production"), the reported process only resulted in minor quantities of PHA before limitation of the nitrogen source

(ammonium sulfate); hence, PHA formation is decoupled from biomass synthesis (non-growth associated PHA production). Kinetic analysis of the process revealed that glycerol as a carbon source, on the one hand, inhibits 3-hydroxybutyrate (3HB) synthesis in *A. australica* DSM 1124, but, on the other hand, can be regarded as a suitable carbon source for the first cultivation phase, where the production of high amounts of catalytically active biomass is desired under complete nutrient supply. Most importantly, co-feeding of glycerol seems to be beneficial for conversion of pentanoate into 3HV, as indicated by the achieved yield of 0.55 g 3HV per g of pentanoate, and a specific productivity of 3HV being five times higher than for 3HB [1].

Tanadchangsaeng et al. compared their own data for glycerol-based poly3-R-hydroxybutyrate (PHB) production by *Cupriavidus necator* with the literature data for glucose-based PHB production by the same strain. The authors found that *C. necator* produces PHB on glycerol at a rather low productivity (0.92 $gL^{-1}h^{-1}$) and maxim-um specific growth rate ($\mu_{max.}$) of 0.11 h^{-1} when compared to the glucose-based literature values. Trying to explain these findings, the authors assumed that *C. necator* can synthesize glucose from glycerol, claiming that the lithotrophic utilization of glycerol as a non-fermentative substrate (gluconeogenesis) influences cell growth and PHB accumulation. The presented results also showed that gluconeogenesis affects the reduction of cell mass, PHB productivity, and molar mass [2].

Methylobacterium rhodesianum and *Ralstonia eutropha* (*C. necator*) were cultivated for PHB production in the medium, which contained glycerol as carbon source and casein hydrolysates as carbon and nitrogen sources. *M. rhodesianum* accumulated an average of ca. 0.4 g PHB per g cell dry mass (CDM) during 92 h of cultivation in shaking flasks, and about 0.5 g PHB per g CDM during 45 h of cultivation on bioreactor scale. *C. necator* yielded an average of 0.47 g PHB per g CDM during 67 h of cultivation using casein peptone, and 0.65 g PHB per g CDM during 45 h of cultivation in the medium supplemented with casamino acids. About 65% of the supplied nitrogen was used for the growth of residual non-PHB biomass. The conversion yield of glycerol into PHB amounted to 0.17 g PHB per g substrate for both strains [3].

Cavalheiro et al. exploited two strategies to enhance the efficiency of PHA production on glycerol: high density cultures to increase PHA volumetric productivity and the use of crude glycerol phase (CGP) as primary carbon source for cell growth and polymer synthesis. *C. necator* DSM 545 was used to accumulate PHB from CGP and from commercial pure glycerol as control substrate. Using pure glycerol, productivities between 0.6 and 1.5 $gL^{-1}h^{-1}$ were attained. The maximum CDM and the PHB content in CDM amounted to 82.5 gL^{-1} and 0.62 gg^{-1}, respectively. When CGP was used, a CDM of 68.8 gL^{-1} with a PHB accumulation of 0.38 gg^{-1}, resulting a final PHB productivity of 0.84 $gL^{-1}h^{-1}$, was obtained. By decreasing the biomass concentration at which accumulation was triggered, a PHB productivity of 1.1 $gL^{-1}h^{-1}$, corresponding to 0.50 g PHB per g CDM, was achieved using CGP [4].

Scl-PHA copolyesters poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (PHB4HB) and poly(3-hdroxy-butyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate) (PHBHV4HB) terpolyesters were produced by *C. necator* DSM 545 in high-cell density fed-batch cultures as presented by the same research group. CGP from biodiesel production was used as a carbon source for growth and PHB production. Incorporation of 4-hydroxybutyrate (4HB) monomers was accomplished using γ-butyrolactone (GBL) as a precursor compound. By adding propionic acid as a stimulator of 4HB production, the incorporation of 4HB into the polyester was doubled; in addition, propionic acid acted as precursor for incorporation of 3HV, resulting in the formation of PHBV4HB terpolyesters. By adjusting the dissolved oxygen concentration and cultivation time, PHB4HBs with the molar fractions of 4HB between 0.11 and 0.22 mol/mol were attained. Similarly, PHBHV4HB was obtained with the molar fractions of 4HB between 0.25 and 0.44 $molmol^{-1}$, and of 3HV between 0.06 to 0.10 $molmol^{-1}$ [5].

Apart from CGP from biodiesel production, Ramachandran and Amirul used “glycerine pitch”, a by-product stemming from oleochemistry, as raw material for cultivation of a yellow-pigmented *Cupriavidus* sp. USMAHM13, isolated from natural samples in Malaysia. The authors investigated different feed mixtures containing “glycerine pitch” plus 4HB precursors GBL or 1,4-butanediol (1,4BD) in one- and two-stage shaking flask-scale cultivation processes. Dependent on the feed composition, PHB, copolyesters of 3HB, and differing contents of 4HB were obtained. Cofeeding of 5 gL^{-1} glycerine pitch and 5 gL^{-1} 1,4BD in a one-stage cultivation process resulted in a CDM of 6 gL^{-1}, containing a mass fraction of poly (3HB-*co*-43%-4HB) of 0.49 gg^{-1} [6]. Similar to the setups described before, also this copolyester was rather of low molar mass [7]. Later, the same authors optimized the incorporation of 4HB building blocks by fine-tuning the supply of ammonia acetate, again using “glycerine pitch” as the main carbon source. This way, a broad range of different PHB4HB copol-yesters with tailored 4HB content between 0.03 and 0.40 $molmol^{-1}$ was produced in the batch fermen-tation set-ups [7].

Mothes et al. investigated the bacterial production of *Pseudomonas denitrificans* and *C. necator* JMP 134 strains, which accumulate PHB from pure glycerol to a mass fraction between 0.6 g (*C. necator*) and 0.7 g (*P. denitrificans*) PHA per g CDM.

When using CGP containing 5.5 % NaCl, a reduced PHB content of 0.48 gg^{-1} was observed at a CDM of 50 gL^{-1}. Furthermore, the PHB yield coefficient was reduced, obviously due to osmoregulation. The effect of glycerol contaminated with K_2SO_4 on PHA production was less pronounced [8].

By screening for PHA-producing bacteria that can utilize CGP as a sole carbon source, Teeka et al. found out that the isolate *Novosphingobium* sp. THA_AIK7, later identified as *Novosphin-gobium capsulatum*, was able to accumulate a mass fraction of PHB in CDM of up to 0.45 gg^{-1} in shaking flask scale experiments after 72 h cultivation. The conversion yield of PHB from glycerol (YPHA/GLY) amounted to 0.29 gg^{-1}. In addition, by using a combined method of hypochlorite and organic solvent application, the authors succeeded in producing an ultra-pure PHA without detectable amounts of endotoxins, which frequently contaminate PHA production by Gram-negative strains. The authors suggested that this degree of purity could meet the purity requirements for *in vivo* application in the medical and surgical fields. The melting point of the produced homopolyester was reported to be 179°C, atypical value for this type of PHA [9].

Zobellella denitrificans MW1 was used by Ibrahim and Steinbüchel for PHB production from glycerol as the sole carbon source by fed-batch fermentations on 42 L scale. After only 24 h of fermentation, relatively high CDM values of about 30 gL^{-1} were obtained, but with relatively low PHB content of about 0.31 g PHA per g CDM. Higher PHB concentrations of more than 60 gL^{-1} and CDM exceeding 80 gL^{-1} were obtained by further optimization of the fed-batch fermentation strategy, when 20 gL^{-1} NaCl was supplied to the cultivation medium, together with optimized feeding of carbon and nitrogen sources. As a result, outstandingly, high specific growth rate of more than 0.4 h^{-1} and mass fractions of PHB in CDM of up to 0.7 gg^{-1} were obtained. The maximum PHB productivity and yield coefficient for PHB synthesis from glycerol amounted to 1.09 $gL^{-1}h^{-1}$ and Y PHA/GLY 0.25 g PHB per g substrate, respectively. In addition, a simple extraction process for PHB recovery was developed by self-flotation of cell debris after chloroform extraction of PHB, resulting in a convenient separation of a clear PHB-solvent solution from the cell residues. Through this improvement, maximum PHB recovery yield of 85.0% w/w after 72 h of chloroform extraction at 30°C was achieved. Also in this case, the polymer is described to be of extremely high purity [10].

Similar to *Burkholderia sacchari* DSM 17165, *Burkholderia cepacia* ATCC 17759 accepts C5-sugars (pentoses) like xylose as carbon substrates, synthesized PHB from the mixtures of xylose and glycerol with the concentrations of the latter ranging from 3% to 9%ww^{-1}. Increasing the glycerol concentration resulted in a reduction of CDM, PHA yield, and molar mass of PHB. Also in this case, ^{1}H-NMR revealed that molar masses decreased due to the esterification of glycerol with PHB, resulting in chain termination by the before described "end-capping" effect. Melting temperature and glass transition temperature of the end-capped polymers showed no significant difference when compared to the xylose-based PHB [11]. Using biodiesel-derived glycerol as the sole carbon source, the process was successfully scaled up to 200 L for PHB production; here, the obtained concentrations for CDM and PHB amounted to 23.6 and 7.4 gL^{-1}, respectively [12].

Additional isolation experiments were carried out by Kawata and Aiaba, who also screened for CGP-utilizing bacteria. These authors discovered the alkalo- and halophile Gram-negative bacterial species, *Halomonas* sp. KM^{-1}, which produced PHB in a simple medium based on diluted CGP or pure glycerol as sole carbon sources. Different concentrations of glycerol resulted in different growth kinetics and different final concentrations of biomass, PHB and mass fraction of PHB in biomass. The cultures grown in the medium containing 5 % w/w glycerol resulted in the PHB mass fractions in CDM of 0.45 gg^{-1}, with PHB concentrations amounting to 2.3 gL^{-1}. Using waste glycerol as a carbon source, the authors reported a considerable decrease of the output values in terms of biomass and PHB formation, however without specifying any further details [13].

Yangia sp. ND199 constitutes another moderate halophile bacterial species able to accumulate PHBHV with a molar monomer fraction of 0.03 mol 3HV per mol PHA from the unrelated carbon sources in a saline medium. Cultivation with glycerol and yeast extract as a nitrogen source resulted in a maximum CDM of 5.7 gL^{-1} and a PHA mass fraction of 0.53 gg^{-1} after 40 h. The 3HV fraction was the highest at the start of PHA accumulation, and decreased with increasing the PHA content in CDM. In contrast, PHB was synthesized when glutamate was used as carbon source. Fed-batch cultivation of *Yangia* sp. ND199 with glycerol and yeast extract resulted a productivity of 0.44 $gL^{-1}h^{-1}$ for PHBHV and a mass fraction of 0.53 g PHBHV per g CDM. Using CGP as a carbon source, these values decreesed to a productivity of 0.25 $gL^{-1}h^{-1}$ for PHBHV and a mass fraction of 0.41 g PHBHV per g CDM. Both mass fraction and productivity were improved to 0.61 $gL^{-1}h^{-1}$ and 0.56 g PHBHV per g CDM, respectively, by using 1:1 mixture of CGP and fructose-rich corn syrup [14].

The biosynthesis of PHA by *P. putida* JCM6160 cultivated in a medium containing glycerol, nonanoic acid or a glycerol/nonanoic acid mixture as carbon sources was investigated by Miura et al. [15]. Using glycerol as the only carbon source, the mass fraction of PHA in CDM amounted to about

0.2 gg^{-1}. The authors argue that this relatively low content might be due to the "endcapping" effect, and also the absence of enzymes that can directly synthesize PHA from acetyl-CoA as the major product of glycerol catabolism. Fatty acids with even numbers of carbon atoms are well-known substrates for PHA synthesis from acetyl CoA ("*de novo* fatty acid synthesis"). In the described article, also the application of fatty acids as carbon feed stocks resulted in decreased PHA content, because fatty acids are shifted into other pathways not explained in detail by the authors. However, addition of the odd-numbered fatty acid nonanoic acid into a glycerol medium dramatically increased the PHA content in *P. putida* cells, reaching considerably higher PHA amounts than the sum of the values found when glycerol and nonanoic acid were separately used as the sole carbon sources. The PHA synthesized in the glycerol/nonanoic acid medium contained 3HA units of 5, 6, 7, 8, 9, or 10 carbon atoms. The 3HA units with the even numbered carbons were supposed to stem from glycerol catabolism and subsequent fatty acid synthesis, whereas the PHA units with odd numbered carbons were supposed to derive from nonanoic acid breakdown. Pentanoate (C5) units derived from glycerol and nonanoic acid were also found in the polyester; because pentanoate units were surprisingly not found in PHA produced in these set-ups containing only nonanoic acid (it can be assumed that perntanoic acid aundergoes catabolism to propionyl-CoA and acetyl-CoA, whereby propionyl-CoA, in this case, gets rapidly decarboxylyzed to CO_2 and acetyl-CoA), the authors suggested that they were synthesized indirectly via β-oxidation of nonanoic acid with the assistance of glycerol [15].

A further study evaluates the potential of several characterized *P. putida* strains to produce *mcl*-PHA from CGP as the sole carbon source. Among all tested strains, *P. putida* KT2440 turned out to be the most efficient strain able to synthesize *mcl*-PHA under nitrogen limiting conditions reaching the mass fractions of PHA in CDM of 0.34 gg^{-1}. Disruption of the PHA depolymerase gene (*phaZ*) in *P. putida* KT2440 further enhanced the mass fraction to up to 0.47 gg^{-1}. It was assumed that this rather low biomass and PHA concentration (found both in the mutant strain and the wild-type strain of *P. putida* KT2440) might be due to the excessive production of the side-product citrate, which accumulates at a high yield of 0.6 gg^{-1} during the cultivation. The authors suggested further metabolic engineering to be focused on reducing the production of citrate in order to increase the carbon flux towards *mcl*-PHA biosynthesis [16].

Recently, glycerol turned out to be an effective carbon source for *mcl*-PHA production by *Pseudomonas* spp. *P. mediterranea* 9.1 (CFBP 5447). This organism synthesizes an amorphous, elastic *mcl*-PHA, when grown on CGP, whereas on a reagent grade and partially refined glycerol, it produces two very similar unique *mcl*-PHAs that can be processed into transparent films [17].

1.2. Archaeal PHA Production from Glycerol

A restricted number of reports are available on archaeal PHA production. PHB production from glycerol was investigated and optimized in *Haloarcula* sp. IRU1, a novel archaeon isolated from Urmia Lake, Iran. Using Taguchi methodology for statistic optimization of the cultivation medium, the impact of glycerol, yeast extract and KH_2PO_4 concentration was evaluated for their individual and interactive effects on PHB production. It was shown that the glycerol concentration was the most significant factor affecting the PHB yield. The optimum factor levels were a glycerol concentration of 8% w/w, yeast extract 0.8% w/w, and KH_2PO_4 0.002% w/w. The predicted value obtained for PHB content in CDM under these conditions was about 0.82 gg^{-1}. The authors concluded that *H.*sp. IRU1 might be another promising candidate for PHB synthesis from glycerol [18].

Also the archaeon *Haloferax mediterranei* turned out to be a promising candidate for production of PHA co- and terpolyesters (PHBHV and PHBHV-4HB, respectively) using CGP as the carbon source. As continuation of the work reported by Koller et al. [19], Hermann-Krauss et al. assessed the application of CGP by direct comparison with pure glycerol. Using pure glycerol, a PHBHV copolyester with a molar fraction of 3HV of 0.10 $molmol^{-1}$ was produced at a volumetric productivity of 0.12 $gL^{-1}h^{-1}$ and an intracellular PHA content of 0.75 gg^{-1}. Application of CGP resulted in the same polyester composition and volumetric productivity, underlining the feasibility of direct application of CGP. The molecular weight of the materials was revealed to be of 150,000 (PDI 2.1) and 253,000 (PDI 2.7) for pure glycerol and CGP, respectively, whereas the melting temperatures ranged between 130°C and 140°C in both setups. Supplying GBL as a 4HB precursor resulted in a PHBHV4HB terpolyester containing 3HV and 4HB 0.12 and 0.05 $molmol^{-1}$. The terpolyester displayed reduced melting (melting endotherms Tm at 122 and 137°C) and glass transition temperature (Tg 2.5°C), increased molar mass (391,000), and a polydispersity (PDI) similar to the PHBV copolyesters. As a particularity, this process was operated without sterilization of the bioreactor equipment; due to the high salinity of the fermen-

tation medium, the culture remained monoseptic during the entire process [20].

1.3. Gram-positive Eubacterial Production Strains

In contrast to the ample reports on glycerol based PHA-production by Gram-negative organisms, the reports on Gram-positive candidates, which can fulfill this task, are very restricted. As one of the rare examples, *Bacillus firmus* NII 0830 was investigated for PHB production on CGP from biodiesel production. Here, a pre-treatment of CGP using strong acids was investigated. It turned out that this pre-treatment did not positively impact PHB productivity, and thus can be avoided. After statistical media optimization, 3 $g.L^{-1}$ of biomass was obtained, harboring a PHB mass fraction of 0.53 gg^{-1} CDM [21].

Furthermore, PHB production under nitrogen-limited conditions by *Bacillus sphaericus* NII 0838 using CGP as a sole carbon source was investigated by Sindhu et al. The effect of various process parameters on PHB production such as glycerol concentration, inoculum size and the medium's pH-value was optimized. The obtained results showed that the bacterial culture can accumulate a mass fraction of PHB in CDM of about 0.31 gg^{-1} in CGP medium [22].

1.4. Enrichment Strategies to Isolate Glycerol Converting PHA Producers From Mixed Culture

Using cultivation dependent and -independent methods, Ciesielski et al. characterized mixed microbial populations capable of converting CGP into PHAs. The authors monitored the enrichment of the microbial community by applying the ribosomal intergenic spacer analysis (RISA). Subsequently, the microbial community was analyzed by 16S rRNA gene sequencing. Molecular analysis showed that the populations consisted of microorganisms belonging to the four bacterial lineages of α-Proteobacteria, γ-Proteobacteria, Actinobacteria, and Bacteroides. Among these, three *Pseudomonas* sp. and *Rhodobacter* sp. also contained PHA synthase genes [23]. RISA was also used by Wattanaphon et al. to analyze the dynamics of microbial communities able to convert CGP into PHA by cultivation on CGP as a sole carbon source. Analyzing the microbial community during 55 days of cultivation by exploiting the obtained gel electrophoresis, three characteristic band patterns were observed. 16S rRNA sequences were analyzed from dominant RISA bands, showing that the members of the CGP-converting bacterial community were closely related to *Azoarcus* sp., *Bacillus cereus*, *Bacillus pseudofirmus*, *Flavobacterium columnare*, and *Thauera* sp. [24].

Selective enrichment of PHA accumulating strains from environmental samples by long-term carbon starvation was already described in 1998 by Renner et al. who supplied the first vessel of a five-stage bioreactor cascade with a carbon-rich and nitrogen-poor medium, and operated the system at extremely low dilution rates. Starting from the second vessel, the cells were exposed to both carbon- and nitrogen-limited conditions, resulting in reduced number of those cells that did not accumulate PHA in the first vessel [25]. Similar considerations were the basis to explore the potential of microbial community engineering for a production of PHA from glycerol, as accomplished by Moralejo Garate et al. Here, a PHA-producing microbial community was enriched based on cultivation in a feast-famine regime, hence, alternating the phases of sufficient and limited nutrient supply, using a glycerol-fed sequencing batch reactor operated at a residence time of 2 days with feast-famine cycles of 24 h. In a subsequent fed-batch PHA production step under growth-limiting conditions, the enriched mixed community produced PHA up to a mass fraction of PHA in CDM of 0.8 gg^{-1}. Since, glycerol was expected to be fully metabolized via the glycolysis pathway, the authors anticipated that, besides PHA, polyglucose (PG) might be formed as a storage polymer as well. In fact, PG was found in CDM at mass fraction of about 0.1 gg^{-1}. The results indicated that the feast-famine-based enrichment strategy might constitute a feasible tool making it possible for the mixed community of microbes to accumulate PHA from glycerol [26]. These works were further continued in order to understand the effect of the cycle length on the bacterial enrichment process with emphasis on the distribution of the glycerol flux towards PHA or PG formation, respectively. Two sequencing batch reactors were operated with the same retention time. It was revealed that a short cycle length (6 h) favored PG production, whereas long cycle length (24 h) was beneficial for predominant PHA accumulation. In both set-ups, the same dominating microorganisms were detected indicating that different metabolic pathways might be the cause for the variable contents of accumulated products rather than an effect of the composition of the microbial community [27].

Similar experiments were accomplished by Moita et al. who enriched a microbial culture with CGP as a sole carbon source in a sequencing batch reactor. The selected culture, pre-adapted on bio-oil, had the ability to consume both glycerol and methanol fraction present in CGP. Similar to the works described right before [26,27], the glycerol content of CGP was converted by the community into PHB and PG. The culture reached a maximum PHB mass fraction in CDM of 0.47 gg^{-1}, and a CDM productivity of 0.27 $gL^{-1}d^{-1}$ [28].

Ashby et al. took advantage of the fact that kinetics of glycerol-based growth and PHA production of the strains *P. oleovorans* NRRL $B^{-1}4682$ and *P. corrugata* 388 are very similar; hence, the organisms grow and accumulate at comparable rates. The authors cultivated both strains in one-pot-fermentations, and succeeded in obtaining natural blends of two different types of PHA: *scl*-PHA (PHB) produced by *P. oleovorans* and *mcl*-PHA stemming from the bio-synthetic action of *P. corrugata*. By time-staggered inoculation of the cultivation medium, and by varying the cultivation time, it was possible to change the contents of *scl*-PHA and *mcl*-PHA in the blends [29].

2. Case Studies with Recombinant Organisms

Glycerol has also been investigated as a substrate for PHA synthesis in various recombinant microorganisms such as engineered *E. coli*, *P. putida*, *R. eutropha*, *A. hydrophila*, or different species of Bacillus. These organisms have been modified to grow faster on different carbon sources, and to produce different types of PHAs at improved kinetics. Therefore, more and more research is devoted to further modifying these organisms for higher PHA yields, ultimately leading to lower production cost. Some of the most intriguing and challenging studies dealing with glycerol conversion into different PHA types using recombinant organisms are summarized in the following text.

Mahishi et al. tested several different carbon sources such as glycerol, glucose, palm oil, sucrose, molasses or ethanol for PHB accumulation in *E. coli* (ATCC:PTA-1579) harboring PHA synthesizing genes *phaC* and *phaB* from *Streptomyces aureofaciens* NRRL 2209. The highest PHB production reaching 60% w/w of CDM was reported for the cells grown in glycerol supplemented with yeast extract and peptone as nitrogen sources. Considering that the original strain, *S. aureofaciens*, from which the PHA synthesizing genes were cloned, only accumulated 0.02 g PHB per g CDM, these results are quite significant. Surprisingly, in the presence of glucose or palm oil, the *E.coli* cells produced only 0.38 and 0.28 g PHB per g CDM, respectively [30].

Lower content of PHB (0.02 to 0.30 gg^{-1}) was reported for the *E. coli* cells grown on glycerol expressing 3-ketothiolase (*phaA*) and NADPH-dependent acetoacetyl-Coa reductase (*phaB*) from *R. eutropha*, and PHA synthase (*phaC*) from several different organisms [31]. The intracellular PHB content depended on which type of *phaC* was expressed due to the synthase´s high substrate specificity towards various monomers. However, by adding dodecanoate and introduction of (*R*)-specific enoyl-CoA hydratase genes (*phaJ*), the recombinant *E.coli* was able to produce *scl-co-mcl*-PHA copolyesters. These novel materials combine the advantages of both *scl*-PHAs and *mcl*-PHAs, and are naturally produced only by a few wild type bacteria such as *Aeromonas caviae* and *Aeromonas hydrophila*. Hence, the authors managed to combine the biosynthetic pathways of *scl*-PHA common in *R. eutropha* and *mcl*-PHA, generally found in *Pseudomonas strains*. Even though these copolyesters showed glass transition temperatures superior to simple PHB homopolyester, the PHA content was measured to be very low, reaching only up to 0.03 g per g CDM [31].

The same *E.coli* strain harboring the *phaARe*, *phaBRe* and *phaCAh* genes of *A. hydrophila* also expressed rather low productivity of 0.14 g PHB per g CDM at 1% $volvol^{-1}$ glycerol [30]. However, with the introduction of *phaJ*, the recombinant cells were able to synthesize the *scl-co-mcl* copolyester poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHB-HHx), which displays desired material properties. Nevertheless, as in the previous case with *scl-co-mcl* PHA copolyesters [31], the PHBHHx content in CDM did not exceed 0.03 gg^{-1} [32].

In another study, an *E. coli* strain carrying the PHA synthesis genes from *Azotobacter* sp. strain FA8 was used [33]. Besides the PHA synthesis genes, the cells also carried a mutation in the *arcA* gene, which represses the genes that encode enzymes involved in aerobic respiration. The mutation allowed the cells to grow micro-aerobically with no repression of the TCA cycle enzymes, thus providing levels of the reducing equivalents NADH and NADPH, comparable to the aerobic conditions. After 48h cultivation in a rich medium on the laboratory bioreactor scale, the cells accumulated 0.24 g PHB per g CDM without aeration, using only slight agitation (75 rpm) [33]. These results correlate well with a study conducted a few years later where the *E. coli* strains carrying the same PHA synthesis genes from *Azotobacter* sp strain FA8 produced 0.30 g PHB per g glycerol when stirred at 125 rpm [34]. Even higher amount (up to 0.35 gg^{-1}) was achieved for the strain carrying leaky arcA2 mutation. In this case, the cells grew with higher maximum specific growth rate, even when a medium poorer in nutrients was used. The authors demonstrated that the *arcA* strains of *E. coli* could accumulate relatively high concentrations of PHB using various carbon sources including glycerol; therefore, they concluded that glycerol could play a role in heterologous PHB synthesis under micro-aerobic conditions [33].

Another group from the same institute also used *E.coli* strains transformed with PHA synthesis genes from *Azotobacter* sp. strain FA8 [35]. However, instead of deleting the genes involved in aerobic metabolism, they expressed *phaP*-encoded phasin,

which regulates granule formation, thus affecting polymer synthesis and the number and size of PHA granules. In the shaking flask experiments, the cells carrying *phaP* accumulated higher amounts of polymer using both glucose and glycerol as the carbon sources. The authors also found that the cells did not only produce more polymer, but also reached higher CDM than control strains lacking *phaP*. These observations were confirmed in bioreactor cultivation, where almost doubled cell densities were achieved with almost triplicated PHB contents [35].

E. coli has also been modified to produce PHAs others than PHB. In the study of Andreessen et al. recombinant *E. coli* was used for glycerol-based Poly (3-hydroxypropionate) (PHP) production. The bacterium was transformed with a plasmid carrying the glycerol dehydratase *dhaB1* gene from *Clostridium butyricum*, the propionaldehyde dehydrogenase pduP from *S. enterica* and PHA synthase *pha C1* from *R. eutropha*. In the two-step fermentation, divided into an aerobic growth phase and an anaerobic production phase, the final PHP content of the cells approached 0.12 g PHB per g CDM in pure glycerol as the sole carbon source, and about 0.05 g PHB per g CDM in crude glycerol, respectively. This low PHA content was attributed either to the rapid loss of the expression plasmid (44% loss after 92 h of cultivation) or changes in the NADH redox balance [36].

For some applications, *mcl*-PHAs exhibit material characteristics superior to those of *scl*-PHAs. Because of their increasing demand, scientists have been trying to develop bacterial strains with high *mcl*-PHA production capacity. As described by Sujatha and Shenbagarathai, *E. coli* expressing the PHA synthase gene (*phaC1*) from indigenous *Pseudomonas* sp. LDC-5 could be one of these candidates. In the shaking flask screening experiments, the recombinant strain was cultivated at 37° C for 48 h in a medium supplemented with different carbon and nitrogen sources such as glycerol, glucose, sucrose, maltose, and different peptones. The results confirmed the ability of the cells to grow on all carbon sources with the highest *mcl*-PHA formation (0.56 g *mcl*-PHA per g CDM) and the cell yields obtained in the medium supplemented with 1% vv^{-1} glycerol [37].

Besides *E. coli*, other recombinant bacteria have been widely studied for PHA production. One of the strains commonly used is *R. eutropha* strain H16, a well-known PHB producer. This strain utilizes various carbon sources such as fructose, gluconate, fatty acids, and plant oils, whereas it is unable to utilize glucose, xylose, and arabinose. That is also a reason why *R. eutropha* strain H16 was subjected to genetic manipulation to allow it to grow on substrates such as glucose [38, 39]. Above these drawbacks, *R. eutropha* is naturally a very efficient PHB producer that can also grow and produce PHA on glycerol. However, growth on glycerol is rather slow and, therefore, Fukui et al. transformed *R. eutropha* strain H16 with the genes involved in the aerobic metabolism of glycerol found in *E.coli*. The genes included aquaglyceroporin (*glpF*), glycerol kinase (*glpK*), and FAD-dependent glycerol 3-phosphate dehydrogenase (*glpD*); these genes were transformed either separately (H16 *glpF* or H16 *glpK*) or together (H16 *glpFK*). In a nitrogen-limited shaking flask experiment supplemented with 0.5 % ($volvol^{-1}$) of glycerol as a sole carbon source, both fast growth and PHB accumulation were observed for the H16-derived strain harboring *glpFK* or even the single gene *glpK*. The PHB content, after 72 hours of incubation, reached about 0.64 gg^{-1} with a PHA concentration of approximately 1.4 gL^{-1}. This increase was rather significant when compared to the wild strain H16 (1.08 gL^{-1} PHB after 268 h) [39].

Overexpressing the *glp* genes involved in glycerol metabolism has been shown to be an effective method to enhance PHA accumulation on glycerol [39]. In the study of Escapa et al. using *P. putida* KT2440, the authors took a different approach, and instead of overexpressing the *glp* genes, they deleted their transcription repressor gene *glpR*. Deletion of this key regulator (controlling glycerol catabolism) resulted in elimination of the lag-phase, and enhanced *mcl*-PHA accumulation. The authors also found that the long lag-phase can be avoided by co-feeding the wild type strain with small amounts of octanoate, which, on the other hand, induces the *glp* genes [40].

PHP was recently produced from glycerol by recombinant *Klebsiella pneumoniae*. The genes encoding glycerol dehydratase and its reactivation factor derived from *K. pneumoniae* and aldehyde dehydrogenase from *E. coli* were cloned and expressed in *K. pneumoniae* to produce PHP. For PHP synthesis, propionyl-CoA synthetase from *E. coli* and PHA synthase (*phaC*) from *R. eutropha* were introduced. Under optimized aeration conditions, mass fraction of 0.13 gg^{-1} was obtained, corresponding to 2.03 gL^{-1} PHP after 48 h cultivation. This study, for the first time, shows the feasibility of PHP production by *K. pneumoniae* from glycerol without co-feeding vitam-in B_{12}. The results also suggest that the aeration conditions are pivotal for optimum PHP production [41].

3. Modelling of Glycerol-Based PHA Production

Mathematical modelling of bioprocesses, inter alia of PHA biosynthesis, has become a tool of increasing importance to put data from research into a kinetic frame, to elucidate metabolic bottlenecks and, most importantly, to restrict the necessary

number of experiments during the process development. Only very recently, mathematical models were also developed for PHA production from glycerol and other by-products of the biodiesel industry [42,43].

By developing formal-kinetic mathematical models, Spoljaric et al. investigated the utilization of glycerol by *C. necator* DSM 545 for the production of PHB in fed-batch cultivations on bioreactor scale. From two different experiments, differentiated by the use of different inocula from the exponential or the stationary phase, respectively, maximal specific growth rates, $\mu_{max.}$, (0.12 and 0.3 h^{-1}) and maximal specific non-growth PHB production rate, $qp_{max.}$, (0.16 $gg^{-1}h^{-1}$) were calculated. Saturation constants for nitrogen (0.107 & 0.016 gL^{-1}), glycerol (0.05 gL^{-1}), non-growth related PHB synthesis (0.011 gL^{-1}) and nitrogen/PHB related inhibition constant (0.405 gL^{-1}) were estimated. Five different modeling approaches, encompassing double substrate Monod relations combined with different substrate inhibition terms, were tested to obtain the most appropriate relations for µ. *In silico* performed optimization procedures with varied glycerol/nitrogen ratio and feeding strategy have revealed that a PHB content of 0.71 %w/w CDM, a considerable shortening of cultivation time to only 23 h, and a drastically enhanced PHB yield of 0.347 g PHB per g glycerol should be achievable by the suggested process optimization. According to the developed model, the most important factors to reach this increased productivity were the initial biomass concentration of 16.8 gL^{-1} together with a constant actual glycerol concentration between 3 and 5 gL^{-1} [43].

These works were further extended by developing an advanced metabolic network model. This network consisted of 48 reactions to describe intracellular processes during growth and PHB production by *C. necator* DSM 545, and was especially targeted to finally clarify the question if the Enter-Doudoroff or the Emden-Meyerhof-Parnas pathways constitute the predominant route for glycerol conversion into PHA by this strain. Again, glycerol acted as the sole carbon source during the exponential, steady-state cultivation conditions. Elementary flux modes were obtained by the Metatool program, and analyzed by using yield space analysis. Four sets of elementary modes were obtained, depending on whether the pair NAD/NADH or $FAD/FADH_2$ contributed to the reaction of glycerol-3-phosphate dehydrogenase (GLY-3-P DH), and whether 6-phosphogluconate dehydrogenase (6-PG DH) was involved in the reaction. The established metabolic network and the related system of equations provided multiple solutions for the simultaneous synthesis of PHB and biomass; this number of solutions can be further increased if NAD/NADH or $FAD/FADH_2$ was assumed to act as co-factors in the GLY3P DH-catalyzed reaction. As a major outcome, it was demonstrated that experimentally determined yields for biomass and PHB with respect to glycerol fitted well not only to the values obtained *in silico* when the Enter-Doudoroff dominated over the Emden-Meyerhof-Parnas but also in the case of EMP dominating over the Enter-Doudoroff [44].

The viability of glycerol conversion into PHA homo- and copolyesters by the active sludge isolate, *Bacillus* sp. RER002, was investigated by Zafar et al. in a lab-scale bioreactor. The developed mathematical model included logistic, Luedeking-Piret and Luedeking-Piret-like equations that simulated the growth of residual biomass, PHB synthesis, and glycerol consumption. In order to describe the dynamics of PHB production, the model kinetic parameters were optimized by using a stochastic search-based genetic algorithm. PHB synthesis was found to be in principal highly-growth associated, but partially non-growth associated at glycerol concentration between 10-40 gL^{-1}. The maximum *scl-co-mcl*-copolyester concentration of 3.2 gL^{-1} was observed at a glycerol concentration of 30 gL^{-1} in the synthetic crude glycerol medium with a yield coefficient of 0.16 g PHA per g glycerol. Further-more, the analyses of chemical and thermal properties of the *scl-co-mcl*-copolyester revealed its enhanced material properties when compared to simple *scl*-PHA [45].

4. Conclusion

The article demonstrates the high potential of a huge variety of eu- and archaebacterial wild-type microorganisms for glycerol-based PHA production (see Table 1). For bulk-production of *scl*-PHA, well-established strains like *C. necator*, and, to a lower extent, *H. mediterranei*, seem to be the most prospective candidates though ample kinetic and material data are already available for many other strains. Reports on the genetically engineered strain constructs look promising on laboratory scale; however, they did not yet pass the proof of applicability and stability on bioreactor- or even pilot scale; hence, it is not likely that, in the near future, semi- (industrial) glycerol-based PHA production will be based on recombinant organisms .

What is also of high interest for the future is a more intensive investigation of *mcl*- and *scl-co-mcl*-PHA production from glycerol by selected production strains; data collected for these materials from small-scale experiments appear very promising; a more deep-going investigation and further development should result in smart polymeric biomaterials with new and improved properties regarding, among others, flexibility, tensile strength, and extremely low glass transition temperature.

5. Conflict of interest

The authors declare that there exist no conflicts of any interest.

Table 1. Selected results for the kinetics of PHA bio-production by various microbial strains and reported data for polymer properties

Strain	$\mu_{max.}$ [h^{-1}]	$q_{P\ max.}$ [h^{-1}]	P [$gL^{-1}h^{-1}$]	*m*[g g^{-1}]	PHA	M_w,M_n*PDI*	Tm [°C]	Tg [°C]	Note	Ref.
Methylomonas extorquens	0.05	0.23	n.r.	0.28	PHB	*n.r.*	*n.r.*	*n.r.*	Diauxic conversion of methanol &glycerol	[46]
Methylobacterium rhodesianum MB 126	n.r.	0.23	0.22	0.50	PHB	*n.r.*	*n.r.*	*n.r.*	Casein hydrolysate cofeeding	[3]
Azohydromonas australica DSM 1124	0.075	0.05 (3HB); 0.01 (3HV)	0.08	0.53	PHBHV (*m* 3HV: 0.85)	*n.r.*	*n.r.*	*n.r.*	Co-feeding of pentanoate	[1]
Cupriavidus necator ATCC 17699	0.11	ca. 0.1(for linear accumulation phase)	0.92	0.71	PHB	M_w 550000; M_n28000; PDI 2.0	161 / 176.4 *	3.2	Comparison of glycerol-based production to data on glucose	[2]
C. necator DSM 11348	n.r.	n.r.	0.25	0.65	PHB	*n.r.*	*n.r.*	*n.r.*	Casein hydrolysate cofeeding	[3]
Cupriavidus necator DSM 545	0.3	0.13 (CGP) 0.09 (CGP)	Pure glycerol: 0.6 – 1.5 CGP: 0.84 – 1.1	0.62 (pure glycerol) 0.50 (CGP)	PHB	M_w 957,000; M_n304,00 PDI 3.15 (pure glycerol) M_w 786,000; M_n215,00 PDI 3.15 (CGP)	*n.r.*	*n.r.*	First glycerol-based PHA production by *C. necator*	[4]
C. necator DSM 545	PHB4HB: 0.15 (high DOC, 0.15 (low DOC) PHBV4HB: 0.17 (high DOC, (low DOC: *n.r.*)	PHB4HB: 0.10 (high DOC, 0.04 (low DOC) PHBV4HB: 0.06 (high DOC, 0.09 (low DOC)	PHB4HB: 0.33 (high DOC, 0.15 (low DOC) PHBV4HB: 0.29 (high DOC, 0.35 (low DOC)	PHB4HB: 0.36 (high DOC, 0.18 (low DOC) PHBV4HB: 0.37 (high DOC, 0.18 (low DOC)	PHB4HB PHBV4HB	M_w550,000 – 1.370,000; PDI2.6 – 4.0	*n.r.*	*n.r.*	Different DOC-levels; co-feeding of GBL	[5]
C. necator DSM 545	1.21	0.05	0.76	0.65	PHB	M_w302,500 M_n63,560 PDI4.72	173	*n.r.*	Co-feeding glucose and glycerol	[47]
C. necator DSM 545	0.12	0.16	0.93	0.65	PHB	*n.r.*	*n.r.*	*n.r.*	Inoculum from exponential growth phase	[43]
Cupriavidus necator JMP 134	0.13	*n.r.*	0.24	0.70	PHB	M_w 750,000; M_n 290,000; PDI 2.6	169	+10	Impact of different impurities in CGP	[8]
Cupriavidus sp. USMAHM13	*n.r.*	*n.r.*	*n.r.*	Pure glycerol: 1-stage: 0.10 2-stage: 0.26 Glycerol pitch: 1-stage: 0.48 2-stage: 0.50	PHB	Pure glycerol: (1-stage: M_w 94,000, PDI 2.2); (2-stage: M_w 23,000, PDI 1.7) &Glycerol pitch: (1-stage: M_w 37,000, PDI 1.9), (2-stage: M_w36,000, PDI 1.9	*n.r.*	*n.r.*	Application of glycerol pitch from oleochemistry	[6]
*C.*sp. USMAHM13	*n.r.*	*n.r.*	*n.r.*	Pure glycerol: + GBL: 0.30 + 1,4-BD: 0.28 Glycerol pitch: + GBL: 0.29 + 1,4-BD: 0.43	PHBV4HB	Pure glycerol:+ GBL: (M_w 94,000, PDI2.2); + 1,4-BD: (M_w23,000, PDI 1.7); Glycerol pitch: (+ GBL: M_w 28,000, PDI2.7); + 1,4-BD: M_w 29,000, PDI 1.9	*n.r.*	*n.r.*	Co-feeding of glycerol pitch or pure glycerol, and 4HB-precursors GBL or 1,4BD	[6]
*C.*sp. USMAHM13	0.008 – 0.092	0.0001 – 0.0065	0.005 – 0.023	0.14 – 0.30	PHB and PHB4HB	PHB: (M_w 418,000; M_n 210,000, PDI 2.0) PHB4HB: (M_w186,000 – 416,000, M_n 73,000 – 107,000, PDI 2.1 – 3.0)	PHB: 157.9 PHB4HB: 147.8 – 158.4	PHB: -8.4 PHB4HB: -11.3 - -27.7	Co-feeding of glycerol pitch, acetate, and 1,4BD	[7]

Novosphingobium sp. THA_AIK7 (*Novospingobium capsulatum*)	*n.r.*	*n.r.*	*n.r.*	0.45	PHB	M_w 133,000 / 700 M_n 23,800 / 755 PDI 5.6	179	*n.r.*	Ultrapure product	[9]
Zobellella denitrificans MW1	0.42	*n.r.*	0.39 – 1.09	0.31 – 0.67	PHB	*n.r.*	*n.r.*	*n.r.*	Optimization for salinity and feeding strategy	[10]
Burkholderia cepacia ATCC 17759	*n.r.*	*n.r.*	0.20	0.31	PHB	M_n:138.400 (2% glycerol)–174,000 (5% glycerol) after 24h, 114.000 (2% glycerol)–133.600 (5% glycerol) after 48h	181.9	1.6	200 L scale	[12]
Burkholderia sacchari DSM 17165	0.42	0.002	0.08	0.10	PHB	M_w 200,000 M_n 80,000 PDI 2.50	173	*n.r.*	Co-Feeding glucose and glycerol	[47]
Halomonas sp. KM-1	*n.r.*	*n.r.*	*n.r.*	0.45	PHB	M_w 140,000; M_n32,000 PDI 4.2 (3% glycerol) M_w 87,000; M_n 29,000 PDI 3.0 (5% glycerol)	*n.r.*	*n.r.*	Osmophile organism	[13]
Yangia sp. ND199	*n.r.*	*n.r.*	0.44 (pure glycerol), 0.25 (CGP), 0.61 (CGP + corn syrup)	0.53 (pure glycerol) 0.41 (CGP) 0.56 (CGP + corn sirup)	PHBHV	*n.r.*	*n.r.*	*n.r.*	Osmophile organism	[14]
Pseudomonas putida JCM6160	*n.r.*	*n.r.*	*n.r.*	0.18 (without cofeeding of nonanoic acid); 0.47: pure nonanoic acid; Mix glycerol/nonanoic acid: *n.r.*	Glycerol: *scl-mcl*-PHA(C6, C8, C10, C12:0, C12:1) Nonanoic acid: *mcl*-PHA(C6, C8) Mix: *scl-co-mcl*-PHA(C5, C6, C7, C8, C9, C10, C12:0, C12:1)	Glycerol: M_w 58,000; M_n 40,000 PDI 1.4 Nonanoic acid: M_w150,000; M_n91,000 PDI 1.7 Mix: M_w150,000; M_n 92,000 PDI 1.6	*n.r.*	*n.r.*	Co-feeding of nonanoic acid	[15]
P. putida KT2440 after disrupture of depolymerase gene	*n.r.*	0.005 0.01	0.02 0.03	0.34 0.47	*mcl*-PHA (mainly C10)	*n.r.*	*n.r.*	*n.r.*	*mcl*-PHA production from glycerol	[16]
Pseudomonas spp. P. *mediterranea* 9.1 (CFBP 5447)	*n.r.*	*n.r.*	*n.r.*	0.23 (pure glycerol) 0.21 (partially refined glycerol)	*mcl*-PHA	Pure glycerol:(M_w 55,480; M_n 41,400; PDI 1.34); Partially refined glycerol:(M_w 63,200; M_n 45,800; PDI 1.38)	*n.r.*	*n.r.*	*mcl*-PHA production from glycerol	[17]
Haloarcula sp. IRU1	*n.r.*	*n.r.*	*n.r.*	0.73 (experimental) 0.82 (predicted)	PHB	*n.r.*	*n.r.*	*n.r.*	Osmophile organism	[18]
Haloferax mediterranei DSM 1411	0.06	0.081	0.12	0.76	PHBHV	M_w 253,000; M_n 93,700 PDI 2.7	128.7 / 138.8 *	7.0	1st report on archaeal PHA production from CGP	[19]
.DSM 1411	0.10	0.	0.12	0.75	PHBV	M_w 150,000 M_n 71,400 PDI 2.1	130.2 / 140.6 *	4.3	Pure glycerol	[20]
*H.*DSM 1411	0.14	0.01	0.14	0.88	PHBV4HB	M_w 986,000 M_n 657,000 PDI 1.5	122 / 137 *	2.5	4HB precursor GBL; 1st archaeal terpolyester; unsterile process	[20]
Paracoccus. denitrificans	0.25	*n.r.*	0.16	0.65	PHB	M_w 620,000; M_n 170,000; PDI 3.7	173	+10	Impact of different impurities in CGP	[8]
Bacillus firmus NII 0830	*n.r.*	*n.r.*	*n.r.*	0.31	PHB	*n.r.*	*n.r.*	*n.r.*	Gram-positive production strain	[21]

Bacillus sphaericus NII 0838	*n.r*	*n.r*	*n.r*	0.53	PHB	n.r	165.24	n.r	Gram-positive production strain	[22]
*Bacillus*sp. RER002	0.082	*n.r.*	0.07 (t 42h)	0.3	*scl-co-mcl*-PHA	*n.r.*	*n.r.*	*n.r.*	Predicted by modelling	[45]
Rec. E. coli ATCC:PTA1579 exp *S. aureofaciens* gene	*n.r*	*n.r*	*n.r*	0.60	PHB	*n.r*	*n.r*	*n.r*	Predicted by modelling	[30]
Rec. E. coli (exp *R. eutropha* genes)	*n.r*	*n.r*	*n.r*	PHB: 0.30 *scl-co-mcl*-PHA: 0.032	PHB *scl-co-mcl*-PHA (C4 – C10) (supplemented with DD)	PHB:(M_w 260,000, M_n 140,000, PDI 1.9) P(3HB-*co*-3HHx):M_w 260,000; M_n 45,000; PDI 5.7); P(3HB-*co*-3HHx-*co*-3HO):(M_w 170,000; M_n25,000; PDI 6.9)	PHB: 175 P(3HB-*co*-3HHx): 149 /165 P(3HB-*co*-3HHx-*co*-3HO): 146 / 163	PHB: 7 P(3HB-*co*-3HHx): 4 P(3HB-*co*-3HHx-*co*-3HO): -1	*scl-co-mcl*-PHA production	[31]
Rec. E. coli E. coli-ABC$_{Ah}$and *E. coli*-ABC$_{Ah}$J$_{Ah}$ (exp*A. hydrophila* genes)	*n.r*	*n.r*	*n.r*	*E. coli*-ABC$_{Ah}$: (CGP + DD: 0.40 Pure glycerol+ DD: 0.15) *E. coli*-ABC$_{Ah}$J$_{Ah}$: (CGP: 0.07, Pure glycerol: 0.16, CGP + DD: 0.03, Pure glycerol+ DD: 0.04	P(3HB-*co*-3HHx) P(3HB-*co*-3HHx) PHB PHB P(3HB-*co*-3HHx) P(3HB-*co*-3HHx)	*E. coli*-ABC$_{Ah}$J$_{Ah}$: CGP + DD: M_w 110,000; M_n 52,400 PDI 2.1) Pure glycerol + DD:M_w130,000; M_n 65,000 PDI 2.0	*E. coli*-ABC$_{Ah}$J$_{Ah}$: CGP + DD: 174 Pure glycerol + dodecanoate: 159 7 171	*E. coli*-ABC$_{Ah}$J$_{Ah}$: CGP + DD:7 Pure glycerol + DD:6	*scl-co-mcl*-PHA production	[32]
Rec. E. coli (exp *Azotobacter* sp.Genes; exp *phaP* phasin)	0.6	*n.r.*	0.2	0.52	PHB	*n.r.*	*n.r.*	*n.r.*		[34]
Rec. E. coli exp Dhab1, *pdu*P, and *pha*C1	*n.r.*	*n.r.*	0.18	0.12 (pure glycerol) 0.05 (CGP)	PHP	*n.r.*	*n.r.*	*n.r.*		[36]
Rec. E. coli exp*pha*C1 from *P*.sp. LDC-5	*n.r.*	*n.r.*	*n.r.*	0.56	PHA	*n.r.*	*n.r.*	*n.r.*		[37]
R. eutropha H16exp *glpK* and *glpD*	*n.r.*	*n.r.*	*n.r.*	0.64	PHB	M_w 225,000; M_n 92,000; PDI 2.4	*n.r.*	*n.r.*		[39]
Rec. Klebsiella pneumonia	*n.r.*	*n.r.*	0.04	0.13	PHP	*n.r.*	*n.r.*	*n.r.*	PHP production;vitamin B_{12}supplementation	[41]

* Two melting endotherms were observed.DD: Dodecanoate; *m*: mass fraction PHA in CDM;M_n: number average molar mass; M_w: weight average molar mass; $\mu_{max.}$: max. specific growth rate; P: volumetric productivity; PDI: Polydispersity index; PHB: poly(3-*R*-hydroxybutyrate); PHBHV: poly(3-*R*-hydroxybutyrate-*co*-3-*R*-hydroxyvalerate); PHB4HB: poly(3-*R*-hydroxybutyrate-*co*-4-hydroxybutyrate); PHBHV4HB: poly(3-*R*-hydroxybutyrate-*co*-3-*R*-hydroxyvalerate-*co*-4-hydroxybutyrate); PHP: poly(3-hydroxypropionate); P(3HB-*co*-3HHx): poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate);$q_{Pmax.}$: max. specific productivity; *n.r.*: not reported in the original literature; GBL: γ-butyro lactone; 1,4BD: 1,4-butandiol; Tg: Glass transition temperature; Tm: Melting temperature

References

1. Haage G, Wallner E, Bona R, Schellauf F, Braunegg G. Production of poly-3-hydroxybutyrate-co-3-hydroxy-valerate with Alcaligenes latus DSM 1124 on various carbon sources. In: Chiellini E: Biorelated Polymers. Springer US, 2001: 147-155.
2. Tanadchangsaeng N, Yu J. Microbial synthesis of polyhydroxybutyrate from glycerol: Gluconeogenesis, molecular weight and material properties of biopolyester. Biotechnol Bioeng. 2012; 109(11): 2808-2818.
3. Bormann EJ, Roth M. The production of polyhydroxy-butyrate by Methylobacterium Rhodesian-um and Ralstonia eutropha in media containing glycerol and casein hydrolysates. Biotechnol Lett. 1999; 21(12): 1059-1063.
4. Cavalheiro JM, de Almeida MCM, Grandfils C, Da Fonseca MMR. Poly (3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol. Process Biochem. 2009; 44(5): 509-515.
5. Cavalheiro JM, Raposo RS, de Almeida MCM, Cesario MT, Sevrin C, Grandfils C, da Fonseca MMR. Effect of cultivation parameters on the production of poly (3-hydroxybutyrate-co-4-hydroxy-butyrate) and poly (3-hydroxybutyrate-4-hydroxy-butyrate-3-hydroxyvalerate) by Cupriavidus necator using waste glycerol. Bioresour Technol. 2012; 111: 391-397.
6. Ramachandran H, Amirul AA. Yellow-pigmented Cupriavidus sp., a novel bacterium capable of utilize-ing glycerine pitch for the sustainable production of P (3HB-co-4HB). J Chem Technol Biotechnol. 2013; 88(6): 1030-1038.
7. Ramachandran H, Amirul AA. Bioconversion of glycerine pitch into a novel yellow-pigmented P (3HB-co-4HB) copolymer: Synergistic effect of amm-onium acetate and polymer characteristics. Appl Biochem Biotech. 2014; 172(2): 891-909.
8. Mothes G, Schnorpfeil C, Ackermann JU. Production of PHB from crude glycerol. Eng Life Sci. 2007; 7(5): 475-479.
9. Teeka J, Imai T, Reungsang A, Cheng X, Yuliani E, Thiantanankul J, Poomipuk N, Yamaguchi J, Jeenan-ong A, Higuchi T, Yamamoto K, Sekine M. Charact-erization of polyhydroxyalkanoates (PHAs) biosynth-esis by isolated Novosphingobium sp. THA_AIK7 using crude glycerol. J Ind Microbiol Biotech. 2012; 39(5): 749-758.
10. Ibrahim MH, Steinbüchel A. Poly (3-hydroxybutyrate) production from glycerol by *Zobellella denitrificans* MW1 via high-cell-density fed-batch fermentation and simplified solvent extraction. Appl Environ Microb. 2009; 75(19): 6222-6231.
11. Cesario MT, Raposo RS, de Almeida MCM, van Keulen F, Ferreira BS, da Fonseca MMR. Enhanced bioproduction of poly-3-hydroxybutyrate from wheat straw lignocellulosic hydrolysates. New Biotechnol. 2014; 31: 104-113.
12. Zhu C, Nomura CT, Perrotta JA, Stipanovic AJ, Nakas JP. Production and characterization of poly-3-hydroxybutyrate from biodiesel-glycerol by *Burkholderia cepacia* ATCC 17759. Biotechnol Prog. 2010; 26(2): 424-430.
13. Kawata Y, Aiba SI. Poly(3-hydroxybutyrate) production by isolated Halomonas sp. KM-1 using waste glycerol. Biosci Biotech Biochem. 2010; 74(1): 175-177.
14. Van-Thuoc D, Huu-Phong T, Minh-Khuong D, Hatti-Kaul, R. Poly (3-hydroxybutyrate-co-3-hydroxy-valerate) production by a moderate halophile Yangia sp. ND199 using glycerol as a carbon source. Appl Biochem Biotech. 2015; 175: 3120-3132.
15. Miura T, Ishii D, Nakaoki T. Production of Poly (3-hydroxyalkanoate)s by *Pseudomonas putida* cultivated in a glycerol/nonanoic acid-containing medium. J Polym Environ. 2013; 21(3): 760-765.
16. Poblete-Castro I, Binger D, Oehlert R, Rohde M. Comparison of mcl-Poly (3-hydroxyalkanoates) synthesis by different *Pseudomonas putida* strains from crude glycerol: Citrate accumulates at high titer under PHA-producing conditions. BMC Biotechnol. 2014; 14(1): 962-972.
17. Pappalardo F, Fragala M, Mineo PG, Damigella A, Catara AF, Palmeri R, Rescifina A. Production of filmable medium-chain-length polyhydroxyalkanoates produced from glycerol by *Pseudomonas mediterr-anei*. Int J Biol Macromol. 2014; 65: 89-96.
18. Taran M, Azizi E, Taran S, Asadi N. Archaeal poly 3-hydroxybutyrate polymer production from glycerol: optimization by Taguchi methodology. J Polym Environ. 2011; 19(3): 750-754.
19. Koller M, Bona R, Braunegg G, Hermann C, Horvat P, Kroutil M, Martinz J, Neto J, Pereira L, Varila P. Production of polyhydroxyalkanoates from agricult-ural waste and surplus materials. Biomacromolecules. 2005; 6(2): 561-565.
20. Hermann-Krauss C, Koller M, Muhr A, Fasl H, Stelzer F, Braunegg G. Archaeal production of polyhydroxyalkanoate (PHA) co and terpolyesters from biodi-esel industry-derived by-products. Archaea. 2013; 2013: 1-10.
21. Deepthi SK, Binod P, Sindhu R, Pandey A. Media engineering for production of poly-β-hydroxybutyrate by Bacillus firmus NII 0830. J Sci Ind Res. 2011; 70(11): 968-975.
22. Sindhu R, Ammu B, Binod P, Deepthi SK, Ramachan-dran KB, Soccol CR, Pandey A. Production and characterization of poly-3-hydroxybutyrate from crude glycerol by *Bacillus sphaericus* NII 0838 and improve-ing its thermal properties by blending with other polymers. Braz Arch Biol Techn. 2011; 54(4): 783-794.
23. Ciesielski S, Pokoj T, Klimiuk E. Cultivation dependent and independent characterization of microbial commun-ity producing polyhydroxyalkan-oates from raw glycerol. J Microbiol Biotechnol. 2010; 20(5): 853-861.
24. Wattanaphon HT, Ciesielski S, Pisutpaisal N. Determ-ining microbial dynamics of polyhydroxyalkanoates-producing consortium in waste glycerol using RISA technique. Science 2011; 19: 181-185.
25. Renner G, Schellauf F, Braunegg G, Rodriguez F. Selective enrichment of bacteria accumulating polyhy-

droxyalkanoates in multistage continuous culture. Food Technol Biotech. 1998; 36(3): 203-208.

26. Moralejo-Gárate H, Mar'atusalihat E, Kleerebezem R, van Loosdrecht MC. Microbial community engineering for biopolymer production from glycerol. Appl Microbiol Biotechnol. 2011; 92(3):631-639.
27. Moralejo-Gárate H, Palmeiro-Sánchez T, Kleerebezem R, Mosquera-Corral A, Campos JL, van Loosdrecht M. Influenceof the cycle length on the production of PHA and polyglucose from glycerol by bacterial enrichments in sequencing batch reactors. Biotechnol Bioeng. 2013; 110(12): 3148-3155.
28. Moita R, Freches A, Lemos PC. Crude glycerol as feedstock for polyhydroxyalkanoates production by mixed microbial cultures. Water Res. 2014; 58: 9-20.
29. Ashby RD, Solaiman DK, Foglia TA. Synthesis of short-/medium-chain-length poly (hydroxyl alkanoate) blends by mixed culture fermentation of glycerol. Biomacromolecules. 2005; 6(4): 2106-2112.
30. Mahishi LH, Tripathi G, Rawal SK. Poly(3-hydroxybutyrate) (PHB) synthesis by recombinant Escherichia coli harbouring *Streptomyces aureofaciens* PHB biosynthesis genes: effect of various carbon and nitrogen sources. Microbiol Res. 2003; 158(1): 19-27.
31. Phithakrotchanakoon C, Champreda V, Aiba S, Pootan-akit K, Tanapongpipat S. Engineered Escherichia coli for short chain length, medium chain length polyhydroxyalkanoate copolymer biosynthesis from glycerol and dodecanoate. Biosci Biotech Bioch. 2013; 77(6): 1262-1268.
32. Phithakrotchanakoon C, Champreda V, Aiba S, Pootanakit K, Tanapongpipat S. Production of polyhydroxyalkanoates from crude glycerol using recombinant *Escherichia coli*. J Polym Environ. 2015; 23: 38-44.
33. Nikel PI, Pettinari MJ, Galvagno MA, Mendez BS. Poly (3-hydroxybutyrate) synthesis by recombinant *Escherichia coli* arcA mutants in microaerobiosis. Appl Environ Microb. 2006; 72(4): 2614-2620.
34. de Almeida A, Giordano AM, Nikel PI, Pettinari MJ. Effects of aeration on the synthesis of poly(3-hydroxybutyrate) from glycerol and glucose in recombinant *Escherichia coli*. Appl Environ Microb. 2010; 76(6): 2036-2040.
35. de Almeida A, Nikel PI, Giordano AM, Pettinari MJ. Effects of granule-associated protein PhaP on glycerol-dependent growth and polymer production in poly(3-hydroxybutyrate)-producing *Escherichia coli*. Appl Environ Microb. 2007; 73(24): 7912-7916.
36. Andreessen B, Lange AB, Robenek H, Steinbuchel A. Conversion of glycerol to poly(3-hydroxypropionate) in recombinant *Escherichia coli*. Appl Environ Microb. 2010; 76(2): 622-626.
37. Sujatha K, Shenbagarathai R. A study on medium chain length-polyhydroxyalkanoate accumulation in *Escherichia coli* harbouring phaC1 gene of indigenous Pseudomonas sp. LDC-5. Lett Appl Microbiol. 2006; 43(6): 607-614.
38. Orita I, Iwazawa R, Nakamura S, Fukui T. Identification of mutation points in *Cupriavidus necator* NCIMB 11599 and genetic reconstitution of glucose-utilization ability in wild strain H16 for polyhydroxyalkanoate production. J Biosci Bioeng. 2012; 113(1): 63-69.
39. Fukui T, Mukoyama M, Orita I, Nakamura S. Enhancement of glycerol utilization ability of *Ralstonia eutropha* H16 for production of polyhydroxyalkanoates. Appl Microbiol Biotechnol. 2014; 98(17): 7559-7568.
40. Escapa IF, del Cerro C, Garcia JL, Prieto MA. The role of GlpR repressor in *Pseudomonas putida* KT2440 growth and PHA production from glycerol. Environ Microbiol. 2013; 15(1): 93-110.
41. Feng X, Xian M, Liu W, Xu C, Zhang H, Zhao G. Biosynthesis of poly (3-hydroxypropionate) from glycerol using engineered *Klebsiella pneumoniae* strain without vitamin B12. Bioengineered. 2015; 6(2): 77-81.
42. Spoljaric IV, Lopar M, Koller M, Muhr A, Salerno A, Reiterer A, Malli K, Angerer H, Strohmeier K, Schober S, Mittelbach M, Horvat P. Mathematical modeling of poly [(R)-3-hydroxyalkanoate] synthesis by *Cupriavidus necator* DSM 545 on substrates stemming from biodiesel production. Bioresour Technol. 2013; 133: 482-494.
43. Spoljaric IV, Lopar M, Koller M, Muhr A, Salerno A, Reiterer A, Horvat P. In silico optimization and low structured kinetic model of poly [(R)-3-hydroxybutyrate] synthesis by *Cupriavidus necator* DSM 545 by fed-batch cultivation on glycerol. J Biotechnol. 2013; 168(4): 625-635.
44. Lopar M, Spoljaric IV, Cepanec N, Koller M, Braunegg G, Horvat P. Study of metabolic network of *Cupriavidus necator* DSM 545 growing on glycerol by applying elementary flux modes and yield space analysis. J Ind Microbiol Biotech. 2014; 41(6): 913-930.
45. Zafar M, Kumar S, Kumar S, Agrawal J, Dhiman AK. Valorization of glycerol into polyhydroxyalkanoates by sludge isolated Bacillus sp. RER002: Experimental and modeling studies. Chem Prod Process Model. 2014; 9(2): 117-131.
46. Braunegg G, Genser K, Bona R, Haage G, Schellauf F, Winkler E. Production of PHAs from agricultural waste material. Macromol Symp. 1999; 144: 375-383.
47. Rodriguez-Contreras A, Koller M, Miranda-de Sousa Dias M, Calafell-Monfort M, Braunegg G, Marqués-Calvo MS. Influence of glycerol on poly (3hydroxybutyrate) production by *Cupriavidus necator* and Burkholderia sacchari. Biochem Eng J. 2015; 94: 50-57.