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A modified pathway for the production of acetone in *Escherichia coli*

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ABSTRACT

A modified synthetic acetone operon was constructed. It consists of two genes from *Clostridium acetobutylicum* (*thlA* coding for thiolase and *adc* coding for acetoacetate decarboxylase) and one from *Bacillus subtilis* or *Haemophilus influenzae* (*teII*$_{srf}$ or *ybgC*, respectively, for thioesterase). Expression of this operon in *Escherichia coli* resulted in the production of acetone starting from the common metabolite acetyl-CoA via acetoacetyl-CoA and acetoacetate. The thioesterases do not need a CoA acceptor for acetoacetyl-CoA hydrolysis. Thus, in contrast to the classic acetone pathway of *Clostridium acetobutylicum* and related microorganisms which employ a CoA transferase, the new pathway is acetate independent. The genetic background of the host strains was crucial. Only *E. coli* strains HB101 and WL3 were able to produce acetone via the modified plasmid based pathway, up to 64 mM and 42 mM in 5-ml cultures, respectively. Using glucose fed-batch cultures the concentration could be increased up to 122 mM acetone with HB101 carrying the recombinant plasmid pUC19ayt (thioesterase from *H. influenzae*). The formation of acetone led to a decreased acetate production by *E. coli*.

1. Introduction

In times of increasing oil prices and shortage of fossil resources the biological production of valuable chemicals from biomass is of steadily increasing significance in today's economy. Acetone (also known as propanone or dimethyl ketone) is a colorless, low viscosity liquid widely used as a solvent for various polymers (Sifniades et al., 2011). Global production and consumption of acetone in 2009 was approximately 5.1 million metric tons and it is expected to increase on average by 4.8% per year from 2009 to 2014, declining to 2.5% per year from 2014 to 2019 (WP report, 2010; 〈http://www.sriconsulting.com/WP/Public/Reports/acetone/〉). The largest volume applications of acetone are solvent uses and generation of methyl methacrylate and bisphenol-A, which is used for production of polycarbonate plastics.

The biological formation of acetone and other solvents from renewable sources is not new. Acetone was the first desired product in the classical ABE (acetone, butanol, ethanol) fermentation by *Clostridium acetobutylicum* developed during World War I (Jones and Woods, 1986). In *C. acetobutylicum,* acetone is formed starting from acetyl-CoA by the action of thiolase (encoded by *thlA*), acetoacetyl-CoA:acetate/butyrate CoA-transferase (encoded by *ctfAB*), and acetoacetate decarboxylase (encoded by *adc*). Similar as with the butanol pathway (Nielsen et al., 2009), the acetone pathway can be transferred to other bacteria. Introduction of these genes into *Escherichia coli* resulted in acetone production in comparable amounts as in *C. acetobutylicum* (Bermejo et al., 1998).

The CoA-transferase transfers the CoA moiety from acetoacetyl-CoA to either acetate or butyrate. Thus, its activity requires the availability of one of these acids in their non-dissociated form. Additionally, the CoA transferase from *C. acetobutylicum* has a high K_m of 1200 mM for acetate requiring relatively high concentrations of this acid (Wiesenborn et al., 1989; Bermejo et al., 1998). Thus, it was suggested that a CoA-transferase with a lower K_m for acetate might improve the overall conversion of acetyl-CoA to acetone. Indeed, Hanai et al. (2007) observed higher acetone titers, when the CoA-transferase of *E. coli* (AtoDA, K_m of 53.1 mM) instead of CtfAB from *C. acetobutylicum* was used in the acetone pathway.

Here, we report the construction of a modified acetone pathway, replacing the CoA-transferase by a thioesterase. The thioesterase yields acetoacetate (and CoA-SH) simply by hydrolysis of the thioester acetoacetyl-CoA, independent of the presence of acetate or butyrate (Fig. 1).

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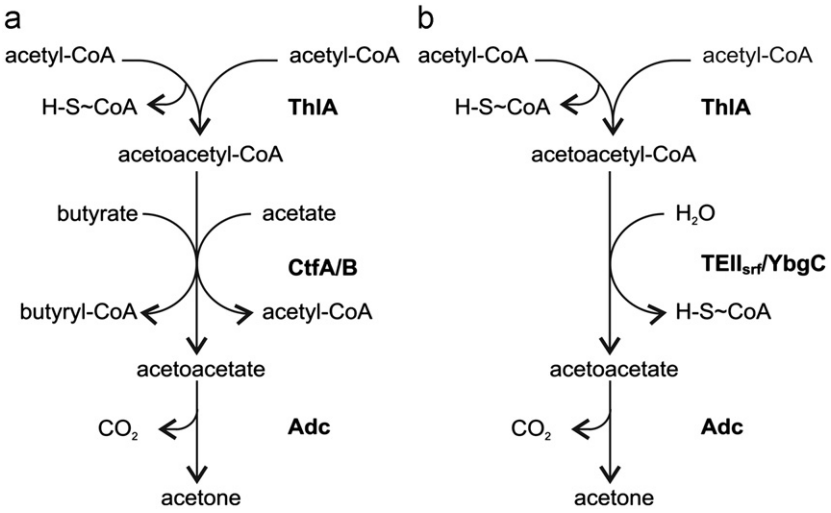

Fig. 1. Pathway for acetone production in *C. acetobutylicum* (a) and newly constructed way with thioesterases $TEII_{srf}$ or YbgC (b) starting from acetyl-CoA. ThlA, thiolase; CtfAB, acetoacetyl-CoA:acetate/butyrate:CoA-transferase; Adc, acetoacetate decarboxylase; $TEII_{srf}$, thioesterase II from *B. subtilis*; YbgC, thioesterase from *H. influenzae*.

Thus, we searched for thioesterases able to hydrolyze acetoacetyl-CoA. Acetoacetyl-CoA hydrolases (EC 3.1.2.11) have been found only in vertebrates so far and are involved in ketogenesis in the liver and synthesis of fatty acids in the cytoplasm (Schomburg et al., 2002; ⟨www.brenda-enzymes.org⟩). The Pfam-Database (Finn et al., 2010; ⟨http://pfam.sanger.ac.uk⟩) comprises the thioesterase (PF00975) and the acyl-CoA thioesterase (PF02551) families. The first includes the thioesterase domain II, which often occurs integrated in or associated with peptide synthetases, which are involved in the non-ribosomal synthesis of peptide antibiotics and have similarity to the type II fatty acid thioesterases (TEII) of vertebrates. Schwarzer et al. (2002) characterized two TEII enzymes from *Bacillus subtilis* that are associated with the synthetases of the peptide antibiotics surfactin ($TEII_{srf}$) and bacitracin ($TEII_{bac}$). For $TEII_{srf}$ the authors could detect a slow hydrolysis of acetyl-CoA. The YbgC protein from *Haemophilus influenza*, which has similarity to a cytoplasmic Tol–Pal system associated protein of *E. coli* (important for cell envelope integrity and part of the cell division machinery), was identified as a member of the acyl-CoA thioesterase family (Zhuang et. al., 2002). This enzyme exhibits a significant activity towards short chain acyl-CoA esters such as propionyl-CoA with K_m values between 11 mM and 24 mM. These results led us to the assumption that both enzymes ($TEII_{srf}$ and YbgC) might also hydrolyze other short chain acyl-CoA esters like acetoacetyl-CoA. Furthermore, the acetoacetyl-CoA synthetase of *Sinorhizobium meliloti* (Aneja et al., 2002) was another promising candidate for our studies, since the reverse reaction would yield acetoacetate from acetoacetyl-CoA without the involvement of acetate or butyrate.

In this study we have analyzed the thioesterase activity of the above mentioned enzymes with acetoacetyl-CoA as substrate and have combined the respective genes of suitable candidates with the *C. acetobutylicum thlA* and *adc* genes to construct a modified acetone synthesis pathway in an inducible synthetic operon. Expression of this operon in *E. coli* resulted in significant acetone production.

2. Materials and methods

2.1. DTNB [5,5′-Dithiobis(2-nitrobenzoic acid)] assay

Hydrolysis of acetyl-CoA and acetoacetyl-CoA as substrates was monitored by measuring the absorbance of 5-thio-2-nitrobenzoate (TNB) at 412 nm, released from the reaction of DTNB with free thiols. As acetoacetyl-CoA shows auto-hydrolysis in assay buffer without enzyme, the rate was measured and subtracted from the assay data with added enzyme.

$TEII_{srf}$ from *B. subtilis* (15 μg) was incubated in a glass cuvette (1 cm) with between 100 μM and 3 mM acetyl-CoA or acetoacetyl-CoA and DTNB (stock solution 10 mM in DMSO; final concentration 0.2 mM) in assay buffer (50 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, pH 7) in a total volume of 400 μl at 37 °C.

Purified YbgC (30 μg) was incubated in a glass cuvette (1 cm) with between 100 μM and 3 mM acetyl-CoA or acetoacetyl-CoA and DTNB (stock solution 10 mM in DMSO; final concentration 0.2 mM) in assay buffer (10 mM K^+–HEPES, 0.2 M KCl, pH 7) in a total volume of 400 μl at room temperature.

2.2. Analysis of fermentation products

The concentrations of acetone and acetate in the cell free supernatant were analyzed by use of a gas chromatograph (CP9001; Chrompack, Frankfurt/Main, Germany) equipped with a flame ionization detector (FID) and a Chromosorb 101 (80/100 mesh, 2.0 m × 2.0 mm) glass column. 1 ml of a 1:10 dilution of the supernatant was acidified with 0.1 ml 2 N HCl containing 55 mM isobutanol as internal standard (final concentration in the sample was 5 mM). For detection 0.5 μl of the sample was injected. Products were analyzed under following conditions: injector temperature, 195 °C; detector temperature, 230 °C; column temperature, 155 °C–195 °C; rate of temperature increase, 9 °C/min; carrier gas, N_2 (flow rate 30 ml/min).

2.3. Measurement of OD

The optical density at 600 nm (OD_{600}) was determined in a spectrophotometer (Spekol 1100; Analytik Jena, Jena, Germany) using plastic cuvettes (1 cm). During growth experiments in 5 ml volume the OD_{600} was measured directly in the test tubes without a dilution. During experiments in larger volumes the taken samples were diluted to an OD_{600} below 0.3 and measured against medium.

2.4. Determination of glucose

Glucose concentration was determined by an optic-enzymatical assay (Bergmeyer, 1983). In a coupled assay using a mixture of the enzymes hexokinase and glucose-6-phosphate dehydrogenase (HK/G6P-DH from yeast/*Leuconostoc spec.*, respectively; Roche Diagnostics,

Mannheim, Germany) NADPH production, which is directly related to glucose concentration, was measured at 340 nm in a spectrophotometer (Ultrospec 3000, Analytik Jena, Jena, Germany). 900 µl assay buffer (0.2 M Tris–HCl, 2 mM $MgSO_4$, pH 7.6) were mixed with 10 µl ATP (96 mg/ml), 10 µl NADP (44 mg/ml) and 10 µl cell free supernatant (diluted as necessary). After mixing and reading the absorbance (blank), 10 µl enzyme solution was added and the absorbance was read after the reaction stopped (approx. 5 min).

2.5. Expression and purification of $TEII_{srf}$

The plasmid $pTEII_{srf}$ containing the $TEII_{srf}$ gene from *B. subtilis* transformed in *E. coli* M15 was kindly provided by M. A. Marahiel (Philipps-Universität Marburg, Germany) (Schwarzer et al., 2002). For expression of the protein, cells were grown in 2 × YT medium (with 100 µg/ml ampicillin and 25 µg/ml kanamycin for selection purposes) with 20 mM $MgCl_2$ at 30 °C (150 rpm). At an OD_{600} of 0.6–0.8 recombinant protein expression was induced by addition of 1 mM IPTG and cells were harvested 2 h later by centrifugation (6000 × *g*, 15 min). The pellet was washed twice and suspended in 50 mM HEPES, 300 mM NaCl, pH 7.8 (3 ml/g wet weight). Crude extract was obtained by sonication on ice with the sonication tip MS64 (Ultraschall Desintegrator Sonopuls HD60, Medizin- und Labortechnik, Hamburg, Germany) for 3 × 1 min with intermittent cooling between the sonication cycles followed by centrifugation (30,000 × *g*, 30 min, 4 °C). The purification was carried out using Ni^{2+}-NTA-Agarose (Qiagen Superflow; Qiagen GmbH, Hilden, Germany; bed volume 5 ml) and a FPLC apparatus (Pharmacia Biotech, Freiburg, Germany). The column was equilibrated with 50 mM HEPES, 300 mM NaCl, 30 mM imidazole, pH 7.8 and 3–4 ml crude extract was applied. The column was washed with 50 ml of the buffer used for equilibration. The His-tagged protein was then eluted with a linear gradient of 30 mM–300 mM imidazole (in 50 mM HEPES, 300 mM NaCl, pH 7.8). Protein concentration in the individual protein-containing fractions was determined by the absorbance at 280 nm as well as Bradford assay and the fractions were subsequently pooled. According to Linne et al. (2004) glycerol was added to a final concentration of 10% to the protein solution and aliquots were frozen in liquid nitrogen and stored at −70 °C.

2.6. Expression and purification of AACS

The plasmid pRD112 containing the gene *acsA2* cloned in vector pET30 Xa/LIC from *S. meliloti* and transformed in *E. coli* BL21 (DE3) pLys was kindly provided by T. Charles (University of Waterloo, Ontario, Canada) (Aneja et al., 2002). The expression of the His-tagged protein was carried out in LB medium supplemented with 34 µg/ml chloramphenicol and 30 µg/ml kanamycin at 37 °C (180 rpm). For induction of protein expression, IPTG (1 mM final concentration) was added at an OD_{600} of 0.5–0.6. Cells were harvested 3 h after induction by centrifugation (5000 × *g*, 10 min) and suspended in 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8 (10 ml/g wet weight). Crude extract was obtained by sonication on ice (sonication tip MS66, Ultraschall Desintegrator Sonopuls HD60, Medizin- und Labortechnik, Hamburg, Germany) for 3 × 1 min with intermittent cooling between the sonication cycles followed by centrifugation (30,000 × *g*, 30 min, 4 °C). The purification was carried out using Ni^{2+}-NTA-Agarose (Qiagen Superflow; Qiagen GmbH, Hilden, Germany; bed volume 5 ml) and a FPLC apparatus (Pharmacia Biotech, Freiburg, Germany). The column was equilibrated with buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8) and 5 ml crude extract were applied. The column was washed with 50 ml buffer 1 and 50 ml buffer 2 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8), respectively. The His-tagged protein was then eluted with a linear gradient of 20–250 mM imidazole (in 50 mM NaH_2PO_4, 300 mM NaCl, pH 8). Protein containing fractions were pooled, dialyzed (MWCO 12–14 kDa; against 50 mM NaH_2PO_4, 100 mM NaCl, pH 8) and concentration was determined by Bradford assay. For storage (−70 °C) glycerol was added to a final concentration of 10%.

2.7. Cloning, expression and purification of YbgC

The gene *ybgC* encoding the YbgC protein from *H. influenzae* was kindly provided by D. Dunaway-Mariano (University of New Mexico, Albuquerque, New Mexico, USA) (Zhuang et al., 2002). Using the plasmid pET3a-ybgC as template, the gene was amplified with concurrent introduction of restrictions sites for *Nde*I and *Xho*I via PCR and subcloned in vector pJET (Fermentas GmbH, St.-Leon-Rot, Germany) according to the manufacturer's instructions. The resulting pJET-ybgC was digested with endonucleases *Nde*I and *Xho*I, and the gel-eluted *ybgC* fragment (Gel extraction Kit, peqlab Biotechnologie GmbH, Erlangen, Germany) was ligated into *Nde*I/*Xho*I-digested vector pTYB1 (New England Biolabs GmbH, Frankfurt/Main, Germany) using the 'Rapid Ligation Kit' (Fermentas GmbH, St.-Leon-Rot, Germany) according to the manufacturer's instruction. The resulting plasmid pTYBybgC was transformed into *E. coli* BL21 (DE3). For recombinant protein expression cells were grown in LB medium (100 µg/ml ampicillin) at 37 °C, induced with 0.5 mM IPTG at an OD_{600} of ∼0.5 and incubated for additional 6 h at 20 °C (150 rpm). Cells were harvested by centrifugation (7000 × *g*, 10 min) and suspended in 50 mM K^+–HEPES, 500 mM KCl, pH 7.5 (10 ml/g wet weight). To obtain crude extract for purification the cells were sonicated (sonication tip MS64, Ultraschall Desintegrator Sonopuls HD60, Medizin- und Labortechnik, Hamburg, Germany) for 5 × 1 min with intermittent cooling between the sonication cycles followed by centrifugation (20,000 × *g*, 30 min, 4 °C). 15 ml crude extract were applied to chitin-beads containing columns (bed volume 2 ml). The columns were washed with 30 ml buffer (50 mM K^+–HEPES, 0.5 M NaCl, pH 8). On-column cleavage was carried out by addition of 6 ml cleavage buffer (50 mM K^+–HEPES, 0.5 M KCl, 50 mM DTT, pH 7.5) and incubation at RT overnight (∼16 h). Protein was eluted by addition of 10 × 1 ml 10 mM K^+–HEPES, 0.2 M KCl, pH 7.5. Fractions were analyzed using the Bradford assay. Protein containing fractions were pooled, dialyzed (MWCO 3.5 kDa) against 10 mM K^+–HEPES, 0.2 M KCl, pH 7.5 and concentrated with PEG 8500.

2.8. Determination of protein concentration

Protein concentration was determined using the method described by Bradford (1976).

2.9. Bacterial strains and plasmids

Table 1 lists the bacterial strains and plasmids used in this study.

2.10. Growth conditions and media

Except when otherwise mentioned, *E. coli* strains were grown at 30 °C or 37 °C with shaking at 150 rpm–180 rpm. 5-ml cultures were inoculated with a single colony from fresh LB plates grown overnight. If 5-ml cultures were used for growth experiments, an inoculum of 1% of a same medium overnight liquid culture was used. Larger volume cultures were inoculated with 1%–2% from an overnight liquid culture. To grow cultures LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) or 2 × YT (16 g/l tryptone, 10 g/l yeast extract,

Table 1
Bacterial strains and plasmids.

Bacteria strain or plasmid	Relevant characteristics	Reference/source
Strains		
C. acetobutylicum	Type strain (ATCC 824)	American Type Culture Collection
***E. coli* strains**		
B	Wild type (DSM 613)	German Collection of Microorganisms and Cell Cultures
BL21(DE3)	F-ompT *gal dcm lon hsd*SB(rB- mB-) λ(DE3 [lac^I *lacUV5-T7 gene 1 ind1 sam*7 *nin*5])	Studier and Moffat, 1986/Laboratory collection
BL21 (DE3)pLys	F-*omp*T, *hsd*SB (rB-, mB-), *dcm*, *gal*, λ (DE3), pLysS, Cmr	Promega GmbH, Mannheim, Germany
C600	F-*ton*A21 *thi*-1 *thr*-1 *leu*B6 *lac*Y1 *gln*V44 *rfb*C1 *fhu*A1 λ-	Appleyard, 1954/Laboratory collection
ER2275	*trp*31 *his*1 *ton*A2 *rps*L104 *sup*E44 *xyl*-7 *mtl*-2 *met*B1 $e14^+$ Δ(*lac*)*U169 end*A1 *rec*A1 *R(zgb-210::*Tn*10)* Tet^S Δ*(mcr-hsd-mrr)114::IS10*/F′ *proAB lacI ZΔ*M15*zz::min*-Tn*10* (K_m^r)	Mermelstein and Papoutsakis, 1993/Laboratory collection
HB101	F-*mcrB mrr hsd*S20(rB-mB-) *rec*A13 *leu*B6 *ara*-14 *proA2 lacY1 gal*K2 *xyl*-5 *mtl*-1 *rpsL*20(Sm^R) *gln*V44 λ-	Boyer and Roulland-Dussoix, 1969/Laboratory collection
JM109	*end*A1 *gln*V44 *thi-1 rel*A1 *gyrA*96 *rec*A1 $mcrB^+$ Δ(*lac-pro*AB) *e14-* [F′ *traD*36 $proAB^+$ $lacI^q$ *lacZ*ΔM15] *hsd*R17(rK^-mK^+)	Yanisch-Perron et al., 1985/Laboratory collection
K-12	Wild Type (DSM 498)	German Collection of Microorganisms and Cell Cultures
M15	nal^S str^S rif^S thi^- lac^- ara^+ gal^+ mtl^- $recA^+$ uvr^+ lon^+	Qiagen GmbH, Hilden; Germany
N99	F-*gal*K Sm^r λ-	Laboratory collection
RR1	HB101 $recA^+$	Bolivar et al., 1977/Laboratory collection
WL3	*adhC*81 *fadR adhE* (F^+ *mel supF*)	Lorowitz and Clark, 1982/Laboratory collection
W3110	F-*λ-rph-1 INV*(*rrnD*, *rrnE*)	American Type Culture Collection
XL1-B	*rec*A1 *endA*, *gyrA*96 *thi-1 hsdR*17 *supE*44 *rel*A1 *lac* [F, *proAB*, $lacI^qZDM15$, Tn*10* (Tet^r)]^c	Bullock et al., 1987/Laboratory collection
Plasmids		
$pTEII_{srf}$	pQE60 with $teII_{srf}$ (*B. subtilis*)	Schwarzer et al., 2002
pRD112	pET30 Xa/LIC expressing His-tagged *acsA2*	Aneja et al., 2002
pETybgC	pET-3a with *ybgC* (*H. influenzae*)	Zhuang et al., 2002
pTYB1	7477 bp; Amp^r	New England Biolabs GmbH; Frankfurt/Main, Germany
pUC19	2686 bp; Amp^r	
pUC18	2686 bp; Amp^r	

5 g/l NaCl) were used. For growth of recombinant strains the appropriate antibiotic was added (see Table 1).

2.11. Batch cultures and glucose fed batch cultures for acetone formation in E. coli

Growth experiments concerning the acetone formation were carried out in 5-ml cultures (1.5 × 15 cm test tubes) or in 100-ml cultures (1000-ml Erlenmeyer shake flasks with baffles; Glasgerätebau Ochs, Bovenden, Germany) in LB medium supplied with 100 μg/ml ampicillin. Tubes and flasks were shaken at 180 rpm in a rotary shaker. For expression of the synthetic acetone operons, 1 mM IPTG was added when an OD_{600} between 0.4 and 0.5 was reached. After further incubation for 3 h 20 g/l glucose (from a 500 g/l stock solution) was added. In glucose fed-batch cultures the glucose content of the cultures was regularly checked and occasionally supplemented to maintain glucose concentration above 2 g/l.

2.12. Cloning of the acetone operon

DNA was manipulated by standard molecular cloning techniques (Sambrook and Russell, 2001). Restriction endonucleases and DNA ligase (Fermentas GmbH, St. Leon-Rot, Germany) were used according to the manufacturer's instructions. PCR amplification of the fragments was performed using Taq-Mastermix (Qiagen GmbH, Hilden, Germany) or Synergy polymerase (GeneCraft GmbH, Lüdinghausen, Germany) according to the manufacturer's specifications.

As starting point of all other plasmids constructed for expression of the acetone pathway, clostridial genes were cloned in plasmid pUC18. The *adc* gene was amplified by PCR with the *adc*-primers and ligated into the pUC18 vector using generated *Acc*65I and *Eco*RI restriction sites. With the *thlA*-primers the *thlA* gene was amplified generating the restriction sites *Sal*I and *Bam*HI and finally the *ctfAB* genes were amplified with the *ctfAB*-primers and ligated with the provided *Bam*HI and *Acc*65I. The resulting plasmid pUC_adc_ctfAB_thlA was verified by restriction enzyme analysis and sequencing by Eurofins MWG Operon (Ebersberg, Germany). For gene expression controlled by the *lac* promoter, pUC_adc_ctfAB_thlA were restricted by *Sal*I and *Eco*RI and the operon was ligated into pUC19, obtaining pUC19act. Plasmids pUC19att and pUC19ayt were constructed by replacing *ctfAB* with *teIIsrf* or *ybgC*, restricted with *Bam*HI and *Acc*65I. To maintain expression under the control of the clostridial *thlA* promoter, the *thlA* gene in pUC18 variants was replaced by a *thlA* gene including the *thlA* promoter, followed by *ctfAB* replacement with *teIIsrf* or *ybgC* obtaining pUC18att or pUC18ayt as described above. The resulting plasmids were verified by restriction enzyme analysis and sequenced by Eurofins MWG Operon (Ebersberg, Germany). All plasmids used for acetone production are summarized in Table 2.

3. Results

3.1. Thioesterase activity of $TEII_{srf}$ from B. subtilis, YbgC from H. influenzae, and AACS from S. meliloti on acetoacetyl-CoA

To analyze the thioesterase activity of selected enzymes with acetoacetyl-CoA as substrate, the genes for $TEII_{srf}$ from *B. subtilis*, YbgC from *H. influenzae*, and AACS from *S. meliloti* were cloned

and expressed in *E. coli*, and the recombinant proteins purified via their affinity tags.

The gene encoding the thioesterase II from *B. subtilis* (*teII$_{srf}$*) cloned in plasmid pQE30 was obtained from Schwarzer et al. (2002). The C-terminal His$_6$-tag fusion protein was purified from *E. coli* M15 cells to homogeneity to a concentration of 1 mg/ml (Fig. 2a) as described in Materials and methods.

The YbgC protein was cloned in plasmid pTYB1 of the IMPACT^{TM}-System (Intein Mediated Purification with an Affinity Chitin-binding-Tag; New England Biolabs GmbH, Frankfurt/Main, Germany) developed for expression of recombinant proteins in *E. coli*. pTYB1 is a vector in which the C-terminus of the target protein is fused to the intein tag. The IMPACT^{TM} system utilizes the DTT-inducible self-cleavage activity of engineered protein splicing elements termed inteins to purify recombinant proteins by a single affinity chromatography step and provides the ability to separate the recombinant protein from the affinity tag without the use of a protease (Chong et al., 1998). As shown in Fig. 2b, the eluated fractions show the desired YbgC protein (15 kDa) and in low concentration the uncleaved fusion protein and intein. The fractions were pooled, concentrated to 0.5 mg/ml and used for the determination of thioesterase activity.

Protein AACS from *S. meliloti* (72 kDa) was purified via the fused His$_6$ tag by Ni^{2+}-agarose affinity chromatography to homogeneity at a concentration of 0.6 mg/ml (Fig. 2c).

The purified recombinant proteins were tested for their thioesterase activity with acetoacetyl-CoA and for comparison with acetyl-CoA as substrates in a DTNB assay. For TEII$_{srf}$ K_m values of 0.2 mM for acetyl-CoA and 0.77 mM for acetoacetyl-CoA as substrates were observed. This means that TEII$_{srf}$ has a higher substrate affinity for acetyl-CoA. In contrast, the YbgC protein from *H. influenzae* showed higher affinity for the substrate acetoacetyl-CoA. The K_m was 0.14 mM for acetoacetyl-CoA and 0.53 mM for acetyl-CoA. The K_m of AACS from *S. meliloti* was 0.7 mM for acetyl-CoA. The specific activity of the protein with the acetoacetyl-CoA substrate was very low (0.0038 μmol min^{-1} mg^{-1}), so that no K_m was determinable under given conditions. Due to their acceptable thioesterase activity with acetoacetyl-CoA the genes *ybgC* and *teII$_{srf}$*, but not the *aacs* gene were chosen to be integrated in to the new acetone operon together with *thlA* and *adc* from *C. acetobutylicum*.

Table 2
Summary of constructed plasmids used for acetone production.

Name of plasmid	Vector	Promoter	CoA-Transferase/Thioesterase
pUC19act	pUC19	*lac*	*ctfAB* from *C. acetobutylicum*
pUC19att	pUC19	*lac*	*teII$_{srf}$* from *B. subtilis*
pUC19ayt	pUC19	*lac*	*ybgC* from *H. influenzae*
pUC18att	pUC18	*thlA*	*teII$_{srf}$* from *B. subtilis*
pUC18ayt	pUC18	*thlA*	*ybgC* from *H. influenzae*

3.2. Construction of the acetone pathway with thioesterases

The cloning strategy is described in Section 2.12. At first clostridial genes were cloned consecutively in pUC18. Variants with thioesterase genes were obtained by replacing *ctfAB* gene. All plasmids used for acetone production are summarized in Table 2.

3.3. Acetone production by E. coli via the modified pathway

The genes of the selected thioesterases *teII$_{srf}$* (*B. subtilis*) and *ybgC* (*H. influenzae*) were cloned in combination with *thlA* and *adc* from *C. acetobutylicum* into the expression vectors pUC18 or pUC19 either under the control of the constitutive clostridial *thlA* promoter or the inducible *lac* promoter, respectively (Table 2). The plasmid pUC19act containing the classic acetone operon with the *ctfAB* genes (encoding acetoacetyl-CoA:acetate/butyrate CoA-transferase equivalent to the thioesterase function to the novel pathway) served as control. The plasmids were electroporated into several *E. coli* strains (B, C600, ER2275, HB101, JM109, K-12, N99, RR1, WL3, W3110 and XL1-B) and the transformants checked for acetone production.

Expression of the modified synthetic acetone operon led to significant production of acetone (up to 63 mM) only in the *E. coli* strains HB101 and WL3 (Table 3). Thus, the genetic background of the host was crucial. Furthermore, using the constitutive *thlA* promoter from *C. acetobutylicum*, acetone formation was only observed in strain HB101 carrying plasmid pUC18att.

Exemplarily, Fig. 3 shows growth and product formation in 5-ml cultures (LB$_{Amp}$) of strain HB101 carrying pUC19 and different derivatives. Cultures were induced with 1 mM IPTG and 3 h after

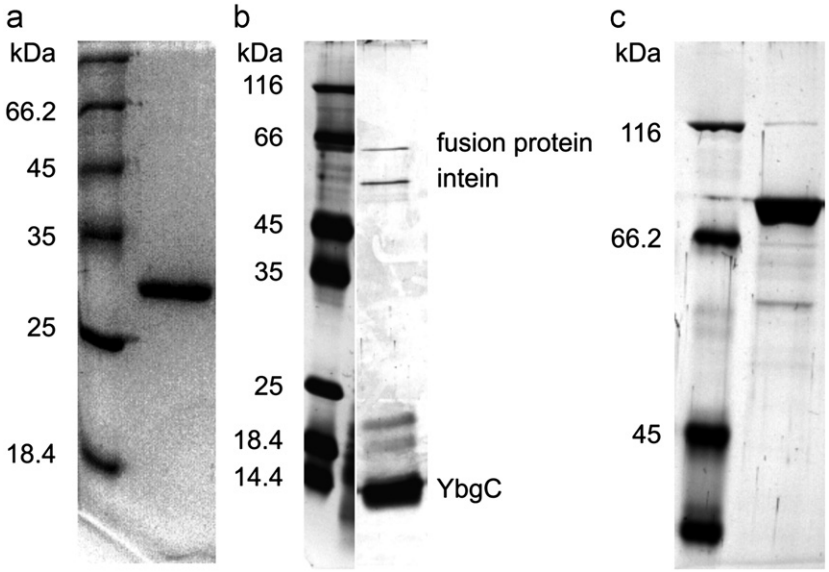

Fig. 2. Purification of recombinant thioesterases. (a) Elution fraction of thioesterase II (TEII$_{srf}$), 1 μg protein/lane, 12% SDS-PAGE, Coomassie-stained; (b) Elution fraction of thioesterase YbgC, 2 μg protein/lane, 10%–20% gradient SDS-PAGE, silver-stained; (c) Elution fraction of acetoacetyl-CoA synthetase (AACS), 1 μg protein/lane, 7.5% SDS-PAGE, silver-stained.

Table 3
Maximal acetone production (mM) in different *E. coli* strains in 5-ml cultures (LB medium) transformed with constructed plasmids.

	B	C600	ER2275	HB101	JM109	K12	N99	RR1	WL3	W3110	XL-1B
pUC19act	23.7	26.9	80.9	35.8	47.0	0.34	24.5	1.90	41.3	1.05	68.4
pUC19att	0.47	1.45	0.2	31.6	0	0.14	0.56	0.81	25.8	0	0.64
pUC19ayt	0.84	1.75	0.5	63.7	0.59	0.84	0.66	0.55	42.4	0.63	0.6
pUC18att	0.77	0	0.79	32.2	0.42	0.64	0.27	0.20	0	0.96	0
pUC18ayt	0.79	0	n.d.	3.70	0.40	n.d.	1.5	0.44	0	0	0

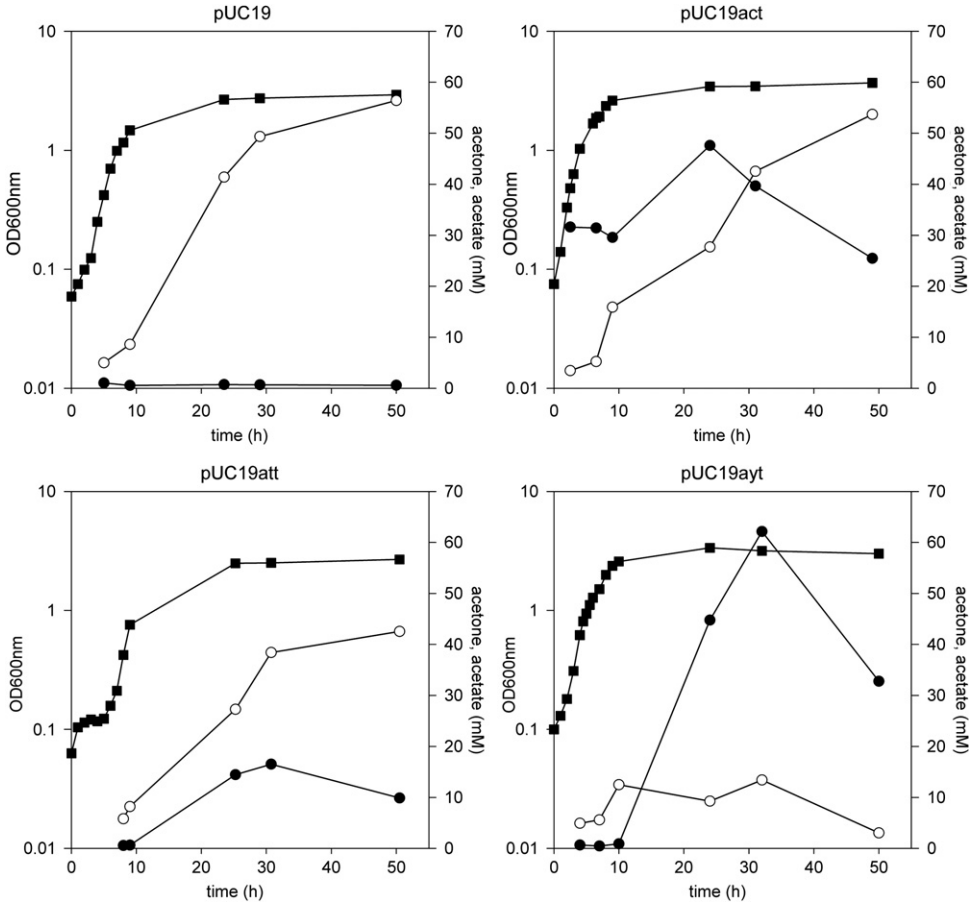

Fig. 3. Acetone production in *E. coli* HB101 after transformation with pUC19 constructs in 5-ml cultures (LB medium), 37 °C, 180 rpm. $OD_{600\ nm}$ (■); concentration of acetone (●) and acetate (○) in mM.

induction 20 g/l glucose was added. All strains grew comparably. As expected the strain with the control plasmid pUC19act (classical acetone pathway) produced acetone (up to 48 mM or 2.8 g/l) together with acetate (54 mM) whereas the strain with the parental plasmid pUC19 did not produce any acetone and 56 mM acetate during cultivation. Expression of the modified acetone pathway also resulted in high acetone production. HB101 (pUC19att) produced a maximum of 26 mM (1.5 g/l) acetone, whereas in the culture medium of HB101 (pUC19ayt) 62 mM (3.6 g/l) acetone were measured. This strain also showed the lowest acetate production (13 mM). The amount of acetone decreased after 24 h–30 h of cultivation due to evaporation of acetone from the aerated culture.

Since strain HB101 (pUC19ayt) showed the highest acetone titer it was chosen for a scale-up experiment from a 5-ml to a 100-ml culture in baffled shake flasks (37 °C, 180 rpm). Growth, acetone and acetate production, glucose consumption and pH were analyzed. As shown in Fig. 4a, after addition of glucose the acetone titer increased continuously to a maximum of 76 mM (4.4 g/l) at 48 h. At this time the glucose was exhausted. The pH dropped down to a minimum of 4.7 and the average acetate concentration was 22 mM. To test if continued supplementation of glucose would increase the acetone titer, a fed-batch culture was performed (Fig. 4b). In this case the pH of the culture was kept above 5 by addition of NaOH and glucose was added four times during the entire cultivation time of 80 h. The maximum of acetone production was reached at 48 h with a titer of 122 mM (7.1 g/l).

4. Discussion

In this study a modified metabolic pathway for the production of acetone was established in *E. coli.* This pathway depends on a thioesterase which produces the acetone precursor acetoacetate by hydrolysis of the thioester acetoacetyl-CoA. Three thioesterases from different sources with unknown activity on acetoacetyl-CoA as substrate were tested. In an in-vitro assay,

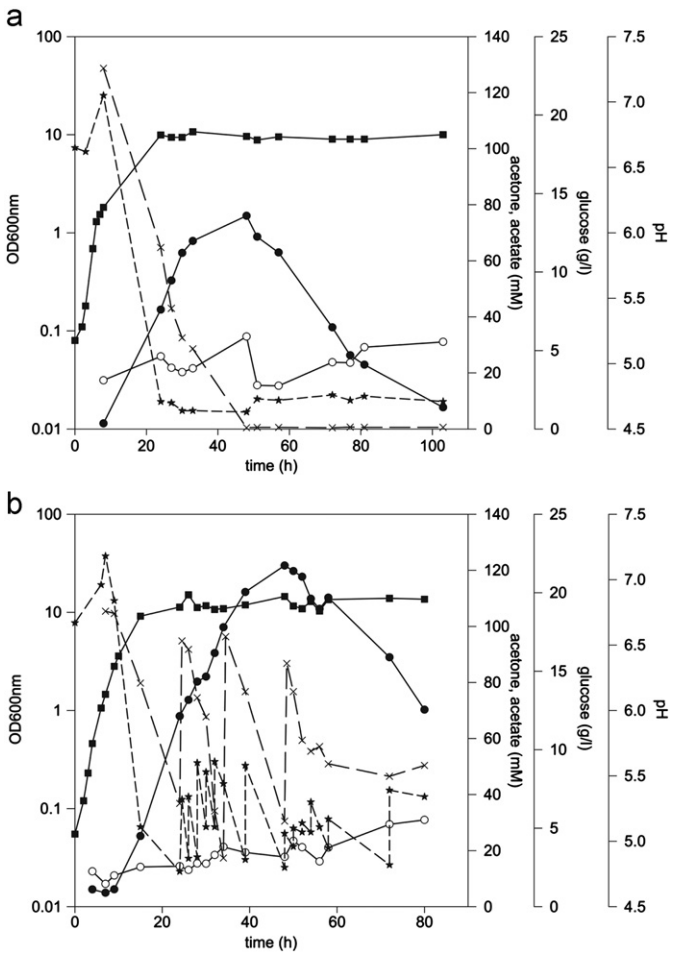

Fig. 4. Acetone production of *E. coli* HB101 (pUC19ayt) in 100-ml batch and glucose fed-batch culture. Expression of acetone operon was induced by addition of 1 mM IPTG at 5 h (a) and 4 h (b). (a) Batch culture with single addition of glucose at 7 h. (b) Glucose fed-batch culture with four times glucose addition. pH was checked externally and adjusted above 5 by addition of 0.2 M NaOH, if indicated. $OD_{600\ nm}$ (■), acetone (●), acetate (○), glucose (×), pH (★).

the enzymes YbgC from *H. influenzae* und $TEII_{srf}$ from *B. subtilis* exhibited reasonable activity with acetoacetyl-CoA as a substrate. However, it was not clear whether these enzymes were indeed suitable for replacing acetoacetyl-CoA:acetate/butyrate CoA-transferase in the new acetone pathway in vivo. Especially in the case of $TEII_{srf}$, the K_m value for acetyl-CoA was lower than for acetoacetyl-CoA (0.2 mM and 0.77 mM, respectively). The synthetic acetone pathway competes with the endogenous acetate and ethanol pathways for the common precursor acetyl-CoA. Thus, in the recombinant *E. coli* acetone producers, acetyl-CoA is a potential substrate for several enzymes: the thiolase (leading to acetone), the acetaldehyde dehydrogenase (leading to ethanol), the phosphotransacetylase (leading to acetate), or the thioesterase (leading to acetate). Furthermore, dependent on the supply of oxygen, acetyl-CoA is oxidized to CO_2 via the tricarboxylic acid cycle. Therefore, it was not surprising that not in all *E. coli* strains tested acetone was produced after the induction of the acetone pathway. However, from the known genetic background it is not easy to explain why only *E. coli* HB101 and WL3 exhibited significant acetone formation. It can only be speculated that in these strain the character of the acetyl-CoA pool and the actual activity of the above mentioned enzymes make it possible that the carbon flow is directed towards acetone. Thus, the overexpression of a thioesterase in the acetone pathway which is not highly specific for acetoacetyl-CoA needs a specific host for high acetone production. *E. coli* HB101 proved to be optimal for this purpose. Interestingly, in all *E. coli* strains with an acetone operon encoding the classical acetone pathway from *C. acetobutylicum*, acetone production could be observed (Bermejo et al., 1998), but also in varying amounts (strains ATCC11303 and ER2275: 40 mM; MC1060: 2 mM). With this pathway under the control of the *lac* promoter we found acetone concentrations of 1 mM in *E. coli* K-12 (pUC19act) up to 81 mM in strain ER2275 (pUC19act) confirming that both the choice of promoter as well as of the plasmid backbone and host strain influence the production of acetone.

A direct comparison of acetone production in *E. coli* HB101 indicated higher acetone concentrations (62 mM) when the modified acetate-independent pathway (pUC19ayt) was present instead of the clostridial pathway (48 mM, pUC19act). This indicates an advantage of the acetate-independent pathway. The lower K_m of 0.021 mM and therefore the higher affinity to its substrate acetoacetyl-CoA of the CoA-transferase CtfAB should in theory result in higher acetoacetate and thereby acetone production as compared to the thioesterase YbgC (K_m 0.14 mM). However, for its activity CtfAB requires acetate as co-substrate and the high K_m of 1200 mM for acetate was considered to be the limiting factor in this acetone pathway (Bermejo et al., 1998). With the new acetone pathway the yield (g acetone/g glucose) was 0.19 which is 60% of the theoretical maximum (0.32). Again, with the clostridial pathway introduced in *E. coli* the yield was lower, below 50% of the maximum (Bermejo et al., 1998). In a fed-batch culture, acetone concentrations of up to 122 mM (7.1 g/l) were obtained. In comparison, the classical ABE fermentation with *C. acetobutylicum* resulted in acetone titers of about 5 g/l (Bahl and Gottschalk, 1988).

In all *E. coli* strains producing acetone, lower amounts of acetate were formed, e.g., a decrease from 56 mM to 13 mM acetate was observed in *E. coli* HB101 (pUC19ayt) as compared to the control strain. Under the conditions tested, ethanol was always produced in low concentrations, below 10 mM (data not shown), probably due to the presence of oxygen as electron acceptor. The use of *E. coli* as host for the production of fermentation products from carbohydrates in general requires anaerobic conditions. This prevents complete oxidation of the substrate to CO_2. On the other hand, if all acetyl-CoA is converted to acetone, the cells might be in trouble to re-oxidize $NADH+H^+$ generated during glycolysis. The pathway from acetyl-CoA to acetone does not involve a redox reaction. Thus, it can be assumed that acetone production can be optimized by a limited oxygen supply, which does not prevent the formation of high amounts of the desired fermentation product but, if necessary, allows the cells to balance their redox state. The fact that in our shaken test tube or flask cultures high concentrations of fermentation products were present is an indication that these cultures were far away from fully oxygenated. A controlled aeration of the culture, e.g., in a fermenter, is certainly one way to optimize acetone production via this pathway. Naturally, anaerobic bacteria can achieve a redox balance by adjusting the ratio of more reduced to more oxidized products, e.g., by the production of ethanol (reduced) or acetate (oxidized). If a hydrogenase is present an excess of electrons can be released as H_2 (Lütke-Eversloh and Bahl, 2011; Sillers et al., 2008; Fischer et al., 2008).

Another target to improve acetone production in *E. coli* might be the acetyl-CoA pool, the precursor of acetate and many other products. There exist already comprehensive analyses concerning the reduction of acetate formation (De Mey et al., 2007; Eiteman and Altman, 2006; Nicolaou et al., 2010), representing some promising starting points for gene deletion in the acetone producing HB101 strain. It has been shown that an increase of the acetyl-CoA pool by inactivation of acetyl-CoA consuming

reactions lead to the production of poly-β-hydroxybutyrate (PHB) in a phosphotransacetylase mutant (Δ*pta*) of strain *E. coli* W3110 (Chang et al., 1999). The deletion of host pathways that compete with an introduced pathway for improving the desired product was, for example, already described for the production of 1-butanol in *E. coli* by Atsumi et al. (2008).

5. Conclusions

A modified pathway for acetone production in *E. coli* was established. Instead of a CoA transferase, several thioesterases were cloned in combination with thiolase and acetoacetate decarboxylase to catalyze the formation of acetone from acetyl-CoA via acetoacetyl-CoA and acetoacetate. Thus, this pathway is acetate-independent. Highest amounts of acetone were obtained when using the thioesterase YbgC from *H. influenzae*. Without further optimization of culture conditions or strain improvement a maximum of 122 mM acetone was obtained with *E. coli* HB101, exceeding the concentration obtained in the classical industrial ABE fermentation with *C. acetobutylicum*.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2012.08.001.

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