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# Improved Polyhydroxybutyrate Production by *Saccharomyces cerevisiae* Through the Use of the Phosphoketolase Pathway

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**ABSTRACT:** The metabolic pathways of the central carbon metabolism in *Saccharomyces cerevisiae* are well studied and consequently *S. cerevisiae* has been widely evaluated as a cell factory for many industrial biological products. In this study, we investigated the effect of engineering the supply of precursor, acetyl-CoA, and cofactor, NADPH, on the biosynthesis of the bacterial biopolymer polyhydroxybutyrate (PHB), in *S. cerevisiae*. Supply of acetyl-CoA was engineered by over-expression of genes from the ethanol degradation pathway or by heterologous expression of the phophoketolase pathway from *Aspergillus nidulans*. Both strategies improved the production of PHB. Integration of *gapN* encoding $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase from *Streptococcus mutans* into the genome enabled an increased supply of NADPH resulting in a decrease in glycerol production and increased production of PHB. The strategy that resulted in the highest PHB production after 100 h was with a strain harboring the phosphoketolase pathway to supply acetyl-CoA without the need of increased NADPH production by *gapN* integration. The results from this study imply that during the exponential growth on glucose, the biosynthesis of PHB in *S. cerevisiae* is likely to be limited by the supply of NADPH whereas supply of acetyl-CoA as precursor plays a more important role in the improvement of PHB production during growth on ethanol.

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**KEYWORDS:** polyhydroxybutyrate; acetyl-CoA; NADPH; phosphoketolase pathway

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## Introduction

Energy and carbon metabolism are highly interconnected, and the medium composition and the availability of oxygen during cultivation therefore impact the physiology and overall metabolism of most cells. In *Saccharomyces cerevisiae*, a widely used cell factory for production of fuels, chemicals, and materials, glucose is metabolized via the Embden-Meyerhof-Parnas (EMP) pathway or the pentose phosphate (PP) pathway, which split at the glucose-6-phosphate branch point. The split ratios towards the PP pathway range from 2 to 44.2 moles per 100 moles of consumed glucose depending on the cellular state and culture conditions (Bruinenberg et al., 1983; Gombert et al., 2001; van Winden et al., 2005). However, almost 90% of the carbon that enters the PP pathway re-enters the lower glycolytic pathway (Gombert et al., 2001). In Crabtree negative yeasts like *Pichia stipitis*, the flux through the PP pathway is generally higher than in Crabtree positive yeasts like *S. cerevisiae* (Bruinenberg et al., 1983; Papini et al., 2012; Siso et al., 1996). Due to the relatively low catabolic fluxes through this pathway in Crabtree positive yeasts, the PP pathway is believed to be used mainly for NADPH production but not for biomass production or catabolic reactions (Blank and Sauer, 2004). The major sources of cytosolic NADPH production in *S. cerevisiae* grown on glucose has been attributed to the glucose-6-phosphate dehydrogenase reaction in the PP pathway, with the cytosolic aldehyde dehydrogenase (Ald6p) as a secondary source (Grabowska and Chelstowska, 2003), and the flux through the PP pathway is therefore directly related to NADPH production. The availability of NADPH influences the production of several biotechnologically relevant compounds such as $\alpha$-santalene (Scalcinati et al., 2012) and polyhydroxybutyrate (PHB) (Carlson and Srienc, 2006; Dawes and Senior, 1973), and it is therefore often necessary to engineer the central carbon metabolism such that there is increased supply of NADPH.

In this study, the PHB biosynthesis pathway (Fig. 1) including $\beta$-ketothiolase (PhaA), NADPH-dependent acetoacetyl-CoA

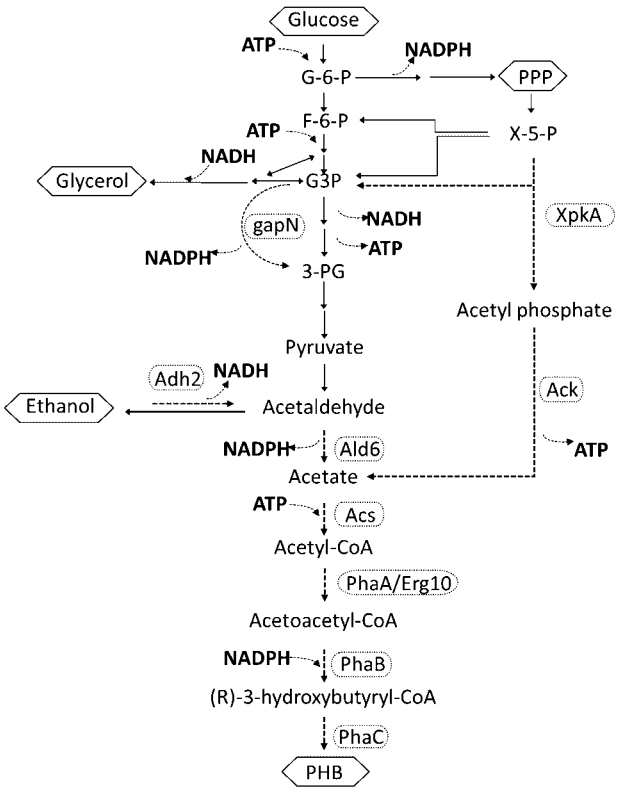


**Figure 1.** Schematic pathway representing metabolic engineering strategies for improving PHB production in *S. cerevisiae*. PPP, pentose phosphate pathway; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; G3P, glyceraldehyde-3-phosphate; 3-PG, 3-phosphoglycerate; PHB, polyhydroxybutyrate. Dashed arrows represent engineered pathway steps.

reductase (PhaB), and polyhydroxyalkanoate synthase (PhaC) was introduced into *S. cerevisiae*. In the PHB biosynthesis pathway, thiolase activity is mediated through the AcCoA/CoA SH ratio and the acetoacetyl-CoA reductase activity is mediated by the NADPH/$NADP^+$ ratio, and these two ratios are therefore considered as the major regulators influencing PHB production in a native PHB producer, *Ralstonia eutropha* (Leaf and Srienc, 1998). Earlier, a mathematical model of recombinant *S. cerevisiae* expressing the PHB pathway from *R. eutropha* was constructed by Carlson et al. (2002) in order to investigate the possible modes of PHB production in *S. cerevisiae*. From analysis of the model it was suggested that the theoretical yield of PHB in *S. cerevisiae* can be improved by including a combination of ATP citrate-lyase to supply acetyl-CoA and a transhydrogenase reaction to catalyze the interconversion of NADH and $NADP^+$ to $NAD^+$ and NADPH (Carlson et al., 2002).

Increasing the production of cytosolic to acetyl-CoA by over-expressing enzymes of the pathway from ethanol to acetyl-CoA was recently shown to improve PHB production in *S. cerevisiae* (Kocharin et al., 2012). An alternative route for providing cytosolic acetyl-CoA through expression of the phosphoketolase pathway from *Aspergillus nidulans* was recently shown to be able to shunt carbon through the PP pathway and contribute to acetyl-CoA production in *S. cerevisiae* (Papini et al., 2012). This strategy is attractive as it involves both increased production of NADPH and possible increased supply of cytosolic acetyl-CoA, and in this study we therefore investigated the effect on PHB production of heterologous expression of xylulose-5-phosphate phosphoketolase (XpkA) (EC: 4.1.2.9) and acetate kinase (Ack) (EC: 2.7.2.1) from *A. nidulans* and an acetyl-CoA synthetase variant ($Acs^{L641P}$) from *Salmonella enterica* on a single episomal plasmid.

We also evaluated the combination of our earlier described strategy for increased cytosolic acetyl-CoA supply with engineering of the central carbon metabolism to increase production of NADPH. For this we introduced a non-phosphorylating, $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase encoded by *gapN* from *Streptococcus mutans*, which has earlier been shown to increase ethanol yields on glucose under anaerobic conditions and at the same time reduce glycerol production (Bro et al., 2006). Finally, we also combined the two strategies for improving PHB production in *S. cerevisiae*.

## Materials and Methods

### Strains, Media, and Culture Conditions

Plasmids were maintained and propagated in *E. coli* DH5α. The preparation of competent *E. coli* cells and their transformation were performed according to standard protocols (Sambrook and Russell, 2006). Luria-Bertani medium was used for routine culturing with 80 mg/L ampicillin. *S. cerevisiae* strain CEN.PK113-11C (*MATa SUC2 MAL2-8$^c$ ura3–52 his3-Δ1*; provided by P. Kötter, University of Frankfurt, Germany) was used as the background strain for evaluation of the PHB pathway. Engineered yeast strains were selected on synthetic dextrose medium, prepared with 6.7 g/L yeast nitrogen base without amino acids (YNB-AA) (Formedium, Hunstanton, UK) and 20 g/L glucose with complete supplement mixture (CSM) lacking uracil and/or histidine (Formedium) where appropriate.

### Plasmid Construction and Yeast Transformation

Plasmid pIYC08 carries genes from the ethanol degradation pathway aiming for increased supply of cytosolic acetyl-CoA. Briefly, pIYC08 contains genes for alcohol dehydrogenase (*ADH2*) under control of the $P_{HXT7}$ promoter, acetaldehyde dehydrogenase (*ALD6*) under control of the $P_{PGK1}$ promoter while acetyl-CoA acetyltransferase (*ERG10*) and acetyl-CoA synthetase variant ($acs^{L641P}$) from *S. enterica* are controlled by the $P_{TEF1}$ promoter. The detailed construction of pIYC04 as a background plasmid and

pIYC08 has been described by Chen et al. (2013). The PHB biosynthesis pathway genes *phaA*, *phaB*, and *phaC* were synthesized based on the genes from *R. eutropha* H16 and *gapN* encoding non-phosphorylating $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase was synthesized based on the gene from *S. mutans* by DNA 2.0 (Menlo Park, CA). The gene *xpkA* encoding a phosphoketolase and the gene *ack* encoding acetate kinase were synthesized based on the sequence from *A. nidulans* by GenScript (Piscataway, NJ). All genes were codon optimized for optimal expression in *S. cerevisiae*. The PHB biosynthesis pathway was introduced into the *S. cerevisiae* strain CEN.PK 113-11C by using a multi-copy plasmid, pKK001, based on pSP-GM2 with a $P_{TEF1}$-$P_{PGK1}$ bidirectional promoter (Partow et al., 2010). The detailed construction of pKK001 has been described (Kocharin et al., 2012).

Plasmid pJC7 contains genes responsible for the utilization of the intermediate xylulose-5-phosphate from the pentose phosphate pathway to supply acetyl-CoA as a precursor for PHB production. Two of the genes, *xpkA* and *ack*, were used to construct the vector pMPa2 (Papini et al., 2012; Partow et al., 2010). The *URA3* marker of pMPa2 was changed to *HIS3* derived from pESC-HIS (Agilent Technologies, Santa Clara, CA) through restriction with *Mfe*I and *Sac*I and subsequent ligation (Gionata Scalcinati, pers. commun.). The resulting plasmid, pIGS11, was verified by PCR using the HIS-check primers (Supplementary Table I). Plasmid pIGS11 was further ligated with the PCR amplicon of the $Acs^{L641P}$ expression cassette derived from pIYC08 using primers JC7-ACS1/JC7-ACS2 and cloned into the *Mfe*I site (Jichen Bao, pers. commun.). The resulting plasmid was named pJC7.

Plasmid pJEF1105 containing the bacterial *neo*$^r$ gene was kindly provided by Dr. Jef D. Boeke (Boeke et al., 1988). Plasmid pBluescript SK II (+) (Stratagene, La Jolla, CA) was digested with *Aat*II and ligated with the sequence of the *his3Δ1* allele, which had been amplified from genomic DNA of *S. cerevisiae* CEN.PK 113-11C using primers KK001/KK002. All primers used for strain construction are listed in Supplementary Table I. The resulting plasmid, pBluSKP-his, was used as template for PCR amplification with primers KK003/KK004 to obtain a fragment containing origin of replication, ampicillin-resistance marker and the *his3Δ1* allele with restriction sites for *Kpn*2I and *Mfe*I at the respective ends. Plasmid pSP-GM2 was digested with *Kpn*2I and *Mfe*I and ligated with the PCR fragment derived from pBluSKP-his. The resulting plasmid, pSP-GM2-his, was digested with *Asc*I and *Mfe*I and ligated with the *neo*$^r$ marker, which had been isolated from plasmid pJEF1105 using primers KK005/KK006, generating pCIChE-KK001. pCIChE-KK001 was further digested with *Asc*I and ligated with a second *his3Δ1* sequence amplified from the same source of genomic DNA using primers KK078/KK079. The derived plasmid, pCIChE-KK004, was used as an integration plasmid for introducing *gapN* into the chromosome. Yeast transformation was performed by using the lithium acetate/single-stranded carrier DNA/polyethylene glycol method (Gietz and Woods, 2002). *S. cerevisiae* transformation with the integration cassette was performed with the following modifications. pCIChE-KK004-*GAPN* was linearized with *Ale*I and *Sal*I prior the yeast transformation. Single-stranded salmon sperm DNA (50 μg per transformation) was added together with 5–10 μg of linearized integrative plasmid, and the heat shock time at 42°C was extended from 5 to 15 min. After the heat shock, the cell suspension (ca. 0.4 mL) was mixed with 2.7 mL of YPD medium and incubated at 30°C for 18 h with shaking; 50–100 μL of the culture were then plated on a selective YPD-G418 plate (0.1 g/L G418). All *gapN* integration strains were verified by PCR with primers KK039/KK082 and KK083/KK042. Strains used in this study are summarized in Table I. The schematic metabolic pathway and plasmid maps are illustrated in Figures 1 and 2, respectively.

## Shake Flask Cultivation

The pre-cultures for shake flask cultivations were prepared by inoculation of 5 mL modified minimal media in a 14 mL culture tube and grown at 30°C and180 rpm in an orbital shaking incubator. The modified minimal medium for shake flask cultivations was prepared as follows (per liter): $(NH_4)_2SO_4$, 7.5 g; $KH_2PO_4$, 14.4 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; 2 mL of trace metal solution and 1 mL of vitamin solution with an initial pH of 6.5. Glucose was autoclaved separately from the minimal medium and later added to media at a

**Table I.** Yeast strains used in this study.

| Strain | Genotype or relevant feature(s) | Plasmid | Source |
|---|---|---|---|
| CEN.PK 113-11C | *MATa SUC2 MAL2-8*$^c$ *ura3-52 his3-Δ1* | — | P. Kötter[a] |
| SCKK005 | *MATa SUC2 MAL2-8*$^c$ *ura3-52 his3-Δ1* | pIYC04/pKK01 | Kocharin et al. (2012) |
| SCKK006 | *MATa SUC2 MAL2-8*$^c$ *ura3-52 his3-Δ1* | pIYC08/pKK01 | Kocharin et al. (2012) |
| SCKK032 | *MATa SUC2 MAL2-8*$^c$ *ura3-52 his3-Δ1* | pJC7/pKK01 | This study |
| SCKK033 | *MATa SUC2 MAL2-8*$^c$ *ura3-52 his3-Δ1::gapN*[b] | pIYC04/pKK01 | This study |
| SCKK034 | *MATa SUC2 MAL2-8*$^c$ *ura3-52 his3-Δ1::gapN* | pIYC08/pKK01 | This study |
| SCKK035 | *MATa SUC2 MAL2-8*$^c$ *ura3-52 his3-Δ1::gapN* | pJC7/pKK01 | This study |
| SCKK036 | *MATa SUC2 MAL2-8*$^c$ *ura3-52 his3-Δ1::gapN* | | This study |

[a]Institute of Microbiology, J.W. Goethe Universität, Frankfurt, Germany.
[b]*gapN* here represents the integration cassette including *neo*$^r$ and *gapN* under control of the *PGK1* promoter and *ADH1* terminator.

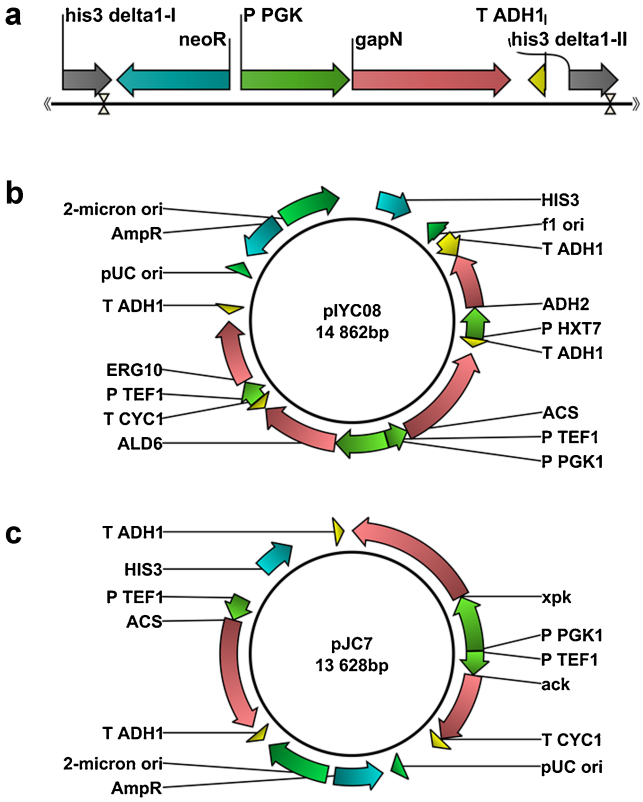


**Figure 2.** Plasmid maps. (**a**) Integration cassette derived from pCIChE-KK004-GAPN containing the *his3-Δ1* allele at both ends of the cassette; (**b**) plasmid pJC7 containing genes for xylulose-5-phosphate phosphoketolase (*xpkA)*, acetate kinase (*ack*), and acetyl-CoA synthetase ($Acs^{L641P}$); (**c**) plasmid pIYC08 containing genes for alcohol dehydrogenase (*ADH2*), aldehyde dehydrogenase (*ALD6*), acetyl-CoA synthetase ($Acs^{L641P}$) variant, and acetyl-CoA acetyltransferase (*ERG10*).

concentration of 20 g/L. The trace metal solution consisted of the following (per liter): EDTA (sodium salt), 15 g; $ZnSO_4 \cdot 7H_2O$, 0.45 g; $MnCl_2 \cdot 2H_2O$, 1 g; $CoCl_2 \cdot 6H_2O$, 0.3 g; $CuSO_4 \cdot 5H_2O$, 0.3 g; $Na_2MoO_4 \cdot 2H_2O$, 0.4 g; $CaCl_2 \cdot 2H_2O$, 0.45 g; $FeSO_4 \cdot 7H_2O$, 0.3 g; $H_3BO_3$, 0.1 g; and KI, 0.1 g. The pH of the trace metal solution was adjusted to 4.0 with 2 M NaOH. The vitamin solution contained (per liter): biotin, 0.05 g; ρ-amino benzoic acid, 0.2 g; nicotinic acid, 1 g; Ca-pantothenate, 1 g; pyridoxine–HCl, 1 g; thiamine–HCl, 1 g; and myo-inositol, 25 g. The pH of the vitamin solution was adjusted to pH 6.5 prior filter sterilization. Histidine and uracil stock solutions were filter sterilized and added to the medium when needed at the final concentration of 20 mg/L of each amino acid. For anaerobic cultivations, the medium was supplemented with 420 mg/L Tween 80 and 10 mg/L ergosterol, which were first dissolved in boiling pure ethanol. The supplementation of Tween 80 and ergosterol is necessary for anaerobic growth of *S. cerevisiae* (Verduyn et al., 1990). Anaerobic shake flasks were sealed with a rubber stopper equipped with a U-tube containing water to ensure anaerobic conditions. After inoculation and each sampling, the flasks were sparged with nitrogen for 5 min prior to the incubation.75 mL of modified minimal medium in a 250-mL unbaffled flask were inoculated with an amount of pre-culture that resulted in a final optical density of 0.02 at 600 nm ($OD_{600}$). The culture was grown at 30°C and 180 rpm in an orbital shaking incubator. Samples were taken to determine biomass, extracellular metabolites and PHB concentration.

### Cell Mass Determination

Culture samples of 10 mL volume were centrifuged at 5,000 rpm and 4°C for 5 min and the pellets were washed once with distilled water and centrifuged at 14,000*g* for 1 min. To lyophilize the biomass, the recovered cell pellet was immediately frozen by immersion in liquid nitrogen followed by lyophilization under vacuum (Christ Alpha 2–4 LSC, Shropshire, UK). The dry cell weight was determined and the pellet kept at 4°C for further analysis.

### Metabolite Analysis

Glucose and extracellular metabolites including ethanol, glycerol, and acetate were quantified in the culture supernatant using an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA) equipped with an Aminex HPX 87H ion exclusion column (300 mm × 7.8 mm, Bio-Rad Laboratories, Hercules, CA) which was operated at 45°C and a flow rate of 0.6 mL/min of 5 mM $H_2SO_4$ using a refractive index detector and UV detector for analysis of sugars and organic acids, respectively.

PHB was analyzed as described previously (Karr et al., 1983; Tyo et al., 2006); 10–20 mg of dried cells were weighed and boiled in 1 mL of concentrated sulfuric acid for 60 min and then diluted with 4 mL of 14 mM $H_2SO_4$. Samples were centrifuged (15 min, 16,000*g*) to remove cell debris, and the supernatant was analyzed using an Ultimate 3000 HPLC (Dionex) equipped with an Aminex HPX-87H ion exclusion column (300 × 7.8 mm; Bio-Rad Laboratories) and UV detector. Commercially available PHB (Sigma-Aldrich, St. Louis, MO), processed in parallel with the samples, was used as a standard. The HPLC was operated at 60°C and a flow rate of 0.6 mL/min of 5 mM $H_2SO_4$.

## Results

### Genomic Integration of *gapN*

In our earlier work on increasing acetyl-CoA supply by overexpression the ethanol degradation pathway we found that the glycerol yield on glucose had increased (Kocharin et al., 2012). Glycerol is produced as a way of handling excess NADH, and as a strategy to reduce glycerol production and improve PHB production in *S. cerevisiae* the bacterial *gapN* gene encoding non-phosphorylating $NADP^+$-dependent

glyceraldehyde-3-phosphate dehydrogenase was introduced into the yeast genome. The gene was integrated at the *his3-Δ1* allele of strain CEN.PK 113-11C under control of the strong constitutive *PGK1* promoter. When *S. cerevisiae* is grown under anaerobic cultivation, apart from biomass, three major metabolites are produced from glucose: $CO_2$, ethanol, and glycerol. Therefore, in order to investigate the effect of *gapN* expression on the growth behavior of *S. cerevisiae*, SCKK036 (harboring *gapN*) and the parental strain, CEN.PK 113-11C, were characterized in anaerobic shake flask cultivation. The growth curves of both strains are shown in Figure 3. SCKK036 showed a growth pattern similar to the parental strain with a specific growth rate of 0.38/h and a slightly higher ethanol yield on glucose (Table II). The major difference between SCKK036 and the parental strain was a 45% reduction in glycerol production by the strain expressing *gapN*, which is consistent with earlier findings (Bro et al., 2006). This reduction is due to the by-pass of the two reactions from glyceraldehyde-3-phosphate to 3-phospho-D-glycerate resulting in reduced NADH production in the EMP pathway (replaced by production of NADPH).

PHB production in the strain with chromosomal *gapN* integration, SCKK033, was evaluated in aerobic shake flask cultivation and compared to SCKK005 harboring the PHB pathway without *gapN* integration. The kinetic parameters are reported in Table III. A decrease in the specific growth rate was observed in SCKK033 compared to SCKK005. There was no significant difference in the glycerol yield on glucose. However, the ethanol yield on glucose in SCKK033 was higher than in SCKK005. Due to the fact that PHB is mainly produced during growth on ethanol, the yield of PHB on substrate was calculated to be much lower during growth on glucose compared to the ethanol consumption phase. The effect of *gapN* integration on PHB production was clearly observed as the PHB yields on glucose and on ethanol of SCKK033 were substantially higher than for SCKK005 (Table III). The maximum cellular PHB content obtained at 100 h in SCKK033, 14.87 mg/gDW, was 3.7 times higher than in the reference strain, SCKK005.

**Table II.** Yields and specific growth rates of the parental strain CEN.PK 113-11C and the recombinant strain SCKK036 with *gapN* integration.

| Strain | CEN.PK 113-11C | SCKK036 |
|---|---|---|
| $\mu_{max}$ (per h) | $0.39 \pm 0.00$ | $0.38 \pm 0.01$ |
| $Y_{SEtOH}$(g/g glc) | $0.50 \pm 0.02$ | $0.53 \pm 0.03$ |
| $Y_{SGly}$ (g/g glc)* | $0.10 \pm 0.01$ | $0.05 \pm 0.01$ |

The values reported were calculated for the exponential growth phase during anaerobic shake flask cultivation in minimal media with 20 g/L glucose as sole carbon source. These values were calculated from at least triplicate shake flasks ($n \geq 3$) and are represented as mean ± SD.

*The values are significant difference at $P$-value $\leq 0.05$.

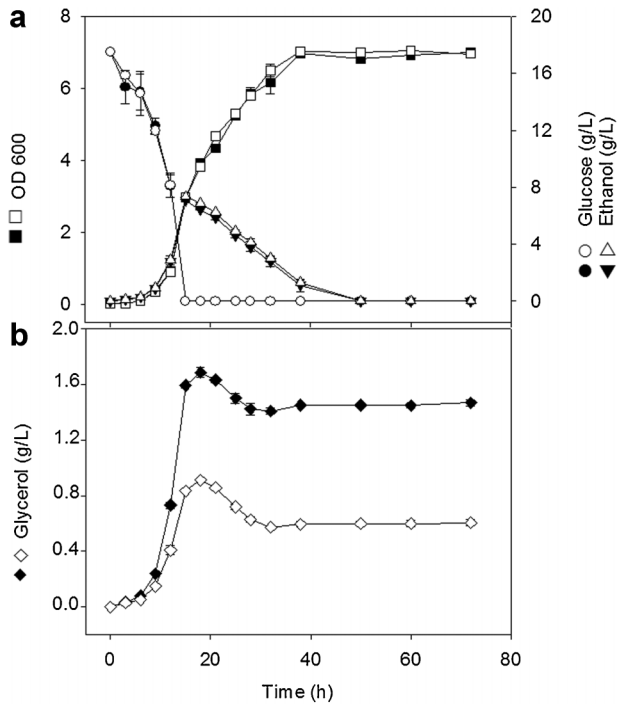


**Figure 3.** Fermentation profile of *S. cerevisiae* CEN.PK 113-11C and SCKK036 during growth in anaerobic shake flask cultivation. Closed and open symbols represent the reference strain (CEN.PK 113-11C) and the reference strain with *gapN* integration (SCKK036), respectively.

## Alternative Pathway for Acetyl-CoA Supply

Heterologous expression of the phosphoketolase pathway from *A. nidulans* enables the utilization of xylulose-5-phosphate, an intermediate from the PP pathway, to be converted to acetyl-CoA via acetyl phosphate (acetyl-P). In order to investigate the possibility of employing the phosphoketolase pathway compared with the ethanol degradation pathway for increasing cytosolic acetyl-CoA concentration and improving PHB production, strain characterization was carried out in aerobic shake flask cultivations. The kinetic parameters are reported in Table III. The strain that carries the phosphoketolase pathway, SCKK032, showed an improved PHB production not only compared with the reference strain but also compared with SCKK006 that over-expressed the ethanol degradation pathway to increase cytosolic acetyl-CoA supply as reported previously (Kocharin et al., 2012). SCKK032 showed a decrease in the specific growth rate, 0.21/h, as well as the biomass yield on glucose compared with SCKK005 as a reference strain and SCKK006. The lower biomass yield in SCKK006 and SCKK032 is associated with a higher glycerol yield on glucose. Although the PHB yield on glucose in SCKK032 was lower than SCKK006 the PHB yield on ethanol in SCKK032 was higher than SCKK006. This resulted in a cellular PHB content in SCKK032 at 100 h of 27.86 mg/g DW.

**Table III.** Physiological parameters obtained during growth on minimal media with 20 g/L glucose in shake flask cultivations.

| | Strains without *gapN* integration | | | Strains with *gapN* integration | | |
|---|---|---|---|---|---|---|
| Strain | SCKK005 | SCKK006 | SCKK032 | SCKK033 | SCKK034 | SCKK035 |
| $\mu_{max}$ (per h) | $0.27 \pm 0.02$ | $0.28 \pm 0$ | $0.21 \pm 0.01$ | $0.22 \pm 0$ | $0.23 \pm 0.02$ | $0.22 \pm 0.02$ |
| $r_s$ (g/g DW · h) | $1.80 \pm 0.09$ | $2.24 \pm 0.33$ | $2.00 \pm 0$ | $1.37 \pm 0$ | $1.31 \pm 0.18$ | $1.30 \pm 0.12$ |
| $Y_{SX}$ (g/g glc) | $0.15 \pm 0.01$ | $0.13 \pm 0.02$ | $0.11 \pm 0$ | $0.16 \pm 0$ | $0.18 \pm 0.02$ | $0.17 \pm 0.02$ |
| $Y_{SEtOH}$ (g/g glc) | $0.35 \pm 0.05$ | $0.35 \pm 0.07$ | $0.39 \pm 0.01$ | $0.48 \pm 0.01$ | $0.35 \pm 0.01$ | $0.43 \pm 0$ |
| $Y_{SGly}$ (g/g glc) | $0.05 \pm 0$ | $0.07 \pm 0$ | $0.07 \pm 0$ | $0.04 \pm 0.01$ | $0.05 \pm 0.01$ | $0.04 \pm 0$ |
| $Y_{SPHB}$ (mg/g glc) | $0.02 \pm 0.01$ | $0.13 \pm 0.02$ | $0.03 \pm 0$ | $0.27 \pm 0.01$ | $0.12 \pm 0$ | $0.06 \pm 0$ |
| $Y_{EtOH\cdot PHB}$ (mg/g EtOH) | $0.22 \pm 0.04$ | $6.09 \pm 1.44$ | $56.40 \pm 0.77$ | $16.11 \pm 2.38$ | $14.50 \pm 2.90$ | $12.48 \pm 0.81$ |
| PHB content at 100 h (mg/g DW) | $4.02 \pm 0.16$ | $15.89 \pm 0$ | $27.86 \pm 0$ | $14.87 \pm 0.4$ | $27.52 \pm 4.82$ | $21.41 \pm 0.69$ |

These values were calculated from at least triplicate shake flasks ($n \geq 3$) and are represented as mean $\pm$ SD. The values for SCKK005 and SCKK006 are taken from a previous study (Kocharin et al., 2012).

All strains listed in this table harbor the PHB plasmid (pKK01). SCKK005 and SCKK032 carry pKK001 and pIYC04. SCKK032 and SCKK035 express the phosphoketolase pathway. SCKK006 and SCKK034 overexpress the ethanol degradation pathway.

$\mu_{max}$, maximum specific growth rate on glucose; $r_s$, specific glucose consumption; $Y_{SX}$, biomass yield on substrate (glucose); $Y_{SEtOH}$, ethanol yield on substrate (glucose); $Y_{SGly}$, glycerol yield on substrate (glucose); $Y_{SPHB}$, PHB yield on substrate (glucose); $Y_{EtOH\cdot PHB}$, PHB yield on ethanol.

## Combined Effect of Cofactor Enhancement and Pathway Engineering

The combination of integrating *gapN* into the genome and the utilization of the acetyl-CoA boost strategies to improve PHB production in *S. cerevisiae* was characterized in aerobic shake flask cultivation and physiological parameters of SCKK033, SCKK034, and SCKK035 were calculated. The values are shown in Table III. Figure 4 shows the concentration of PHB measured at three different time points, 50, 75, and 100 h of cultivation. In general, strains engineered to have an increased supply of acetyl-CoA, SCKK006, SCKK032, SCKK034, and SCKK035, showed an improved PHB production compared to the respective reference strains, SCKK005 and SCKK033. The maximum specific growth rates for all strains used in this experiment, range from 0.21 to 0.28/h depending on the heterologous pathway used to manipulate cytosolic acetyl-CoA supply and *gapN* integration into the genome (Table III). There was no difference in the specific growth rate when *gapN* was integrated into the genome of SCKK035 expressing the phosphoketolase pathway whereas *gapN* integration resulted in a decrease in the specific growth rate of a strain that utilizes the ethanol degradation pathway to supply cytosolic acetyl-CoA, SCKK034. The glucose consumption rate in strains without *gapN* integration was higher than in the strains with *gapN* integration. Among the *gapN* integration strains, SCKK034 showed the lowest ethanol yield on glucose, 0.35 g/g glc, due to the over-expression of genes from the ethanol degradation pathway in this strain. A slight decrease in the glycerol yield on glucose was observed for all strains expressing *gapN*. A positive effect of *gapN* expression on the PHB yield was clearly observed in strains that do not carry any acetyl-CoA boost plasmids. A more than tenfold increase in PHB yield on glucose was observed in SCKK033 compared to SCKK005, producing $0.27 \pm 0.01$ and $0.02 \pm 0.01$ g/g glc, respectively. However, only a twofold higher PHB yield on glucose was observed in SCKK035 compared with SCKK032 whereas no effect of *gapN* integration on $Y_{sPHB}$ was observed in strains with over-expression of the ethanol degradation pathway, SCKK006 and SCKK034. The combination of *gapN* integration and the ethanol degradation pathway was able to improve the yield of PHB on ethanol from $6.09 \pm 1.44$ to $14.50 \pm 2.9$ mg/g EtOH in SCKK034. The combination of *gapN* integration and expression of the phosphoketolase pathway, however, revealed an antagonistic effect on PHB production on ethanol. The strain without *gapN* integration but expressing the phosphoketolase pathway (SCKK032) showed the highest yield of PHB on ethanol, 56.4 mg/g EtOH, which decreased to 12.5 mg/g EtOH when the phosphoketolase pathway was combined with *gapN* integration.

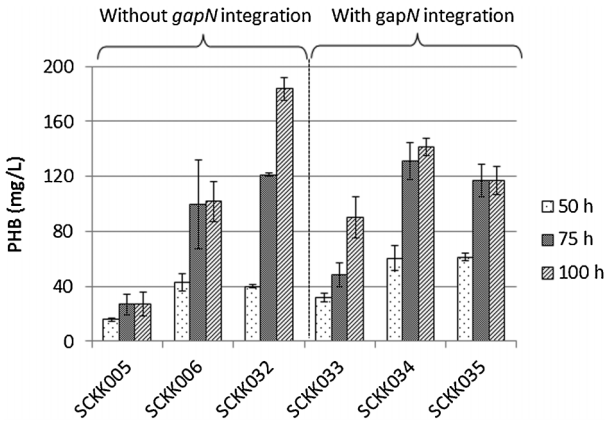


**Figure 4.** Polyhydroxybutyrate production in *S. cerevisiae* employing different strategies to improve PHB production. SCKK005 and SCKK033 harbor only the PHB plasmid (pKK01). SCKK032 and SCKK035 carry pJC7 (phosphoketolase pathway) whereas SCKK006 and SCKK034 carry pIYC08 (ethanol degradation pathway). Samples for PHB measurement were taken at 50 h (the glucose phase), 75 h (the ethanol phase), and 100 h (the end of fermentation where all the glucose and ethanol were depleted).

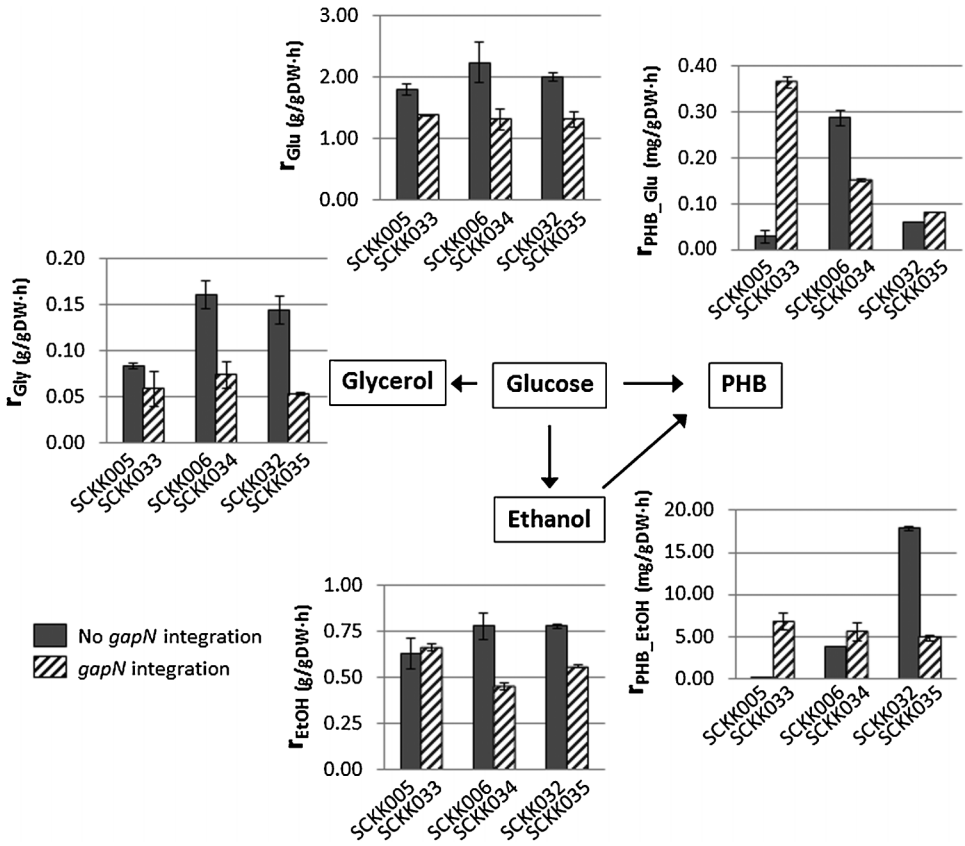


**Figure 5.** Specific productivity of ethanol, glycerol, and PHB obtained from shake flask cultivations. The specific product formation rates were calculated by using the equation: $r_p = \mu_{max} Y_{sp}/Y_{sx}$. $r_{EtOH}$, specific productivity of ethanol; $r_{Gly}$, specific productivity of glycerol; $r_{PHB\_Glu}$, specific productivity of PHB in the glucose consumption phase; $r_{PHB\_EtOH}$, specific productivity of PHB in the ethanol consumption phase.

Figure 5 shows the glucose consumption rate, the specific productivity of ethanol, glycerol, and PHB during growth on glucose and the specific productivity of PHB during growth on ethanol. All the strains with *gapN* integration showed a decrease in glucose consumption rate compared to the non-*gapN* integration strains. Furthermore, a noticeable decrease in the glycerol flux was observed in the *gapN* integration strains especially in SCKK034 and SCKK035, which revealed an about 50% reduction in the glycerol flux compared to their respective reference strains. Integration of *gapN* has previously been investigated for improving the production of bioethanol by reducing the flux towards glycerol, however, in this study when *gapN* was integrated into the PHB strains (SCKK034 and SCKK035) with the aim to enhance acetyl-CoA production, it resulted in a decrease in the ethanol flux compared to the non-integrated strains. The lowest flux towards ethanol seen for SCKK034 was probably caused by the over-expression of the ethanol degradation pathway, while the lower ethanol flux in SCKK035 might be due the redirection of carbon flux from the EMP pathway to the PP pathway and further to the phosphoketolase pathway. The highest specific productivity of PHB during growth on glucose was obtained for the strain with *gapN* integration and without any acetyl-CoA boost plasmid (SCKK033) whereas the strain without *gapN* integration and with the reconstructed phosphoketolase pathway as the alternative route to produce acetyl-CoA and NADPH (SCKK032) gave the highest specific productivity of PHB during growth on ethanol.

## Discussion

The biosynthesis of PHB in *S. cerevisiae* is initiated by the condensation of two acetyl-CoA molecules to acetoacetyl-CoA that is subsequently reduced by an NADPH-dependent acetoacetyl-CoA reductase resulting into the monomer, 3-hydroxybutyryl-CoA, which is polymerized by polyhydroxyalkanoate synthase yielding PHB. In a previous study, we increased acetyl-CoA supply through over-expression of the genes of the ethanol degradation pathway and showed that this can improve the specific productivity of PHB in *S. cerevisiae* (Kocharin et al., 2012). Here, we investigated an alternative pathway to increase acetyl-CoA supply with the objective to further improve PHB production. We found that by reconstruction of the phosphoketolase pathway from

*A. nidulans* in combination with expression of an acetyl-CoA synthetase variant ($Acs^{L641P}$) from *S. enterica* enabled efficient supply of cytosolic acetyl-CoA required as a precursor for PHB production. A further advantage of using this route via the PP pathway is that excess NADPH is generated, which is needed for PHB biosynthesis. While the strain expressing the heterologous phophoketolase pathway gave the highest PHB production, it showed a decrease in both the specific growth rate and the biomass yield compared with the strain over-expressing the genes in the ethanol degradation pathway to increase the acetyl-CoA supply. This might be due to the split of carbon from the EMP to the PP pathway that likely may result in growth attenuation and reduced biomass yield on glucose. A reduction in the biomass yield of *S. cerevisiae* expressing the phosphoketolase pathway was also observed in previous studies (Papini et al., 2012; Sonderegger et al., 2004).

In the present study, we also investigated the supply of NADPH as a major cofactor required in the reaction catalyzed by NADPH-dependent acetoacetyl-CoA reductase in the PHB biosynthesis pathway. We introduced the bacterial *gapN* gene into the yeast's genome aiming to take advantage of the NADPH formed during the catalytic activity of GapN to increase the production of NADPH and thereby improve PHB production. The effect of increased NADPH supply obtained by *gapN* integration is clearly observed in the strain harboring only the PHB production plasmid, SCKK033, as there was observed a 13-fold increase in PHB yield on glucose and a 70-fold increase in the PHB yield on ethanol. The PHB content at 100 h was increased 3.7 times, from 4 to 14.87 mg/g DW. Besides influencing the redox metabolism by regenerating NADPH, integration of *gapN* may also result in altered carbon fluxes as expression of *gapN* alone results in an increased flux towards ethanol and a reduced flux towards glycerol. However, we believe that integration of *gapN* into strains carrying the PHB biosynthesis pathway helps increase the flux toward PHB production primarily by supplying more NADPH as there was no significant difference in the specific productivity of glycerol and ethanol in SCKK005 and SCKK033 (Fig. 5). Therefore, we believed that *gapN* integration in this study mainly plays a role in production of NADPH required for PHB biosynthesis.

In order to investigate the combined effect of precursor and cofactor supply for improving PHB production in *S. cerevisiae*, we combined over-expression of either the ethanol degradation pathway or the reconstructed phosphoketolase pathway, to increase the supply of cytosolic acetyl-CoA, with *gapN* integration into the chromosome. It is evident that the strategy to enhance acetyl-CoA supply via the ethanol degradation pathway together with provision of NADPH via GapN promotes the production of PHB in *S. cerevisiae* as revealed in the improved PHB production by SCKK034 compared to SCKK033 and SCKK006, respectively (Fig. 3). Moreover, a reduced glycerol yield on glucose was observed as a positive side effect of *gapN* integration. This result suggests that supply of both precursor (acetyl-CoA) and cofactor (NADPH) are important to improve PHB production in *S. cerevisiae*. However, for the yeast strain carrying the reconstructed phosphoketolase pathway to generate acetyl-CoA supply there was no benefit of *gapN* expression as the resulting strain gave an amount of PHB at 75 h of cultivation comparable with the strain not having *gapN* integrated. The reason that no improvement in PHB production was observed in SCKK035 compared with SCKK032 might be due to the lack of a mitochondrial NADPH dehydrogenase in *S. cerevisiae* which otherwise couples the oxidation of cytosolic NADPH to the mitochondrial respiratory chain. Therefore, a direct oxidation of a surplus NADPH generated from GapN and the PP pathway in the respiration chain is not possible (Bruinenberg, 1986; Siso et al., 1996). Furthermore, an increased production of NADPH by the EMP pathway may result in a reduced flux through the PP pathway as the glucose-6-phosphate dehydrogenase flux is very sensitive to the NADP/NADPH ratio (Vaseghi et al., 1999).

From the results in this study, the strain that exhibited the best performance for PHB production was SCKK032 which harbors only the PHB production plasmid and expresses the heterologous phosphoketolase pathway, which may point to that provision of acetyl-CoA is important for ensuring a high PHB production. When *S. cerevisiae* is grown on glucose, the PP pathway is the major source for NADPH production, specifically the dehydrogenase activity during the early steps in the pathway (Grabowska and Chelstowska, 2003; Minard and McAlister-Henn, 2005). Therefore, an increased carbon flux through the PP pathway has a direct consequence on the supply of NADPH required in various anabolic reactions and of certain anabolic precursors (Frick and Wittmann, 2005). For this reason, a noticeable high yield of PHB on ethanol was observed for the strain that can generate acetyl-CoA and NADPH simultaneously through the PP pathway, SCKK032. These results strongly support our hypothesis that the improved PHB production in *S. cerevisiae* required both sufficient precursor and cofactor supply.

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