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Effects of Aeration on the Synthesis of Poly(3-Hydroxybutyrate) from Glycerol and Glucose in Recombinant *Escherichia coli*▽†

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Bioreactor cultures of *Escherichia coli* recombinants carrying *phaBAC* and *phaP* of *Azotobacter* sp. FA8 grown on glycerol under low-agitation conditions accumulated more poly(3-hydroxybutyrate) (PHB) and ethanol than at high agitation, while in glucose cultures, low agitation led to a decrease in PHB formation. Cells produced smaller amounts of acids from glycerol than from glucose. Glycerol batch cultures stirred at 125 rpm accumulated, in 24 h, 30.1% (wt/wt) PHB with a relative molecular mass of 1.9 MDa, close to that of PHB obtained using glucose.

Polyhydroxyalkanoates (PHAs), accumulated as intracellular granules by many bacteria under unfavorable conditions (5, 8), are carbon and energy reserves and also act as electron sinks, enhancing the fitness of bacteria and contributing to redox balance (9, 11, 19). PHAs have thermoplastic properties, are totally biodegradable by microorganisms present in most environments, and can be produced from different renewable carbon sources (8).

Poly(3-hydroxybutyrate) (PHB) is the best known PHA, and its accumulation in recombinant *Escherichia coli* from several carbon sources has been studied (1, 13). In the last few years, increasing production of biodiesel has caused a sharp fall in the cost of its main by-product, glycerol (22). Its use for microbial PHA synthesis has been analyzed for natural PHA producers, such as *Methylobacterium rhodesianum*, *Cupriavidus necator* (formerly called *Ralstonia eutropha*) (3), several *Pseudomonas* strains (22), the recently described bacterium *Zobellella denitrificans* (7), and a *Bacillus* sp. (18), among others. Glycerol has also been used for PHB synthesis in recombinant *E. coli* (12, 15). PHAs obtained from glycerol were reported to have a significantly lower molecular weight than polymer synthesized from other substrates, such as glucose or lactose (10, 23).

Apart from the genes that catalyze polymer biosynthesis, natural PHA producers have several genes that are involved in granule formation and/or have regulatory functions, such as phasins, granule-associated proteins that have been shown to enhance polymer synthesis and the number and size of PHA granules (17, 24). The phasin PhaP has been shown to exert a beneficial effect on bacterial growth and PHB accumulation from glycerol in bioreactor cultures of strain K24KP, a recombinant *E. coli* that carries *phaBAC* and *phaP* of *Azotobacter* sp. FA8 (6).

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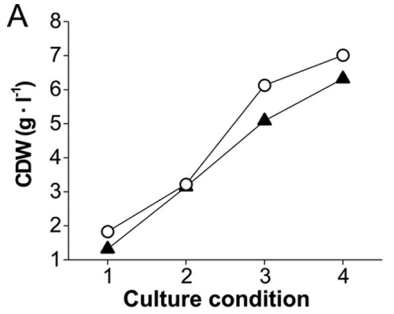
Because the redox state of the cells is known to affect the synthesis of PHB (1, 4, 14), the present study investigates the behavior of this recombinant strain under different aeration conditions, by using two substrates, glucose and glycerol, with different oxidation states.

Aeration conditions affect PHB accumulation differently in shaken-flask cultures grown on glycerol and on glucose. Four different aeration conditions were established using different agitation speeds and medium-to-flask volumes (Fig. 1). Growth and polymer synthesis in 48-h MYA (6) cultures using either 3% (wt/vol) glucose or 3% (wt/vol) glycerol as the carbon source were monitored. As expected, cell growth increased at higher aeration rates, and the maximum biomass production was observed under the highest aeration condition for both substrates. When PHB accumulation was determined, polymer content was observed to accompany growth in glucose cultures, as the highest values were observed for the most-aerated cultures, but in medium with glycerol, the highest PHB content was unexpectedly obtained under a relatively low-aeration condition (half-filled flasks at 125 rpm), and it was lower under the two more-aerated conditions tested (Fig. 1).

PHB production in bioreactor cultures using glycerol is higher at low aeration. In order to further analyze the relationship between carbon source, oxygen availability, and polymer accumulation of the recombinants, 3.0-liter cultures were conducted with a 5.6-liter benchtop bioreactor as previously described (15), using either glucose or glycerol as substrates, at two different agitation speeds (125 and 500 rpm) with a constant air supply rate (3 liters · min^{-1}).

As expected, cultures grew more with stronger agitation, reaching similar cell densities for the two carbon sources under each condition. The maximum growth rates of cultures grown on glucose were slightly higher under both aeration conditions, even when glucose cultures presented a short initial lag phase under low agitation, which was not observed when glycerol was used. Cultures grown with stronger agitation had higher growth rates than those with weaker agitation using either carbon source (Fig. 2, Table 1).

The relationship between PHB content and aeration was completely different with the two carbon sources, similar to what was observed in the flask experiments (Fig. 2, Table 1).



Culture condition	1	2	3	4
Agitation (rpm)	75	125	125	250
Medium-to-flask volume ratio	1:1	1:2	1:10	1:10

FIG. 1. Growth and PHB accumulation by strain KP24KP grown with shaking in 250-ml flasks in MYA medium supplemented with 3% (wt/vol) glucose (triangles) or glycerol (circles) under the four culture conditions indicated in the figure. All experiments were conducted at least twice, and results shown are mean values. Standard deviations were <5% of the means.

Cultures using glucose produced 1.8 times more polymer at 500 rpm than at 125 rpm, while those grown on glycerol produced 1.8 times more polymer under lower aeration. The rate of substrate consumed was higher at low aeration for both carbon sources (Table 1). This could be due to the more-efficient energy obtention under the more-aerated conditions, reflected in the growth differences observed. Specific substrate consumption was also higher with glycerol, the more-reduced carbon source, under both aeration conditions, in accordance with previous results obtained with other *E. coli* strains using carbon sources with different oxidation states (20).

When oxygen consumption was observed, results were consistent with the different oxidation states of the substrates used (0 for glucose and −2 for glycerol), as cultures grown in glucose consumed approximately two times more oxygen than those grown in glycerol (Table 1).

The metabolic profiles of bioreactor cultures grown on glycerol and glucose at different aeration rates reflect differences in carbon fluxes. PHB accumulation and fermentation products were assayed by using 24-h bioreactor cultures grown with each substrate at the two different agitation speeds to determine differences in the metabolic behavior of the recombinants. Results for formate, lactate, and acetate were determined by high-performance liquid chromatography (HP1100; Agilent, Santa Clara, CA) with a reverse phase column (Zorbax SB-Aq; Agilent). Detection was performed at 210 nm, and

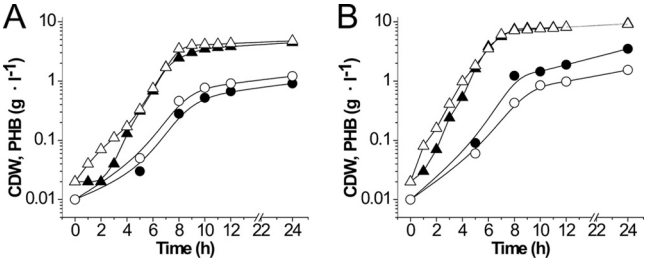


FIG. 2. Fermentation profiles of strain K24KP grown on glycerol or glucose in 24-h bioreactor cultures at low (A) or high (B) aeration. Cell dry weight (CDW) in glycerol cultures, open triangles; CDW in glucose cultures, closed triangles; PHB content in glycerol cultures, open circles; PHB content in glucose cultures, closed circles. All experiments were conducted at least twice, and results shown are mean values. Standard deviations were <5% of the means.

the operating conditions were: eluent, 0.1% (vol/vol) H_3PO_4; flow rate, 0.7 ml · min^{-1}; and column temperature, 39°C. Ethanol was analyzed with an enzymatic kit (Sigma-Aldrich Co., St. Louis, MO).

Both the carbon sources and the aeration conditions used affected carbon distribution among the different metabolic products assayed. As glycerol has a lower oxidation state than glucose, its catabolism produces more reducing equivalents (see Fig. S1 in the supplemental material). This has a significant effect on the intracellular NADH/NAD^+ ratio that causes the cells to direct carbon flow toward the synthesis of more-reduced products when using glycerol compared to glucose in order to maintain redox balance (20). On the other hand, the two different agitation speeds provided the cultures with different levels of oxygen availability, which also affected the oxidation state of the cells, resulting in variations in the product pattern.

The ethanol/acetate ratio can be used as an indicator of the redox state of the cells (16, 20). The values obtained were highest for cultures grown in glycerol at 125 rpm (4.04 ± 0.01 mol · mol^{-1}), followed by cultures grown in glycerol at 500 rpm (2.66 ± 0.01 mol · mol^{-1}) and cells grown in glucose at 125 rpm (1.03 ± 0.05 mol · mol^{-1}), and the lowest ethanol-to-acetate ratio (0.75 ± 0.05 mol · mol^{-1}) corresponded to the cultures grown with the more-oxidized carbon source (glucose) at the highest agitation speed (500 rpm). In cultures stirred at a low speed, lactate and formate concentrations were higher with both carbon sources than in strongly agitated cultures, and higher with glucose, the more-oxidized carbon source, under each aeration condition (see Fig. S2 in the supplemental material). The increase in lactate was more pronounced, probably due to the fact that its formation consumes reducing power, while formate synthesis does not. Accordingly, glucose cultures produced more formate than glycerol cultures.

In cultures grown on glucose, the specific productivity for ethanol was observed to increase 1.5-fold, but that of PHB decreased 1.7-fold, in low agitation compared to high agitation (Table 2), reaching final concentrations for PHB and ethanol of 29.4 ± 0.4 and 19.7 ± 0.3 mM, respectively, at high agitation and 8.5 ± 0.6 and 14.3 ± 0.5 mM at low agitation (see Fig. S2 in the supplemental material). The specific productivity of lactate and formate increased 12.8- and 2.7-fold, respectively, while that of acetate, which does not consume reducing power,

TABLE 1. Fermentation parameters for 24-h bioreactor cultures of strain K24KP grown in glucose or glycerol under different aeration conditions

Culture condition	CDW[a] ($g \cdot liter^{-1}$)	% PHB[b] (wt/wt)	μ_{max}[c] (h^{-1})	O_2 consumption[d] ($\mu mol\ O_2 \cdot g\ CDW^{-1} \cdot h^{-1}$)	$q_{substrate}$[e] ($mmol \cdot g\ CDW^{-1} \cdot h^{-1}$)
Glycerol					
125 rpm	4.75 ± 0.05	30.1 ± 0.4	0.69 ± 0.02	9.6 ± 0.5	1.86 ± 0.12
500 rpm	9.62 ± 0.02	16.9 ± 0.3	0.79 ± 0.02	8.9 ± 1.3	1.05 ± 0.04
Glucose					
125 rpm	4.45 ± 0.02	20.4 ± 0.1	0.75 ± 0.03	4.9 ± 0.5	1.41 ± 0.02
500 rpm	9.43 ± 0.01	37.2 ± 0.5	0.89 ± 0.04	5.1 ± 0.4	0.75 ± 0.18

[a] CDW, cell dry weight.
[b] The amount of PHB is given as a percentage of the weight (average ± standard deviation) of the CDW.
[c] Maximum specific growth rate.
[d] Oxygen consumption was determined as indicated in reference 19.
[e] The specific consumption rate was calculated on the basis of glucose or glycerol consumption.

was almost unchanged. The final amount of acetate produced at high agitation (26.3 ± 0.9 mM) was higher than at low agitation (13.9 ± 0.2 mM). The amount of ethanol produced in the cultures grown in glucose did not suffer major changes, but the decrease in acetate at low aeration caused a significant change in the ethanol-to-acetate ratio, reflecting the reducing state of the cells (see Fig. S2 in the supplemental material). Shalel-Levanon et al. (21) have reported that the specific production rates of ethanol, formate, and lactate are increased when oxygen availability is reduced in glucose-limited chemostat cultures of *E. coli*.

In cultures using glycerol, the reduction in oxygen availability caused a redirection of carbon flow not only toward acids but also toward ethanol and PHB, increasing the specific production rates 4.5- and 3.5-fold, respectively, compared to strongly agitated cultures. An increase in the production rates of acetate, lactate, and formate was also observed (3.0-, 22.0-, and 8.5-fold, respectively) (Table 2). The increase in ethanol production relative to the small decrease in acetate resulted in a very high ethanol-to-acetate ratio, reflecting a very reduced internal state (see Fig. S2 in the supplemental material). The dramatic increase in lactate formation can be due to the fact that poorly agitated cells growing on glycerol resort to lactate synthesis to dispose of the excess reducing power. However, the overall amount of acids produced was lower than in cultures grown on glucose.

In glycerol cultures grown with strong agitation, low quantities of the metabolic products measured correlated with the formation of large amounts of biomass (Table 1), and probably more oxidized products, such as CO_2. This fact was reflected in the residual biomass values, which were 3.32 ± 0.02 $g \cdot liter^{-1}$ at 125 rpm and 7.99 ± 0.03 $g \cdot liter^{-1}$ at 500 rpm. Low-agitation conditions, in which an increase in specific carbon source consumption was observed (Table 1), resulted in an increase in the amounts of the products measured, especially the most reduced of them, PHB and ethanol (Table 2). In glucose cultures, specific carbon consumption also increased at lower agitation speeds (Table 1), but carbon flow was directed toward the synthesis of formate and lactate, while formation of PHB was reduced (Table 2). For these cultures, the residual biomass values were 3.54 ± 0.03 $g \cdot liter^{-1}$ at 125 rpm and 5.92 ± 0.04 $g \cdot liter^{-1}$ at 500 rpm.

TABLE 2. Specific productivity of PHB, ethanol, acetate, formate, and lactate in 24-h bioreactor cultures of strain K24KP grown on glycerol or glucose at high or low aeration

Culture condition	Specific production rate ($\mu mol \cdot g\ CDW^{-1} \cdot h^{-1}$)				
	PHB	Ethanol	Acetate	Formate	Lactate
Glycerol					
125 rpm	194 ± 8	630 ± 8	160 ± 5	220 ± 6	110 ± 24
500 rpm	55 ± 6	141 ± 8	53 ± 1	26 ± 1	5 ± 1
Glucose					
125 rpm	79 ± 6	134 ± 33	130 ± 16	459 ± 2	308 ± 10
500 rpm	130 ± 13	87 ± 15	116 ± 33	171 ± 5	24 ± 1

The molecular mass of PHB produced from glycerol is comparable to that obtained from glucose. Previous studies showed that the molecular masses of PHAs obtained using glycerol were significantly lower than those obtained from other substrates, typically less than 1 MDa. In *Methylobacterium extorquens* and *C. necator*, PHB obtained from glycerol, ethanol, or methanol had a lower molecular mass than that obtained from other substrates (such as succinate, glucose, and fructose), and the molecular mass of the polymer was shown to decrease with increasing glycerol concentrations (23). This effect was further analyzed and attributed to chain termination caused by glycerol (10). In studies performed using *Pseudomonas* strains, increasing glycerol concentrations (from 1% [wt/vol] to 5% [wt/vol]) resulted in a decrease of more than 50% in the M_r of the PHB produced by *P. oleovorans* and medium-chain-length PHA synthesized by *P. corrugata* (2). A recent study performed using *C. necator* describes PHB obtained from commercial glycerol and from waste glycerol with a molecular mass of 957 and 786 kDa, respectively, less than half of that of PHB obtained from glucose (4). In contrast, in a recent report describing PHB-[poly(3-hydroxyvalerate)] (PHB-PHV) accumulation in a *Bacillus* strain, similar molecular masses, lower than 700 kDa, were observed for the polymer obtained from the two carbon sources (18).

A low M_r is undesirable for industrial processing of the polymer, so the results available in the literature pointed to a drawback in the use of glycerol as a substrate for the microbial production of PHAs. When the physical properties of PHB produced by strain K24KP in 24-h bioreactor cultures using glucose or glycerol were determined by viscosimetry as previ-

TABLE 3. Physical properties for PHB extracted from bioreactor cultures of recombinant *E. coli* strains grown on glycerol or glucose at high or low aeration

Strain[a]	Culture condition	M_r[b] (MDa)	T_g[c] (°C)	T_m[d] (°C)	% Crystallinity
K24KP	Glycerol, 125 rpm	1.90 ± 0.05	7.0 ± 0.1	175.1 ± 0.1	57.3 ± 1.0
	Glycerol, 500 rpm	1.71 ± 0.05	4.9 ± 0.1	173.1 ± 0.1	52.6 ± 1.0
	Glucose, 125 rpm	2.21 ± 0.05	3.1 ± 0.1	177.0 ± 0.1	60.8 ± 1.0
	Glucose, 500 rpm	2.28 ± 0.05	5.1 ± 0.1	177.3 ± 0.1	61.7 ± 1.0
	Glycerol, 1,000 rpm	2.04 ± 0.05	ND[e]	177.3 ± 0.5	66.4 ± 1.5
$K24K_{vc}$	Glycerol, 1,000 rpm	1.80 ± 0.05	ND	169.8 ± 0.4	52.6 ± 0.4

[a] K24KP carries *phaBAC* and *phaP* of *Azotobacter* sp. FA8. $K24K_{vc}$ carries *phaBAC* of *Azotobacter* sp. FA8.
[b] M_r, relative molecular mass.
[c] T_g, glass transition temperature.
[d] T_m, melting temperature.
[e] ND, not determined.

ously described (13), the polymer obtained from glycerol had an M_r only 12% and 14% lower than the one obtained from glucose under low- and high-aeration conditions, respectively (Table 3). For cells grown on glycerol, cultures stirred at 125 rpm reached a PHB content of 30.1% (wt/wt) in 24 h, with an M_r approximately 12% higher than cultures stirred at 500 rpm, which accumulated only 16.9% (wt/wt) polymer.

The possible effect of PhaP expression on the M_r of the polymer produced by our recombinants was also studied. PHB produced by strain K24KP was compared to the polymer obtained from the strain without PhaP by using 48-h bioreactor cultures grown in high aeration in MYA medium supplemented with 3% (wt/vol) glycerol (6). The strain bearing PhaP grew more (7.9 versus 3.1 g · liter^{-1}), accumulated more PHB (51.9 versus 38.2% [wt/wt]), and produced PHB with a slightly higher molecular mass (2.04 MDa) than the strain without PhaP (Table 3).

Taken together, these data suggest a possible correlation between the amount of polymer accumulated and its M_r, as the values were slightly higher in cultures accumulating more PHB, opening the possibility of further increasing the M_r of the polymer obtained from glycerol when optimizing PHB yields.

The glass transition temperature, melting temperature, and percentage of crystallinity of the polymer obtained under the different conditions were also determined as previously described (13). Only minor differences were observed for these parameters when comparing PHB obtained in glucose or glycerol or under different aeration conditions (Table 3), and their values were similar to those found in the literature (13, 25).

Conclusions. Small variations in oxygen availability, such as the ones used in this work, can lead to significant changes in the metabolite distribution of *E. coli* cultures. These changes, which reflect the metabolic adjustments that take place in order to optimize cell growth in this facultative aerobe, vary when using glucose or glycerol, affecting the synthesis of products such as PHB and ethanol in different ways. In this work, moderate agitation led to the largest amounts of PHB and ethanol from glycerol, which is desirable in production processes in order to reduce aeration-related problems and associated costs. On the other hand, our results indicate that it is possible to obtain PHB from glycerol with molecular weights similar to those of the polymer obtained from glucose or lactose by using adequate bacterial strains and culture conditions.

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