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Improving poly-3-hydroxybutyrate production in *Escherichia coli* by combining the increase in the NADPH pool and acetyl-CoA availability

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Abstract The biosynthesis of poly-3-hydroxybutyrate (P3HB), a biodegradable bio-plastic, requires acetyl-CoA as precursor and NADPH as cofactor. *Escherichia coli* has been used as a heterologous production model for P3HB, but metabolic pathway analysis shows a deficiency in maintaining high levels of NADPH and that the acetyl-CoA is mainly converted to acetic acid by native pathways. In this work the pool of NADPH was increased 1.7-fold in *E. coli* MG1655 through plasmid overexpression of the NADP^{+}-dependent glyceraldehyde 3-phosphate dehydrogenase gene (*gapN*) from *Streptococcus mutans* (pTrc*gapN*). Additionally, by deleting the main acetate production pathway (*ackA-pta*), the acetic acid production was abolished, thus increasing the acetyl-CoA pool. The P3HB biosynthetic pathway was heterologously expressed in strain MG1655 Δ*ack-pta*/pTrc*gapN*, using an IPTG inducible vector with the P3HB operon from *Azotobacter vinelandii* (pPHB$_{Av}$). Cultures were performed in controlled fermentors using mineral medium with glucose as the carbon source. Accordingly, the mass yield of P3HB on glucose increased to 73 % of the maximum theoretical and was 30 % higher when compared to the progenitor strain (MG1655/pPHB$_{Av}$). In comparison with the wild type strain expressing pPHB$_{Av}$, the specific accumulation of PHB (g$_{PHB}$/g$_{DCW}$) in MG1655 Δ*ack-pta*/pTrc*gapN*/pPHB$_{Av}$ increased two-fold, indicating that as the availability of NADPH is raised and the production of acetate abolished, a P3HB intracellular accumulation of up to 84 % of the *E. coli* dry weight is attainable.

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Introduction

Poly-3-hydroxybutyrate (P3HB) is a biodegradable bio-plastic that shows physiochemical properties that

are analogous to those of petro-plastics (Shishatskaya et al. 2005; Harding et al. 2007), and has been produced at industrial scale since almost 30 years ago (Chen 2009). P3HB is produced by different microorganisms, including the genera *Ralstonia*, *Azotobacter*, *Pseudomonas* and *Bacillus* (Pettinari et al. 2001; Lütke-Eversloh and Steinbüchel 2004; Reinecke and Steinbüchel 2009). These microorganisms accumulate the polymer as intracellular granules—storage material of carbon and energy under stress conditions and/or nutrient limitation (Madison and Huisman 1999). The biosynthesis of P3HB is performed by the concerted catalysis of three enzymes; initially the condensation of two molecules of acetyl-CoA is executed by a β-ketothiolase (PhbA), forming acetoacetyl-CoA; this compound is subsequently reduced to 3-hydroxybutyryl-CoA (3HB-CoA), through a NADPH-dependent reductase (PhbB); finally each 3HB-CoA monomer is polymerized by a PHB synthase (PhbC) to form P3HB (Schubert et al. 1988). The three enzymes are found within an operon in *Azotobacter vinelandii* called *phbBAC* (Pettinari et al. 2001).

This pathway is regulated by the β-ketothiolase, as high levels of CoA inhibit its activity, *i.e.* when glucose is being metabolized at a high rate and there is enough oxygen to maintain fully aerobic growth, an increase in the CoA pool is triggered as the result of an intense dissimilation of acetyl-CoA through the tricarboxylic acid cycle (Senior and Dawes 1973). Due to this enzymatic regulation, the heterologous production of P3HB in *E. coli* is carried out in two stages (Wang and Lee 1997; van Wegen et al. 2001; Tyo et al. 2010): the first stage is designed for cellular proliferation, which is followed by a second stage where low levels of oxygen and/or depleted nitrogen promote the accumulation of P3HB. Under such conditions, the tricarboxylic acid cycle activity decreases, allowing an increase in the levels of the acetyl-CoA pool, reducing the pool of CoA and increasing the synthesis of P3HB (Henderson and Jones 1997; Kessler and Witholt 2001). Hence the challenges to improve the production of P3HB with recombinant *E. coli* consist in raising the availability of the precursor acetyl-CoA and the cofactor NADPH. The increased availability of acetyl-CoA under oxygen limiting conditions, through the deletion of the acetic acid (*ackA-pta*), lactate dehydrogenase (*ldhA*) and ethanol (*adhE*) pathways in strains of *E. coli* has

yielded an improved efficiency of up to 4.7-fold in the accumulation of PHB; however, such strains displayed limited P3HB production as a result of low availability of NADPH (Jian et al. 2010). On the other hand, since the catabolic production of NADPH in *E. coli* is insufficient to sustain the anabolic demand (Sauer et al. 2004; Fuhrer and Sauer 2009), the synthesis of metabolites that require this cofactor is challenging (Kabir and Shimizu 2003b; Martínez et al. 2008). One of the most studied strategies to increase the levels of NADPH has been the metabolic manipulation of stimulating carbon flow into the oxidative branch of the pentose phosphate pathway (PPP) (Shi et al. 1999; Lim et al. 2002; Jung et al. 2004). Although the deletion of the phosphoglucose isomerase (*pgi*) results in the increase of NADPH and the over-flow of carbon into the PPP, this metabolic manipulation reduces the total carbon flux and the glucose consumption rate with a concomitant decrease in the specific growth rate, generating low volumetric productivities (Kabir and Shimizu 2003a, b).

During the metabolism of glucose, NADH generation in *E. coli* is conducted through the Embden–
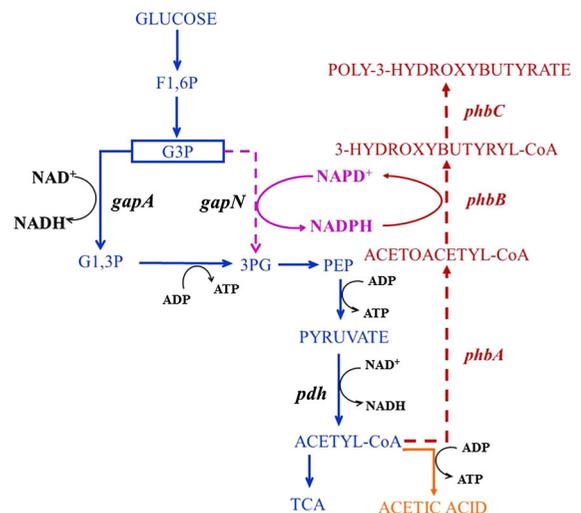
Fig. 1 Glycolysis pathway in wild type *E. coli* (*solid arrows*) and heterologous expression of NADP⁺-dependent glyceraldehyde-3-phosphate-dehydrogenase (*gapN*) and polyhydroxyburyrate pathway (*pointed arrow*). Genes encoding enzymes are indicated by italics. *gapA* NAD⁺-dependent glyceraldehyde-3-phosphate-dehydrogenase, *pdh* pyruvate dehydrogenase, *phbA* β-ketothiolase, *phbB* NADPH-dependent reductase, *phbC* PHB synthase. *F1,6P* fructose 1,6-diphosphate, *G1,3* glyceraldehyde 3-phosphate, *G3P* glyceraldehyde 3-phosphate, *PEP* phosphoenolpyruvate, *TCA* tricarboxylic cid cycle

Meyerhof–Parnas (EMP) pathway by the NAD^+-dependent glyceraldehyde-3-phosphate-dehydrogenase (NAD^+-GAPDH) (E.C. 1.2.1.12) (D'Alessio and Josse [1971]) encoded by *gapA* (Fig. [1]) (Charpentier and Branlant [1994]). However, several microorganisms such as *Bacillus*, *Streptococcus* and *Clostridium* have a $NADP^+$-dependent glyceraldehyde 3-phosphate dehydrogenase ($NADP^+$-GAPDH) (E.C. 1.2.1.9) (Iddar et al. [2005]). Specifically, it has been shown that the $NADP^+$-GAPDH enzyme from *Streptococcus mutans*, coded by *gapN* (Boyd et al. [1995]), has 10-fold higher affinity for G3P than GapA; the substrate affinity constant (Km) of GapN for G3P is 0.046 mM for G3P, while GapA has a Km of 0.89 mM (Nagradova [2001]; Marchal and Branlant [2002]). Also, it has been demonstrated that some genes encoding $NADP^+$-GAPDH enzymes can complement *E. coli* mutants deficient in the native NAD^+-GAPDH (Fillinger et al. [2000]; Iddar et al. [2002], [2003]). These mutants are able to grow under aerobic conditions despite the decrease in the NADH levels necessary for energy generation, but not in anaerobic conditions, possibly due to the inability of the cell to sustain an efficient redox balance (Valverde et al. [1999]; Martínez et al. [2008]). Previously, we reported the substitution of *gapA* of *E. coli* with *gapN* from *S. mutans* (Centeno-Leija et al. [2013]). The alteration in the turnover of NAD(P)H via glyceraldehyde 3-phosphate oxidation increased the NADPH levels with a concomitant decrease in the NADH levels, the respiratory capacity and the ATP levels, which forced the cell to produce more acetic acid through the AckA-Pta pathway (Centeno-Leija et al. [2013]). Furthermore, Martínez et al. ([2008]) have reported the replacement of *gapA* in *E. coli* with *gapC* that codes for a $NADP^+$-GAPDH enzyme from *Clostridium acetobutyricum*, increasing the NADPH yield on glucose around twofold and as a consequence, heterologous lycopene production, a NADPH-dependent metabolite, was increased 2.5-fold in rich medium (Martínez et al. [2008]). In the present work a study was conducted to boost the pool of NADPH, abolish the production of acetate and increase the production of P3HB in *E. coli*, through: the heterologous expression of the $NADP^+$-dependent glyceraldehyde 3-phosphate dehydrogenase ($NADP^+$-GAPDH) gene (*gapN*) from *S. mutans*; the deletion of the main acetate production pathway (*ackA-pta*); and the overexpression of the P3HB synthesis pathway from *A. vinelandii* (*phbBAC*).

Materials and methods

Bacterial strains and plasmids

The wild-type *E. coli* strain MG1655 (GenBank NC_000913) was used as the parental strain. Chromosomal DNA of *A. vinelandii* was generously donated by the Molecular Microbiology Department of Instituto de Biotecnología—Universidad Nacional Autonoma de Mexico. Plasmids and primers used in this work are listed in Table [1]. Standard procedures were employed for plasmid preparations, restriction-enzyme digestions, transformations and gel electrophoresis (Sambrook and Rusell [2001]). Each plasmid construction and mutant strains were verified by restriction pattern analysis in an agarose gel and by sequencing. Gene amplifications were carried out by PCR on a C1000 Touch Thermal Cycler Manual (Bio-Rad Laboratories, Inc., USA) using Pfu DNA polymerase (Thermo Fisher Scientific Inc.). DNA fragments were isolated from agarose gels with the Roche Pure PCR Product Purification kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. The construction of the recombinant strain MG1655Δ*gapA*::*gapN*, the strain MG1655Δ*gapA*::*gapN*/pTrc*gapN* and the plasmid pTrc*gapN* are reported elsewhere (Centeno-Leija et al. [2013]).

The chromosomal DNA from *A. vinelandii* was isolated with the Ultra Clean Microbial DNA Isolation kit (MO BIO Laboratories, Inc., USA). Plasmid pTrc99A was used to overexpress the *phbBAC* operon from *Azotobacter vinelandii*. First, this operon was amplified, from *A. vinelandii* chromosomal DNA, by PCR with oligonucleotides phb1 and phb2, which introduce *Eco*RI and *Hin*dIII flanking sites to facilitate cloning into plasmid pTrc99A. The fragment *phbBAC* was ligated into pTrc99A, previously digested with EcoRI and HindIII, to generate plasmid pPHB$_{Av}$ (Table [1]). The construction was verified by digestion with *Eco*RI and *Hin*dIII and visualized by agarose gel electrophoresis.

The deletion of the *ackA-pta* operon was made using the chromosomal gene inactivation method using PCR products (Datsenko and Wanner [2000]). The primers ackAF and ptaR were designed to amplify region FRT-Kan-FRT from pKD4 with ~50 nucleotides of homology to the chromosome sequence to inactivate the target operon. The Δ*ackA-pta* mutant selected strain was verified by DNA sequencing with

Table 1 Plasmids and primers used in this work

Plasmid	Description	Source
pTrc99A	Designed for IPTG-inducible expression of proteins under hybrid *trp/lac* promoter. Amp[r]	Amann et al. ([1988])
pACYC184	Designed with p15A origin of replication to coexist in cells with plasmids of the ColE1 compatibility group (e.g., pBR322, pUC19). Tc[r] and Cm[r]	Chang and Cohen ([1978])
pTrc*gapN*	pAcyc184 derivative with a constitutive *trc* promoter and *gapN* gene	Centeno-Leija et al. ([2013])
pPHB$_{Av}$	Designed for IPTG-inducible expression of *phbBAC* operon from *A. vinelandii*	This study

Primer	Sequence[a]
phb1	5′GG<u>AATTC</u>CGGATGAGCAATCAACGAATTGCAT 3′
phb2	5′CCC<u>AAGCTT</u>GGGTCAGCCTTTCACGTAACGGCCT 3′
ackAF[b]	5′**GGTACTTCCATGTCGAGTAAGTTAGTACTGGTTCTGA ACTGCGGT**GTGTAGGCTGGAGCTGCTTCG 3′
ptaR[b]	5′**CTGCGGATGATGACGAGATTACTGCTGCTGTGCAGACTGA ATCG**CCATATGAATATCCTCCTTAG 3′
p1	5′GCAGCCTGAAGGCCTAAGTAG 3′
p2	5′CGGGCATTGCCCATCTTCTTG 3′

[a] Restriction sites employed during plasmid construction are underlined

[b] Sequences that flank $\sim$50 pb upstream and downstream of *ackA and pta* gen are indicated in bold

primers p1 and p2, which hybridize at $\sim$230 nucleotides upstream and downstream, respectively, of the *ackA-pta* operon.

Medium and growth conditions

The routine cultivation and genetic manipulations of *E. coli* MG1655 and its mutant were performed in Luria–Bertani medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl, pH 7.2) supplemented with the appropriated antibiotics when it was necessary.

To characterize the strains, *E. coli* MG1655Δ*gapA::gapN*/pTrc*gapN* was compared with *E. coli* MG1655 and MG1655Δ*gapA::gapN* carrying an empty pACYC184 plasmid. Cultures were performed in pH controlled fermenters at 37 °C. Seed cells were prepared in a 2.8 L Fermbach flask containing 0.5 L of M9 medium with 5 g/L glucose. M9 medium contains, per liter: 6 g Na_2HPO_4; 3 g KH_2PO_4; 0.5 g NaCl; 1 g NH_4Cl; 2 mM $MgSO_4$; 0.1 mM $CaCl_2$; and 0.01 g Vitamin B1. The cells from 350 mL of culture were harvested by centrifugation at an OD_{600} of 1 and resuspended with 10 mL of fresh M9 medium; these cells were used as inoculum for the bioreactor. Cultures were performed in mineral M9 medium supplemented with glucose (10 g/L) in a 1-L bioreactor (Applikon Biotechnology, Netherlands), a working

volume of 0.7 L, pH controlled at 7 with NaOH (2 N), an air flow rate of 0.84 L/min, and 600 rpm. The samples were collected in the mid-log phase at an OD_{600} of 3 to determine cofactors and GAPDH enzymatic activities. All experiments and analysis were performed in triplicates, figures and tables show averages and standard deviations.

Metabolite analysis and kinetic and stoichiometric parameters

The samples from cultures were centrifuged (4,000×g, 4 °C, 10 min), and the cell-free culture broth was frozen for subsequent analysis. The concentration of glucose and organic acids in the culture broths were determined by HPLC, as previously reported (Centeno-Leija et al. [2013]).

The cell growth for cells without the plasmid pPHB$_{Av}$ was measured by monitoring the optical density at 600 nm (OD_{600}) in a spectrophotometer DU-70 (Beckman Instruments, Inc., Fullerton, CA, USA). OD_{600} was converted into dry cellular weight (cell concentration) using a calibration standard curve (1 OD_{600} = 0.42 g/L of dry cellular weight: DCW). The specific growth rates (μ) were determined by fitting the biomass data versus time to exponential regressions. The cell mass yield on glucose ($Y_{X/GLC}$)

and the yield of acetate on glucose ($Y_{ACE/GLC}$), were estimated as the coefficient of the linear regression of acetate or cell mass concentration versus the concentration of glucose consumed during the exponential growth phase, in $g_{DCW}/mmol_{GLC}$ and $mmol_{ACE}/mmol_{GLC}$. The specific glucose consumption rate (q_s) was determined as the ratio of μ to $Y_{X/GLC}$. The maximum theoretical yield of P3HB on glucose (0.48 g_{PHB}/g_{GLC}) was calculated from the stoichiometry based on the pathway shown in Fig. 1: 1 mol of 3HB monomer is formed from 1 mol of glucose. For cells producing P3HB the optical density at 600 nm (OD_{600}) is used in graphs only as an indicative of cell growth with intracellular accumulation of P3HB.

Measurements of the intracellular cofactors concentrations

The $NADP^+$-GAPDH and NAD^+-GAPDH enzymatic activities and the intracellular cofactors, NAD^+, NADH, $NADP^+$ NADPH, were assayed as reported elsewhere (Centeno-Leija et al. 2013). One unit of activity is defined as 1 μmol of NAD(P)H formed per min.

Measurements of P3HB

To determinate the P3HB content, the cells were harvested by centrifugation ($4,000 \times g$, 30 min, 4 °C) at the onset of glucose depletion, washed twice with distilled water and then freeze dried. The resulting cell powders were stored at 25 °C until they were processed. 10 mg of freeze-dried cell powder with P3HB-containing biomass was weighted, processed in glass vials (with Teflon caps), and mixed with a hypochlorite solution (2 % vol/vol). The granules containing P3HB were separated from the aqueous fraction (containing the cell debris) by centrifugation. The recovered solids were rinsed twice with distilled water. The granules of P3HB were resuspended with 3 volumes of chloroform and the polymer was dried allowing the total evaporation of chloroform. After drying with chloroform, the P3HB was mixed with 75 μL of solution A (5 mL of H_2SO_4: methanol (10 % vol/vol) + 25 mL of benzoic acid: methanol (4 % wt/vol)). The mixture was heated at 95 °C until total dissolution was observed, then 0.75 mL of solution B (0.5 mL of chloroform + 0.25 mL di-H_2O) was added and vigorously mixed for 1 min five times.

The phases were separated by centrifugation ($3,000 \times g$, 2 min, and room temperature). The organic phase was recovered and quantified using a gas chromatograph (Model 6850, Agilent, CA, USA), equipped with an INNOWax column (initial temperature 50 °C, final temperature 260 °C, increase 20 °C/min) (30 m × 0.25 mm, 0.25 μm), and a flame ionization detector with helium as the mobile phase at 0.1 mL/min. Commercial P3HB was used as standard and benzoic acid (contained in solution A) as internal standard.

Results and discussion

Increasing the NADPH pool by heterologous expression of $NADP^+$-GAPDH

In order to increase the NADPH levels in *E. coli*, the NAD^+-GAPDH gene (*gapA*) was replaced with the $NADP^+$-GAPDH gene from *S. mutans* (*gapN*). Since *E. coli* mutants in the *gapA* gene are unable to grow with hexoses as carbon source (Schubert et al. 1988; van Wegen et al. 2001), the strain MG1655Δ*gapA::gapN*/pTrc*gapN* was constructed, as previously described (Centeno-Leija et al. 2013), in one step by inserting the *gapN* gene at the same locus of the native *gapA* gene and subsequently it was transformed with plasmid pTrc*gapN*, which overexpresses *gapN* from the strong *trc* promoter (Centeno-Leija et al. 2013). Also, the wild-type strain MG1655, having a functional *gapA* gene in the chromosome, was transformed with plasmid pTrc*gapN*, resulting in strain MG1655/pTrc*gapN*. Thus, in strain MG1655/pTrc*gapN*, both NAD^+-GAPDH activity and heterologous $NADP^+$-GAPDH enzyme were synthesized simultaneously (Fig. 1).

Fermenter batch cultures with mineral medium and 10 g/L of glucose were performed under aerobic conditions. Kinetic data showed that strain MG1655Δ*gapA::gapN*/pTrc*gapN* had a specific growth (μ) and glucose consumption (q_{GLC}) rates about 15 and 26 % lower, respectively, when compared to the wild-type strain (Table 2). This result is attributable to redox and energetic perturbations when the turnover of NAD(P)H via glyceraldehyde 3-phosphate oxidation is altered (Martínez et al. 2008; Centeno-Leija et al. 2013). In contrast, the μ and q_{GLC} values for MG1655/pTrc*gapN* were similar to those

Table 2 Growth parameters of glucose fermenter batch cultures in mineral medium of the strains MG1655, MG1655ΔgapA::gapN/pTrcgapN, MG1655/pTrcgapN, MG1655ΔackA-pta and MG1655ΔackA-pta/pTrcgapN

| Strain	μ (h^{-1})	q_s (mmol$_{Glc}$/g$_{DCW}$/h)	$Y_{ACE/GLC}$ (mol$_{ACE}$/mol$_{GLC}$)	NAD$^+$-GAPDH activity (IU/mg$_{PROT}$)	NADP$^+$-GAPDH activity (IU/mg$_{PROT}$)
MG1655	0.52 ± 0.01	7.04 ± 0.05	0.61 ± 0.07	0.522 ± 0.21	ND[a]
MG1655ΔgapA::gapN/pTrcgapN	0.44 ± 0.01	5.24 ± 0.13	0.78 ± 0.02	ND[a]	0.937 ± 0.04
MG1655/pTrcgapN	0.50 ± 0.01	7.57 ± 0.03	0.63 ± 0.05	0.531 ± 0.14	0.788 ± 0.07
MG1655ΔackA-pta	0.45 ± 0.01	6.63 ± 0.02	0.16 ± 0.00	0.562 ± 0.07	ND[a]
MG1655ΔackA-pta/pTrcgapN	0.43 ± 0.01	6.72 ± 0.00	0.08 ± 0.00	0.545 ± 0.03	0.866 ± 0.11

[a] Activity not detected

observed with the wild-type strain (Table 2), suggesting that co-expression of *gapN* along with *gapA* didn't cause a detectable metabolic perturbation. Furthermore, it was possible to observe that the acetic acid was the only sub-product in all strains (Table 2). The acetic acid production was expected, since acetate is generated in *E. coli* cultures under aerobic conditions with high glucose concentrations and high rates of substrate consumption (Andersen and von Meyenburg 1980; Vemuri et al. 2006).

On the other hand, the specific NADP$^+$-GAPDH activity in strain MG1655ΔgapA::gapN/pTrcgapN was 0.937 ± 0.04 IU/mg$_{PROT}$ and no NAD$^+$-GAPDH activity was detected. In agreement, the NADPH/NADH ratio increased threefold in comparison with the wild-type strain (Fig. 2). Likewise, MG1655/pTrcgapN had a specific NADP$^+$-GAPDH activity of 0.788 ± 0.07 IU/mg$_{PROT}$ and a similar specific NAD$^+$-GAPDH activity to the wild-type strain (Table 2), demonstrating that strain MG1655/pTrcgapN had both GAPDH activities. Accordingly, the intracellular NADPH/NADH ratio increased 1.8-fold when compared with the wild-type strain (Fig. 2). This indicates that the activity of the enzyme NADP$^+$-GAPDH was able to increase the intracellular levels of NADPH in both strains even when both enzymes coexist. However, the full replacement of the native NAD$^+$-GAPDH activity by the heterologous NADP$^+$-GAPDH activity increased the NADPH/NADH ratio 1.7-fold compared to the state when both enzymes coexist (Fig. 2).

P3HB production with *ackA-pta$^+$* strains

To test whether the increased levels of NADPH improved the P3HB production, the strains
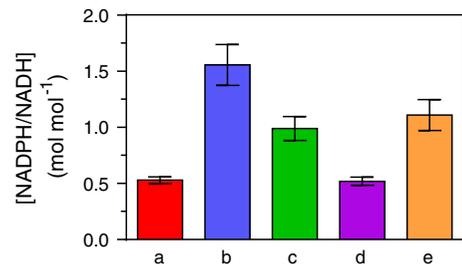
Fig. 2 NADPH/NADH ratios (mol/mol) of the strains MG1655 (*a*), MG1655ΔgapA::gapN/pTrcgapN (*b*), MG1655/pTrcgapN (*c*), MG1655ΔackA-pta (*d*) and MG1655ΔackA-pta/pTrcgapN (*e*)

MG1655ΔgapA::gapN/pTrcgapN and MG1655/pTrcgapN were transformed with plasmid pPHB$_{Av}$. The strains were characterized in fermenter batch cultures with mineral medium supplemented with 10 g/L of glucose. Synthesis of PHB was induced by the addition of 10 μM IPTG when the cultures reached a cell mass of 0.42 g/L (1 OD$_{600}$).

As shown in Figs. 3 and 4, the specific accumulation of P3HB (g$_{PHB}$/g$_{DCW}$) in strains MG1655ΔgapA::gapN/pTrcgapN/pPHB$_{Av}$ and MG1655/pTrcgapN/pPHB$_{Av}$ was 1.38 and 1.84-fold higher, respectively, than the wild type strain; also resulting in the increase of the P3HB yield on glucose (g$_{PHB}$/g$_{GLC}$), 1.17 and 1.39-fold, respectively (Fig. 4). According to these values and based on the theoretical maximum yield of P3HB on glucose (0.48 g$_{PHB}$/g$_{GLC}$), increased levels of NADPH allowed channeling 55 and 68 % of the consumed carbon to P3HB, respectively, which are 17 and 39 % higher than values attained with the wild type strain (Fig. 4). These results indicate that the production of NADPH using the EMP stimulates the synthesis of P3HB in *E. coli*.

Even though the measured intracellular levels of NADPH were higher for strain MG1655ΔgapA::gapN/
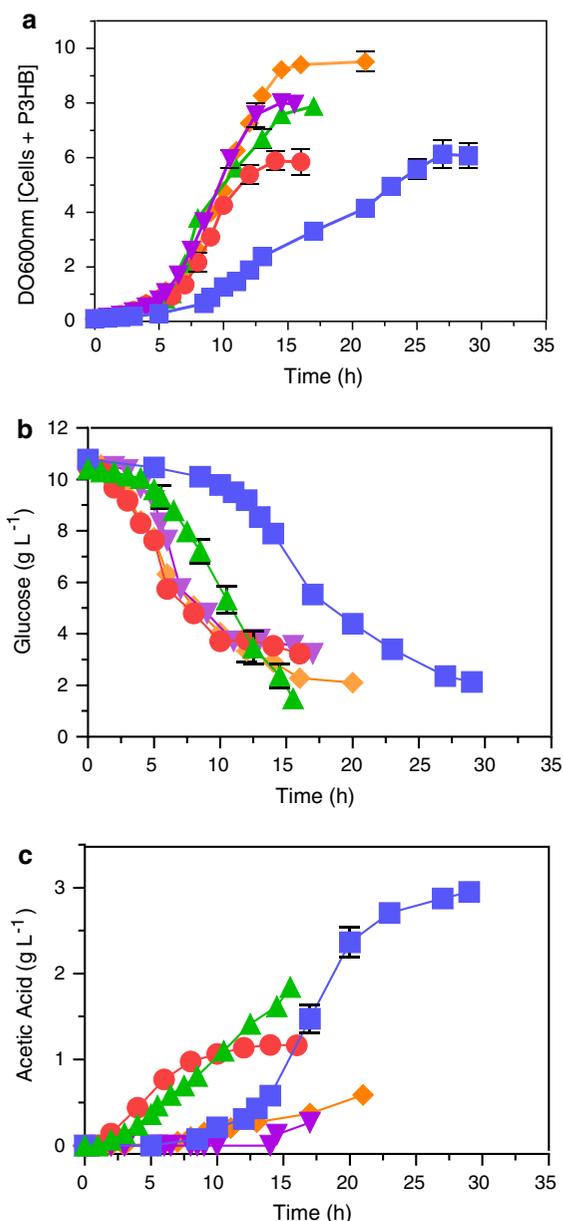
Fig. 3 Kinetic of biomass accumulation (**a**), glucose consumption (**b**) and acetic acid production (**c**) during P3HB production of the strains MG1655/pPHB$_{Av}$ (*circles*), MG1655Δ*gapA::gapN*/pTrc*gapN*/pPHB$_{Av}$ (*squares*), MG1655/pTrc*gapN*/pPHB$_{Av}$ (*triangles*), MG1655Δ*ackA-pta*/pPHB$_{Av}$ (*inverted triangles*) and MG1655Δ*ackA-pta*/pTrc*gapN*/pPHB$_{Av}$ (*diamonds*)

pTrc*gapN*, the pPHB$_{Av}$ derivative strain accumulated 30 % less polymer than strain MG1655/pTrc*gapN*/pPHB$_{Av}$. This result correlates with the loss of carbon due to the acetic acid synthesis, which is 2 times higher in strain MG1655Δ*gapA::gapN*/pTrc*gapN*/pPHB$_{Av}$
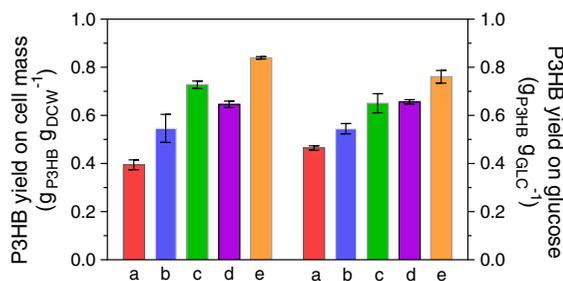
Fig. 4 Yield of P3HB on cells (g$_{P3HB}$/g$_{DCW}$) and yield of P3HB on glucose (g$_{P3HB}$/g$_{GLC}$) of the strains MG1655/pPHB$_{Av}$ (**a**), MG1655Δ*gapA::gapN*/pTrc*gapN*/pPHB$_{Av}$ (**b**), MG1655/ pTrc*gapN*/pPHB$_{Av}$ (**c**), MG1655Δ*ackA-pta*/pPHB$_{Av}$ (**d**) and MG1655Δ*ackA-pta*/pTrc*gapN*/pPHB$_{Av}$ (**e**)

than in strain MG1655/pTrc*gapN*/pPHB$_{Av}$ (Fig. 3). Previously we demonstrated that cells from strain MG1655Δ*gapA::gapN*/pTrc*gapN* have low NADH and ATP levels (Centeno-Leija et al. 2013); *i.e.* the replacement of *gapA* for *gapN* disturbs the turnover of NAD(P)H, which increases NADPH levels, but decreases the NADH levels and hence decreasing the ATP levels. This energy deficiency can be accentuated by expressing multicopy plasmids for the production of heterologous protein (Diaz Ricci and Hernández 2000), and it is likely that the increased synthesis of acetic acid by the AckA-Pta pathway could mitigate part of the ATP deficiency in strain MG1655Δ*gapA::gapN*/ pTrc*gapN*/pPHB$_{Av}$.

P3HB production with *ackA-pta*⁻ strains

To prevent carbon loss and increase the acetyl-CoA pool, we decided to eliminate the acetic acid production by disrupting the *ackA-pta* operon into the chromosome of strains MG1655Δ*gapA::gapN* and MG1655. The mutants MG1655Δ*gapA::gapN* Δ*ackA-pta* and MG1655Δ*ackA-pta* were transformed with plasmids pTrc*gapN* and pPHB$_{Av}$. However, the mutant MG1655Δ*gapA::gapN* Δ*ackA-pta* was unable to grow in mineral medium-glucose or in the presence of one or both plasmids, suggesting that the high energy demand cannot be mitigated in the absence of a functional AckA-Pta pathway; *i.e.* in the absence of the GapA enzyme, the NADH and ATP levels decrease and part of the ATP is replenish through the AckA-Pta pathway (Martínez et al. 2008, Centeno-Leija et al. 2013). When the strain MG1655Δ*gapA::gapN* Δ*ackA-pta* is grown in mineral media-glucose the NADH, required for ATP

production, is only generated by the pyruvate dehydrogenase (Pdh) complex (Fig. 1). Moreover, it has been demonstrated that in *E. coli Δpta* mutants the Pdh complex is inhibited (Castaño-Cerezo et al. 2009); hence our results suggest that the high energy demand of the strain MG1655Δ*gapA::gapN ΔackA-pta* cannot be mitigated in the absence of a functional AckA-Pta pathway. Consequently the MG1655Δ*gapA::gapN ΔackA-pta* mutant was discarded to test the production of P3HB.

Kinetic data of the strain MG1655Δ*ackA-pta* are shown in Table 2, these data indicate that, in comparison with the wild type strain, there is a small decrease in μ and q_{GLC}, but the synthesis of acetic acid was virtually eliminated. When the MG1655Δ*ackA-pta* mutant was transformed with the plasmid pPHB_{Av} (MG1655Δ*ackA-pta*/pPHB_{Av}), the P3HB yield on glucose increased about 48 % compared to the wild-type strain (Fig. 4), and the cells accumulated up to 64 % of their dry weight as P3HB (Figs. 3, 4). These results indicate that by preventing the synthesis of acetic acid the production of P3HB is improved to similar levels of those obtained with the MG1655/pTrc*gapN*/pPHB_{Av}, which has increased levels of NADPH (Figs. 3, 4). Similar results were found in an *E. coli* mutant with several deletions in the mixed acid pathways (Jian et al. 2010).

With the aim of combining the effects of increasing the intracellular levels (pools) of acetyl CoA and NADPH, the strain MG1655Δ*ackA-pta* was transformed with the pTrc*gapN* plasmid. The strain MG1655Δ*ackA-pta*/pTrc*gapN* showed a similar profile for growth, glucose consumption and production of acetic acid when compared to the parent strain MG1655Δ*ackA-pta* (Table 2). Furthermore, the strain MG1655Δ*ackA-pta*/pTrc*gapN* possesses both NAD+-GAPDH and NADP+-GAPDH enzymatic activities (Table 2), which increases the NADPH/NADH ratio about twofold when compared to the parent strain (Fig. 2). When the strain MG1655Δ*ackA-pta*/pTrc*gapN* was transformed with the plasmid pPHB_{Av} (MG1655Δ*ackA-pta*/pTrc*gapN*/pPHB_{Av}) the P3HB yield on glucose increased about 63 % relative to values obtained with the wild-type strain (and 12 % when compared to the isogenic strain—Fig. 4), causing the cells to accumulate 84 % of their dry weight as P3HB and reaching a conversion yield of P3HB on glucose of 73 % of the maximum theoretical (Figs. 3, 4).

Overall, this study shows that the combined co-expression of genes encoding NADP+- and NAD+ glyceraldehyde 3-phosphate dehydrogenases, the deletion of the main acetate production pathway (*ackA-pta*) and the overexpression of the P3HB synthesis pathway from *A. vinelandii* (*phbBAC*), allows to increase the heterologous production of P3HB in *E. coli*.

Conclusions

The results reported in this work, demonstrate that the production of NADPH by an engineered EMP pathway stimulate the synthesis of P3HB. Although the full replacement of the native NAD+-GAPDH activity by the heterologous NADP+-GAPDH activity increased the NADPH/NADH ratio 1.7-fold, when compared to the levels obtained in the strain with both enzymes, the high acetic acid synthesis and probably the high energy demand limited the P3HB yield. However, the simultaneous production of NADPH and NADH during glycolysis and the removal of the main acetic acid synthesis pathway increased the NADPH/NADH ratio and the acetyl-CoA pool, improving the yield of the production of P3HB on glucose by 30 %, using minimal medium and reaching a conversion yield of the carbon source into P3HB of 73 % of the theoretical maximum.

References

Amann E, Ochs B, Abel K (1988) Tightly regulated *tac* promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. Gene 69:301–315

Andersen KB, von Meyenburg K (1980) Are growth rates of *Escherichia coli* in batch cultures limited by respiration? J Bacteriol 144:114–123

Boyd DA, Cvitkovitch DG, Hamilton I (1995) Sequence, expression, and function of the gene for the phosphate dehydrogenase of *Streptococcus mutans*. J Bacteriol 177:2622–2627

Castaño-Cerezo S, Pastor JM, Renilla S et al (2009) An insight into the role of phosphotransacetylase (*pta*) and the acetate/acetyl-CoA node in *Escherichia coli*. Microb Cell Fact 8(54):1–19

Centeno-Leija S, Utrilla J, Flores N et al (2013) Metabolic and transcriptional response of *Escherichia coli* with a NADP$^+$-dependent glyceraldehyde 3-phosphate dehydrogenase from *Streptococcus mutans*. Antonie Van Leeuwenhoek 104(6):913–924

Chang ACY, Cohen SN (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J Bacteriol 134:1141–1156

Charpentier B, Branlant C (1994) The *Escherichia coli gapA* gene is transcribed by the vegetative RNA polymerase holoenzyme Eσ^{70} and by the heat shock RNA polymerase Eσ^{32}. J Bacteriol 176:830–839

Chen G-Q (2009) A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chem Soc Rev 38:2434–2446

D'Alessio G, Josse J (1971) Glyceraldehyde phosphate dehydrogenase of *Escherichia coli*. J Biol Chem 246:4326–4333

Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 97:6640–6645

Diaz Ricci JC, Hernández ME (2000) Plasmid effects on *Escherichia coli* metabolism. Crit Rev Biotechnol 20:79–108

Fillinger S, Boschi-Muller S, Azza S et al (2000) Two glyceraldehyde-3-phosphate dehydrogenases with opposite physiological roles in a nonphotosynthetic bacterium. J Biol Chem 275:14031–14037

Fuhrer T, Sauer U (2009) Different biochemical mechanisms ensure network-wide balancing of reducing equivalents in microbial metabolism. J Bacteriol 191:2112–2121

Harding KG, Dennis JS, von Blottnitz H, Harrison STL (2007) Environmental analysis of plastic production processes: comparing petroleum-based polypropylene and polyethylene with biologically based poly-beta-hydroxybutyric acid using life cycle analysis. J Biotechnol 130:57–66

Henderson R, Jones CW (1997) Poly-3-hydroxybutyrate production by washed cells of *Alcaligenes eutrophus*; purification, characterisation and potential regulatory role of citrate synthase. Arch Microbiol 168:486–492

Iddar A, Valverde F, Serrano A, Soukri A (2002) Expression, purification, and characterization of recombinant nonphosphorylating NAP-dependent glyceraldehyde-3-phosphate dehydrogenase from *Clostridium acetobutylicum*. Protein Expr Purif 25:519–526

Iddar A, Valverde F, Serrano A, Soukri A (2003) Purification of recombinant non-phosphorylating NADP-dependent glyceraldehyde-3-phosphate dehydrogenase from *Streptococcus pyogenes* expressed in *E. coli*. Mol Cell Biochem 247:195–203

Iddar A, Valverde F, Assobhei O et al (2005) Widespread occurrence of non-phosphorylating dehydrogenase among gram-positive bacteria. Int Microbiol 8:251–258

Jian J, Zhang S-Q, Shi Z-Y et al (2010) Production of polyhydroxyalkanoates by *Escherichia coli* mutants with defected mixed acid fermentation pathways. Appl Microbiol Biotechnol 87:2247–2256

Jung Y-M, Lee J-N, Shin H-D, Lee Y-H (2004) Role of *tktA* gene in pentose phosphate pathway on odd-ball biosynthesis of poly-beta-hydroxybutyrate in transformant *Escherichia coli* harboring *phbCAB* operon. J Biosci Bioeng 98:224–227

Kabir M, Shimizu K (2003a) Gene expression patterns for metabolic pathway in *pgi* knockout *Escherichia coli* with and without *phb* genes based on RT-PCR. J Biotechnol 105:11–31

Kabir MM, Shimizu K (2003b) Fermentation characteristics and protein expression patterns in a recombinant *Escherichia coli* mutant lacking phosphoglucose isomerase for poly(3-hydroxybutyrate) production. Appl Microbiol Biotechnol 62:244–255

Kessler B, Witholt B (2001) Factors involved in the regulatory network of polyhydroxyalkanoate metabolism. J Biotechnol 86:97–104

Lim S-J, Jung Y-M, Shin H-D, Lee Y-H (2002) Amplification of the NADPH-related genes *zwf* and *gnd* for the oddball biosynthesis of PHB in an *E. coli* transformant harboring a cloned phbCAB operon. J Biosci Bioeng 93:543–549

Lütke-Eversloh T, Steinbüchel A (2004) Microbial polythioesters. Macromol Biosci 4:166–174

Madison LL, Huisman GW (1999) Metabolic engineering of poly (3-hydroxyalkanoates): from DNA to plastic. Microbiol Mol Biol Rev 63:21–53

Marchal S, Branlant G (2002) Characterization of the amino acids involved in substrate specificity of nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase from *Streptococcus mutans*. J Biol Chem 277:39235–39242

Martínez I, Zhu J, Lin H et al (2008) Replacing *Escherichia coli* NAD-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH) with a NADP-dependent enzyme from *Clostridium acetobutylicum* facilitates NADPH dependent pathways. Metab Eng 10:352–359

Nagradova NK (2001) Study of the properties of phosphorylating D-glyceraldehyde-3-phosphate dehydrogenase. Biochem (Mosc) 66:1067–1076

Pettinari MJ, Vázquez GJ, Silberschmidt D et al (2001) Poly (3-hydroxybutyrate) synthesis genes in *Azotobacter* sp. strain FA8. Appl Environ Microbiol 67:5331–5334

Reinecke F, Steinbüchel A (2009) *Ralstonia eutropha* strain H16 as model organism for PHA metabolism and for biotechnological production of technically interesting biopolymers. J Mol Microbiol Biotechnol 16:91–108

Sambrook J, Rusell D (2001) Molecular cloning a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, New York

Sauer U, Canonaco F, Heri S et al (2004) The soluble and membrane-bound transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of *Escherichia coli*. J Biol Chem 279:6613–6619

Schubert P, Steinbüchel A, Schlegel HG (1988) Cloning of the *Alcaligenes eutrophus* genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in *Escherichia coli*. J Bacteriol 170:5837–5847

Senior PJ, Dawes EA (1973) The regulation of poly-β-hydroxybutyrate metabolism in *Azotobacter beijerinckii*. Biochem J 134:225–238

Shi H, Nikawa J, Shimizu K (1999) Effect of modifying metabolic network on poly-3-hydroxybutyrate biosynthesis in recombinant *Escherichia coli*. J Biosci Bioeng 87:666–677

Shishatskaya EI, Volova TG, Gordeev S, Puzyr P (2005) Degradation of P(3HB) and P(3HB-co-3HV) in biological media. J Biomater Sci Polym Ed 16:643–657

Tyo KEJ, Fischer CR, Simeon F, Stephanopoulos G (2010) Analysis of polyhydroxybutyrate flux limitations by systematic genetic and metabolic perturbations. Metab Eng 12:187–195

Valverde F, Losada M, Serrano A (1999) Engineering a central metabolic pathway: glycolysis with no net phosphorylation in an *Escherichia coli* gap mutant complemented with a plant *gapN* gene. FEBS Lett 449:153–158

Van Wegen RJ, Lee SY, Middelberg P (2001) Metabolic and kinetic analysis of poly(3-hydroxybutyrate) production by recombinant *Escherichia coli*. Biotechnol Bioeng 74:70–80

Vemuri GN, Altman E, Sangurdekar DP et al (2006) Overflow metabolism in *Escherichia coli* during steady-state growth: transcriptional regulation and effect of the redox ratio. Appl Environ Microbiol 72:3653–3661

Wang F, Lee SY (1997) Production of poly(3-hydroxybutyrate) by fed-batch culture of filamentation-suppressed recombinant *Escherichia coli*. Appl Environ Microbiol 63:4765–4769