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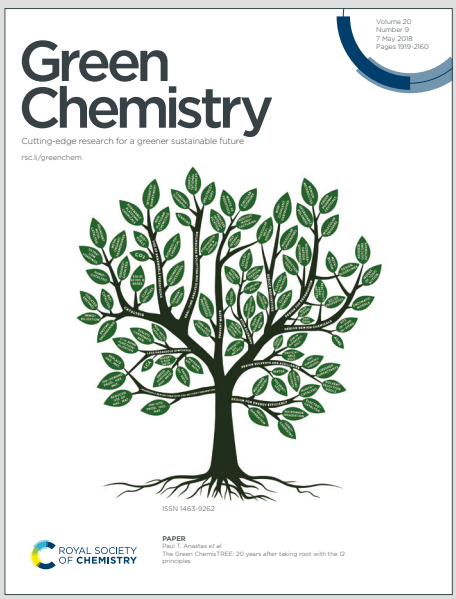

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DOI: 10.1039/C9GC03950C

Methanol-based acetoin production by genetically engineered *Bacillus methanolicus*

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Abstract

Methanol is an attractive alternative non-food feedstock for industrial fermentations that can be used instead of sugar-based raw materials. Here, the thermophilic and methylotrophic bacterium *Bacillus methanolicus* MGA3 was metabolically engineered to produce the platform chemical (*R*)-acetoin from methanol at 50 °C. Three different heterologous *alsSD*/*budAB* operons, each encoding acetolactate synthase and acetolactate decarboxylase, were functionally expressed under control of an inducible promoter in *B. methanolicus* MGA3, resulting in up to 0.26 ± 0.04 g/L of (*R*)-acetoin titer in shake flask cultivations. To further improve acetoin production, a total of six different genes or operons were expressed in the acetoin producing strains to increase supply of the acetoin precursor pyruvate. In particular, expression of a gene coding for malic enzyme from *Geobacillus stearothermophilus* in combination with the isocitrate lyase gene from *B. methanolicus* MGA3 increased acetoin titers 1.6-fold up to 0.42 ± 0.01 g/L which corresponds to 0.07 g/g methanol. This resulted in an MGA3 strain overproducing 4 recombinant enzymes in total from two different plasmids with two distinct antibiotics resistance markers, demonstrating the increased complexity of metabolic engineering allowed by newly developed genetic tools for this organism. To our knowledge, this is the first demonstration of microbial production of acetoin from methanol.

Introduction

Production of bulk chemicals in environmentally friendly biological processes is an area of increasing interest. For example, ketone compounds such as acetoin can replace petroleum-derived compounds which are in high demand as platform chemicals. Acetoin can be used as a food additive, chemical synthesis precursor and as a promoter of plant growth and was highlighted as a second tier building block in a 2004 report by the United States Department of Energy. [1–4] Estimates of global acetoin consumption in food products alone put the number at several thousand tons annually, with prices in the $30-50 per kilogram range. [2]

Acetoin is naturally produced in microbial fermentation processes and it has been suggested that production of acetoin and the closely related 2,3-butanediol help to prevent acidification of the cytoplasm during excessive organic acid production. [5] Biosynthesis of acetoin has been achieved in many species lacking native enzymes by heterologous overexpression of genes coding for acetolactate synthase (*alsS*) and acetolactate decarboxylase (*alsD*) from native acetoin producers, f. ex. *Bacillus subtilis.* [6] Acetolactate synthase (AlsS) catalyzes condensation of two molecules of

pyruvate to acetolactate, which is then decarboxylated in the reaction catalyzed by acetolactate decarboxylase (AlsD) to acetoin. [7] (*R*)-Acetoin is currently produced in high titers in microbial fermentation by species such as *Serratia marcescens* (*S. marcescens*; 75,2 g/L acetoin in fed-batch culture) and *B. subtilis* (53.9 g/L acetoin in batch culture), and enzymatic characterization of acetolactate decarboxylase from *B. subtilis* has shown that it is enantioselective for (*R*)-acetoin synthesis. [5–9] The risk group classification of acetoin producing strains must be considered and while products from *B. subtilis* are generally recognized as safe (GRAS), *S. marcescens* is classified into risk group 2 which can cause complications and additional costs in production processes. [10,11] These processes rely on sugars as carbon sources, leading to undesirable competition with food and feed industry, and indirectly to increasing prices of sugar [12]. Finding substitutes to refined sugars as raw materials for fermentation processes is an area of active research, and alternative carbon sources such as arabinose, xylose, cellulose, hemicellulose, lignocellulose and seaweed have been used in sustainable acetoin production. [13–16] However, the sugar-based carbon sources have some limitation for use as feedstock in bio-industries as they compete with agriculture for use of fertile land resulting in higher costs, strict rules and price regulations. [17] Furthermore, use of lignocellulose may cause technical bottlenecks for bioproduction.

Methanol is a suitable substitute for feedstock currently used in biotechnological processes, and can be utilized by methylotrophs for biomass production and respiration. [12] Methanol occurs naturally in the environment and can be synthesized in large scales from natural gas, biomass, CO_2, coal and oil, which decouples its price from the agricultural market and assures its renewability. [18–21] Methanol has also been receiving increased attention as a potential replacement for fossil fuels, and Patterson et al. (2019) recently proposed a radical idea for renewable production of methanol based on CO_2 and H_2 extracted from sea water, powered by renewable electricity such as floating solar panels or offshore wind turbines. [22] Methanol production on this scale would make fermentation processes using methylotrophs a truly attractive replacement for conventional sugar-based fermentation. As a result, methanol is a promising raw material for bio-based production of value-added compounds such as acetoin.

The pursuit of biologically sourced chemistry which can efficiently and selectively introduce carbon bonds between C1 compounds is critical. Current methods such as the Guerbet reaction can convert short-chain alcohols to longer chain alcohols by addition of methanol, but is not able to form C-C

bonds with methanol as the only substrate. [23] It has been shown that methanol can be converted into longer chain alcohols in the presence of metal acetylides, but the process is non-catalytic and therefore requires a constant supply of metal acetylides. [24] Another approach to producing higher order alcohols from methanol used a cell free biocatalytic system containing purified enzymes to convert methanol into ethanol and *n*-butanol. [25] While this strategy could in theory match the flexibility of cell-based systems, it depends on costly, purified enzymes for new production pathways, which could be a limiting factor for many target compounds. Whole cell biocatalysis can be used for production of methylated organic compounds, which are currently synthesized by use of Grignard reagents, diazomethane, methyl iodine, dimethylcarbonate or methyl sulfate, most of which are toxic, flammable or explosive. [26–28] Fermentative methylamination using monomethylamine in sugar-based processes led to *N*-methylated amino acids, however, methanol only contributed to a minor part of the product e.g. sarcosine or *N*-methyl-L-glutamate and no C-C bond was formed. [29,30] This means that the most adaptable strategy for conversion of methanol into higher order alcohols and other multi-carbon compounds is therefore likely through genetic engineering of methylotrophic hosts, such as the one presented in our study.The Gram-positive, thermophilic methylotroph *Bacillus methanolicus* MGA3 is able to utilize methanol as a sole energy and carbon source during fermentation, it is, therefore, a promising candidate as a production workhorse in the methanol economy. [31] Due to its thermophilic nature, *B. methanolicus* grows optimally at 50 °C. This is a desired feature since its methylotrophic metabolism is highly oxygen intensive and exothermic and the elevated growth temperature leads to a reduction in cooling requirements for large scale fermentations which translates to lower energy consumption as compared to the traditional processes with mesophiles such as *E. coli*.

B. methanolicus MGA3 has previously been used to produce amino acids and amino acid derivatives from methanol. [32–34] These compounds have in common that they are either naturally overproduced in *B. methanolicus* or are direct derivatives of naturally overproduced amino acids, and their synthesis was established through heterologous overexpression of suitable biosynthetic pathways. Significant progress has been made in the utilization of *B. methanolicus* MGA3 as a host for industrial production processes in recent years, f. ex. new genetic tools have been developed such as a theta replicating plasmid backbone (pHCMC04 derivative), a xylose inducible promoter and the discovery of compatible plasmids. [35] The use of a xylose inducible expression system enabled exploiting the

regulated nature of the promoter for transient gene expression, potentially avoiding toxic accumulation of reaction intermediates during biomass accumulation. The possibility of introducing two separate plasmids into the same cell has expanded the possible complexity of genetic engineering projects by accommodating greater numbers of overexpressed genes or pathways in a stable fashion. Additionally, new insights into the transcriptome, proteome and metabolome of *B. methanolicus* have opened up the possibility of more targeted genetic engineering approaches by exploiting naturally abundant metabolites or enzymes. [36–39]

In this study, we have used a methylotrophic host in order to establish methanol-based production of acetoin. We identified the *alsSD* operon from *B. subtilis*, *Bacillus licheniformis* or *Enterobacter cloacae* as suitable candidates to enable (*R*)-acetoin overproduction by *B. methanolicus* MGA3. *B. subtilis* and *B. licheniformis* have successfully been engineered for acetoin production, while *E. cloacae* has been shown to be a natural overproducer of acetoin. [6,13,40,41] The enzyme encoded by operon, acetolactate synthase and acetolactate decarboxylase, convert pyruvate into acetoin through acetolactate. Overexpression of the *alsSD* operon from *B. subtilis* in *B. methanolicus* MGA3 resulted in proof-of-concept for acetoin production from methanol. To improve acetoin titers, we overexpressed genes coding for enzymes involved in pyruvate production reactions in order to increase the cytoplasmic pyruvate availability for the acetolactate synthase reaction. In order to do so, we selected isocitrate lyase and melic enzyme for overexpression, as shown in Figure 1. [42] This improved acetoin titers compared to the control strain to final titer of 0.42 ± 0.01 g/L.

Materials and Methods

Bacterial strains, plasmids, molecular cloning and growth conditions

Bacterial strains and plasmids constructed and used in this study are listed in Table 1. All cloning work was performed in *Escherichia coli* DH5α. *E. coli* cells were made chemically competent by using the rubidium-chloride method. [43] Molecular cloning techniques were performed as detailed in Sambrook and Russel (2001). [44] Recombinant DNA was assembled *in vitro* using Gibson assembly as described by Gibson et al. (2009). [45] DNA sequencing was done by Eurofins Genomics and isolation of plasmid DNA was done with the use of Monarch Plasmid Miniprep Kit (New England Biolabs, T1010L).

Genomic DNA from *B. subtilis* 168, *B. licheniformis* MW3, *B. methanolicus* MGA3, *G. sterarothermophilus* 10, *Lactococcus lactis* ssp. *lactis* IL1403 and *C. glutamicum* ATCC13032 was isolated by use of a modified version of method presented by Eikmanns et al. (1994). [46] PCR products were amplified using Cloneamp HiFi PCR Premix (Takara, 639298) and purified using QIAquick PCR Purification kit (28106) from Qiagen. DNA fragments were separated using 0.8 % SeaKem LE agarose gels (Lonza, 50004) and isolated using QIAquick Gel Extraction Kit (Qiagen, 28706). All primers were purchased from Sigma Aldrich. *B. methanolicus* MGA3 was made electrocompetent and transformed by electroporation as described by Jakobsen et al. (2006). [47]

Shake flask cultivations with recombinant *B. methanolicus* MGA3 strains were cultivated in 250 mL baffled shake flasks at 50 °C and 200 RPM in 40 mL MVcM medium containing 200 mM methanol as described previously (Jacobsen et al 2006). [48] Antibiotics, 5 µg/mL chloramphenicol and 25 µg/mL kanamycin were supplemented as necessary.

For *C. glutamicum* growth experiments, pre-cultivation was performed first in brain heart infusion (BHI, ROTH) liquid medium for 8 hours and cells were transferred to CGXII medium with 1 % (w/v) glucose and 0.5 % potassium acetate for overnight cultivation. *C. glutamicum* main cultures in BioLector (m2p-labs, Baesweiler, Germany) were cultivated in Flowerplates at 1000 rpm, 85 % humidity, 30 °C and backscatter gain 20. Inoculation of CGXII medium was performed to an initial OD_{600} of 1 and 1 % (w/v) glucose and/or 0.5 %/1 % potassium acetate were used as carbon source(s). Isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 mM) was supplemented for induction.

Vector constructions

Plasmid pBV2xp was constructed based on pBV2mp. The *mdh* promoter was removed by digestion with KpnI and XbaI and exchanged with the xylose inducible system which was amplified from pTH1xp by using the primers xp-pBV2_fw and xp-pBV2_rv.

The three different acetoin biosynthetic operons were cloned into the pBV2xp plasmid under control of the xylose inducible xp promoter originating from *Bacillus megaterium.* [35] The six different genes/operons (*mae, odx, pyk-pckA, citM, aceA*) were cloned individually or in pairs into the pTH1mp plasmid under control of the strong and constitutive methanol dehydrogenase promoter P*mdh.* [31] The pTH1mp and pBV2xp plasmids are based on two different and compatible replicons. [35] More specifically, pBV2xp was digested with SacI and BamHI or BspTI, the pTH1mp and pMI2mp plasmids were digested with PciI and BamHI, and the pTH1mp-*mae*^{Gs} was cut with

EcoRI. Plasmid pEC-XT99A was digested with BamHI. Digestion was followed by separation by agarose gel electrophoresis and purification of the vector backbone fragments for cloning.

The *alsSD/budA* encoding regions of *B. subtilis* 168 (Accession numbers: AQR83639.1 and AQR83638.1) and *B. lichenformis* MW3 (Accession numbers: AAU42663.1 and AAU42662.1) were PCR amplified from genomic DNA by using the primer pairs alsSD B. subtilis FW and alsSD B. subtilis RV and alsSD B. licheniformis FW and alsSD B. licheniformis RV, respectively. The *budAB* encoding region of *E. cloacae* (Accession numbers: AFM58913.1 and AFM58914.1) was PCR amplified from the plasmid pET-RABC by using the primer pair budAB E. cloacae FW and budAB E. cloacae RV.

The *citM* gene from *L. lactis* (Accession number: FV54_06325), malic enzyme gene (*mae*) from *G. stearothermophilus* (Accession number: GS458_1402), malic enzyme gene (*mae*) from *B. licheniformis* (Accession number: CXG95_00500), *odx* gene from *C. glutamicum* (Accession number: CYL77_06515), and the *pyk* (Accession number: BMMGA3_13080), *pckA* (Accession number: BMMGA3_13575), *aceA* (Accession number: BMMGA3_01745) genes/operon from *B. methanolicus* were amplified from genomic DNA using primer pairs AC03/AC04, AC11/AC12, AC05/AC06, AC01/AC02, AC09/AC10, AC07/AC08, and AC13/AC14, respectively. For construction of pBV2xp-*aceBA* and pEC-XT99A-*aceBA*, the *aceBA* (Accession number: BMMGA3_01745 and BMMGA3_01750, respectively) operon from *B. methanolicus* was amplified using primers aceB_pBx_fw/aceA_pBx_rv and aceB_pEc_fw/aceA_pEc_rv, respectively.

100 ng of the linearized and purified pBV2mp, pBV2xp, pTH1mp, pMI2mp, pTH1mp-mae^{Gs} vector fragments were then mixed in a 1:3 molar ratio with the different PCR fragments and assembled at 50 °C using isothermal Gibson assembly, resulting in plasmids pBV2xp, pBV2xp-$alsSD^{Bs}$, pBV2xp-$alsSD^{Bl}$, pBVxp-$budAB^{Ec}$, pBV2xp-$aceBA^{Bm}$, pEC-XT99A-$aceBA^{Bm}$, pMI2mp-$citM^{Ll}$, pTH1mp-mae^{Gs}, pTH1mp-mae^{Bl}, pTH1mp-odx^{Cg}, pTH1mp-*pckA*-pyk^{Bm} and pTH1mp-mae^{Gs}-$aceA^{Bm}$. All constructed plasmids were verified with DNA sequencing before transformation to *B. methanolicus* MGA3 or *C. glutamicum* MH20-22b strains. PCR primers are listed in Table 1.

Evaluation of acetoin toxicity on *B. methanolicus*

B. methanolicus MGA3 cells were inoculated from a glycerol stock into pre-warmed MVcMY medium supplemented with 200 mM methanol, and incubated at 50 °C, 200 RPM for 16-18 hours. 25 mL pre-warmed MVcM medium supplemented with racemic acetoin (0, 0.5, 1.0, 5.0, 7.5, 10.0, 15.0, 25.0 or 50.0 g/L), and methanol (200 mM) was inoculated with pre-culture to a starting OD_{600} of 0.2 and incubated at 50 °C, 200

RPM. OD_{600} was measured every 2 hours after inoculation for 10 hours, with a final measurement 24 hours after inoculation. All cultures were grown in triplicates. IC_{50} for acetoin was determined by plotting the growth rates of the MGA3 cultures against their acetoin concentrations and estimating the acetoin concentration which halved the maximal growth rate achieved in this experiment (without acetoin).

Preparation of cell free extracts and coupled α-acetolactate synthase and α-acetolactate decarboxylase enzyme activity assay

Recombinant *B. methanolicus* strains were inoculated to OD_{600} = 0.2 in 40 mL pre-warmed (50 °C) MvCM medium and induced by adding xylose to a final concentration of 1 % at OD_{600} = 0.4. At OD_{600} = 0.8, 20 mL of the culture was collected and centrifuged at 4000 x g for 10 minutes at 4° C. The cells were washed two times with ice cold phosphate buffer (61.5 mM K_2HPO_4, 38.5 mM KH_2PO_4, 10 mM $MgCl_2$, pH 7.0) before storing at -80 °C. The cells were thawed by adding 1 mL ice cold phosphate buffer and incubating in an ice bath. The thawed cell suspension was then lysed by sonication, while still in the ice bath, using a Fisherbrand Sonic Dismembrator (FB-505) with 40 % amplitude and 2 seconds pulse cycle for totally 5 minutes. The lysate was centrifuged for 1 hour at 14000 x g at 4 °C, and supernatant was finally collected as cell free crude extract. The protein concentration of the samples was determined by use of a Bradford assay using bovine serum albumin as the standard.

The assay for coupled α-acetolactate synthase and α-acetolactate decarboxylase activities was adapted from Wiegeshoff and Marahiel (2007). [49] The reaction was started by mixing 50 µL of crude cell extract with 283 µL of pre-warmed (50 °C) assay reaction mix containing pH 7.0 phosphate buffer, 0.2 mM TPP (Sigma), and 10 mM of the substrate Na-pyruvate (Sigma). The reaction was terminated after 5, 10 and 20 minutes as described in McDevitt (2009). [50] The acetoin concentration was determined with the Voges-Proskauer reaction, measured spectrophotometrically at 530 nm (SpectraMax Plus 384 Microplate Reader, Molecular Devices LLC.), as described by McDevitt (2009). [50] Acetoin standard was purchased from Alfa Aesar. 1 U of activity corresponds to 1 µM of acetoin formed per min.

Preparation of cell free extracts and isocitrate lyase enzyme activity assay

Recombinant *B. methanolicus* strains were harvested after overnight cultivation in 50 mL pre-warmed (50 °C) MvCM medium supplemented with 0.25 g/L yeast extract, 200 mM methanol and induced with 5 g/L xylose. The cells were centrifuged at 4000 x g for 10 minutes at 4 °C and the pellet was resuspended in 50 mM morpholinopropanesulfonic acid (MOPS) buffer (pH 7.3). After

sonication with a Hielscher Ultrasound Processor instrument (UP220S, Berlin, Germany; cycle: 0.5, amplitude %: 60) for 9 minutes the lysate was centrifuged for 90 minutes at 20000 x g at 4 °C and the supernatant was used for the isocitrate lyase assay. The protein concentration was determined by using the Bradford method. The isocitrate lyase assay was performed by the method of Reinscheid et al. (1994) at 30 °C and 50 °C. In the cleavage reaction, 1 U of activity corresponds to 1 µM of glyoxylate formed per min. [51]

Detection of acetoin production by recombinant *B. methanolicus* strains

Supernatants from the recombinant *B. methanolicus* cultures were collected by centrifugation at 13000 x g, at 23 °C for 15 minutes. 30 µL of trichloroacetic acid was added to 200 µL supernatant to precipitate proteins. The precipitate was filtered through 0.45 um filters (Acro LC 13 mm, 0.45 µm) before being analyzed by HPLC. HPLC analysis was done on a Waters e2695 separation module using a Symmetry C18 column (4.6 mm x 75 mm). Detection of acetoin was performed using a Waters 2489 UV/Vis detector at 190 nm. The mobile phase composition was 30 mM H_3PO_4, pH 2.5 (pH adjusted with 10 M NaOH):acetonitrile (95:5), with a flow rate of 0.9 mL/min at 30 °C. The sample chamber was kept at 4 °C, and the injection volume was set to 2.5 µL. Retention time of acetoin under these conditions was around 1.5 minutes.

Results and discussion

Evaluation of *B. methanolicus* MGA3 as a host for acetoin production

To evaluate the suitability of *B. methanolicus* MGA3 as a host for acetoin production we investigated whether *B. methanolicus* naturally produces acetoin, potential toxicity effects of acetoin on cell physiology and whether acetoin is degraded by *B. methanolicus*.

First, an investigation of the genome sequence of *B. methanolicus* MGA3 revealed two genes, *ilvH* and *ilvB*, encoding the small and large subunit of acetolactate synthase which catalyzes the conversion of two molecules of pyruvate to one molecule of acetolactate. This particular enzyme is an entry point into the native branched chain amino acid synthesis pathway, and is likely under metabolic regulation by valine (as observed in other species), and so heterologous alternatives were needed for overexpression. [52] No native genes were found that would provide MGA3 with the ability of converting acetolactate to acetoin, despite the finding that small quantities of acetoin were produced by wildtype MGA3. This could result from spontaneous decarboxylation of acetolactate,

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DOI: 10.1039/C9GC03950C

produced by native acetolactate synthase encoded by *ilvH* and *ilvB*, to acetoin, which has been observed previously. [53]

Next, in order to investigate the suitability of *B. methanolicus* MGA3 as a host for acetoin production we analyzed its tolerance to acetoin. An initial experiment was set up to assay tolerance of *B. methanolicus* MGA3 to acetoin by growing *B. methanolicus* MGA3 cultures in minimal medium with 200 mM methanol as a carbon source and increasing concentrations of acetoin. The growth rates of the cultures were monitored by measuring OD_{600} every two hours over the course of 10 hours, and finally after 23 hours, and the growth rates at increasing concentrations of acetoin were compared, resulting in Figure 2. This revealed that the IC_{50} of acetoin in *B. methanolicus* MGA3 was 26 g/L (293 mM), at which point the growth rate was half (0.20 h^{-1}) of the maximal growth rate (0.39 h^{-1}). However, it must be noted that the acetoin used in this experiment was delivered as a dimer, which produces a racemic mix of acetoin upon monomerization by melting. [54] For that reason, an estimate for the IC_{50} of either (*S*)- or (*R*)-acetoin in *B. methanolicus* is in the range of 13 - 26 g/L.

Finally, once we had analyzed the influence of acetoin supplementation on the growth of *B. methanolicus* MGA3, we proceeded to investigate if *B. methanolicus* MGA3 was able to degrade acetoin. Fresh MVcM with 200 mM methanol, MVcM with 50 mM acetoin or MVcM with 200 mM methanol and 50 mM acetoin was inoculated with the overnight cultures of *B. methanolicus* MGA3. The growth of these cultures was monitored by OD_{600} measurements, and supernatants were sampled to measure acetoin concentrations. After 24 hours no growth was detected in the culture containing minimal medium with 50 mM acetoin, while the culture with MVcM supplemented with methanol and acetoin showed the expected growth rates. Additionally, analysis of the supernatants of MGA3 grown in MVcM with methanol and acetoin showed that the acetoin concentration in the medium changed from 6.2 g/L ± 0.15 to 5.3 ± 0.6 g/L over the course of 24 hours (data not shown). This indicates that *B. methanolicus* MGA3 seems to be able to degrade acetoin over long time periods, however, it does not utilize it as a carbon source for growth. In order to understand the metabolic background of this observation, we inspected the genome of *B. methanolicus* in search of genes coding for putative 2,3-butanediol dehydrogenases, but none were identified. A control experiment was performed where crude extracts of wild type *B. methanolicus* cells were tested for 2,3-butanediol dehydrogenase activity, and we could detect low background activity likely due to

(a) promiscuous alcohol dehydrogenase enzyme(s) (0.05 ± 0.04 U/mg; data not shown) which could explain the slow degradation of acetoin observed previously.

Heterologous expression of α-acetolactate synthase and α-acetolactate decarboxylase and production of acetoin in *B. methanolicus*

In this study, we have genetically engineered *B. methanolicus* MGA3 for acetoin biosynthesis. Three *alsSD/budAB* operons from different species were selected for controlled heterologous overexpression in *B. methanolicus* MGA3 to establish production of acetoin. The sources of *alsSD/budAB* operons were selected based on literature review of effective acetoin producers, with a preference for closely related species such as other *Bacilli*. As result, three *alsSD/budAB* operons from *Bacillus subtilis*, *Bacillus licheniformis* and *Enterobacter cloacae* were cloned into a pBV2 backbone under the control of the xylose inducible promoter (xp) from *Bacillus megaterium* and transformed into *B. methanolicus* MGA3.

To analyze the constructed strains, we first performed coupled α-acetolactate synthase and α-acetolactate decarboxylase enzyme assays to evaluate the activity of the enzymes involved in the acetoin biosynthesis pathway. The production and control strains, MGA3 $acet^{Bs}$, MGA3 $acet^{Bl}$, MGA3 $acet^{Ec}$ and MGA3 pBV2xp, were cultivated in shake flasks with minimal medium supplemented with 0.25 g/L yeast extract, 200 mM methanol and 1 % xylose and harvested at OD_{600} ≥ 0.8. The cell material was disrupted by sonication and the enzyme activity was assayed as described by Gerwick et al. (1994), with slight modifications. [55] Due to the fact, that *B. methanolicus* MGA3 is typically cultivated at 50 °C, we conducted the enzyme assays at this temperature so that we could analyze enzyme activity under physiologically relevant conditions. The resulting specific enzyme activities are shown in Table 2.

As depicted in Table 2, the overexpression of the *E. cloacae*-derived *budAB* operon lead to negligible activity of BudAB at 0.3 ± 0.2 U/mg, compared to 0.6 ± 0.3 U/mg for the control. The background enzyme activity observed in the control MGA3 pBV2xp strain is most probably due to spontaneous decarboxylation of acetolactate, that accumulates due to activity of native acetolactate synthase encoded by *ilvH* and *ilvB,* to acetoin. [53] Overexpression of *alsSD* operons derived from *B. subtilis* and *B. licheniformis* led to enzyme activity at levels of 11.3 ± 2.3 and 4.8 ± 1.7 U/mg, respectively. Comparison of the specific enzyme activities of the MGA3 $acet^{Bs}$ strain to that observed for acetolactate synthase overexpressed in *B. subtilis*, shows similar activity levels with specific enzyme

activities in cell extracts of *B. subtilis* at 6.2 ±0.5 U/mg measured at 37 °C. [56] The coupled enzyme activity in the MGA3 acet^{Bs} strain was 2.3-fold higher in comparison to MGA3 acet^{Bl}, despite the fact that the donor organism is mesophilic. *B. licheniformis* is known to be able to grow at higher temperatures in comparison to *B. subtilis*, with optimal growth temperatures around 50 °C. For that reason, our initial expectation was that MGA acet^{Bl} strain would perform better than the other strains tested. However, a study by Sommer et al. (2015) revealed that the optimal temperature for activity of acetolactate synthase AlsS from *B. subtilis* is 50 °C, making it an ideal candidate for use in MGA3. [57] Contrarily, for the acetolactate synthase BudB in *E. cloacae* and AlsS in *B. licheniformis* the optimal temperature has been found to be 37 °C. [58,59] No comparable data is available for the second enzyme of the pathway, acetolactate decarboxylase (AlsD), but our results indicate that AlsD from *B. subtilis* retains activity at 50 °C.

MGA3 acet^{Bs}, MGA3 acet^{Bl} and MGA3 acet^{Ec} were then tested for the production of acetoin. Fresh minimal medium with 200 mM methanol was inoculated with overnight cultures and the strains were allowed to grow for approximately 24 hours after induction. Cleared supernatant was sampled from the culture at the end of the experiment, which was then analyzed by RP-HPLC for acetoin titers. The measured acetoin concentrations for strains MGA3 pBV2xp, MGA3 acet^{Bs}, MGA3 acet^{Bl} and MGA3 acet^{Ec} are shown in Table 2. The best producer among the strains tested was MGA3 acet^{Bs}, with a final titer of 0.26 ± 0.04 g/L acetoin from 6.4 g/L methanol which is consistent with the enzyme assay results and corresponds to 0.04 g/g of methanol (Table 2). The *R* configuration of the produced acetoin can be assigned based on previous characterization of the acetolactate decarboxylase from *B. subtilis* used in this study which has been shown to be enantioselective for (*R*)-acetoin synthesis. [9] The second-best producer among the strains tested was MGA3 acet^{Bl} with a titer at a level of 0.08 ± 0.02 g/L, which is 3.5-fold lower in comparison to MGA3 acet^{Bs} and in accordance with the previously observed enzyme activities (Table 2). Finally, MGA3 acet^{Ec} resulted in acetoin titers comparable to the empty vector control, MGA3 pBV2xp, indicating that the acetoin production pathway was likely inactive *in vivo*, an observation which is supported by the corresponding enzyme activity in Table 2. As depicted in Table 2, the best producer, MGA3 acet^{Bs} exhibited a reduced growth rate compared to the other strains in the first hours of growth and slowed down significantly 6 hours after induction to a specific growth rate of 0.07 h^{-1} (data not shown). The three remaining strains showed similar growth rates to each other.

The acetoin titers achieved by the MGA3 acet^{Bs} strain are lower than titers achieved for acetoin producers where no metabolic engineering for substrate availability or co-factor regeneration was executed. It was shown that the thermotolerant *B. subtilis* IPE5-4 is able to produce up to 12.55 ± 0.28 g/L acetoin in 72-hour shake flask fermentation at 50 °C in LB medium supplemented with 100 g/L glucose as a carbon source, resulting in a yield of 0.13 g/g glucose. [14] The wild type *B. licheniformis* WX-02 strain produces 10.33 ± 0.02 g/L acetoin, along with 18.77 ± 0.33 g/L *meso*-2,3-butanediol and 12.51 ± 0.36 g/L *D*-2,3-butanediol after 24 hours at 37 °C using 120 g/L glucose and 33 g/L of corn steep liquor in flask cultivation (yield of 0.12 g/g glucose). [60] Wild type *E. cloacae* SDM produces 2.72 ± 1.13 g/L of acetoin together with 40.30 ± 0.89 g/L of 2,3-butanediol (BD) in shake flask cultivations in minimal medium supplemented with 90.0 g/L glucose and 5.0 g/L yeast extract. Deletion of the *budC* gene in that strain, coding for acetoin reductase which catalyses the conversion of acetoin to 2,3-butanediol, lead to increased accumulation of acetoin at a level of 27.61 ± 0.92 g/L, with a final yield of 0.3 g/g glucose. [61] Furthermore, *E. coli* engineered for acetoin production by heterologous overexpression of *budRAB* genes from *S. marcescens* H30 accumulated 9.8 g/L of acetoin in batch fermentation in minimal medium supplemented with 60 g/L glucose, 10 g/L of yeast extract and 2 g/L of sodium acetate which corresponds to a yield of 0.13 g/g glucose. [62] However, several points need to be made regarding this comparison. Firstly, in our experiment a defined medium was used, whereas other strains were cultivated on glucose supplemented with additional complex media components such as yeast extract, tryptone and corn steep liquor. Secondly, in our production system, methanol was used as the C-source, which has not been achieved ever before in the case of acetoin production. For this reason, we believe that we have created a promising system for methanol-based acetoin production, and we have taken further steps to improve our production strain.

To examine the regulation of the *alsSD*^{Bs} operon under the control of the xylose inducible promoter (xp), we also compared the coupled AlsS and AlsD enzyme activities in MGA3 acet^{Bs} and MGA3 pBV2xp crude extracts upon induction with 10 g/L xylose and without induction. Crude extracts from MGA3 acet^{Bs} cells induced with 10 g/L xylose showed 22-fold higher enzyme activity compared to uninduced MGA3 acet^{Bs} cells. Furthermore, the AlsSD activity in uninduced MGA3 acet^{Bs} is comparable to the enzyme activity of the empty vector control confirming that the system is tightly regulated. The results of this experiment are shown in Figure 3. We do not have an

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DOI: 10.1039/C9GC03950C

immediate explanation for the discrepancies observed in enzyme activity between the two separate experiments (Table 2 and Figure 3).

We examined the effect of different induction levels in order to analyze the functionality of the xylose inducible promoter. MGA3 acet^{Bs}, the strain that was chosen as the best producer based on the enzyme assay and production data was grown in minimal medium with 200 mM methanol and induced with different concentrations of D-(+)-xylose. We found that the optimal inducer concentration was the previously used 10 g/L. Xylose has previously been tested as a carbon source in *B. methanolicus* MGA3, and is metabolized by *B. methanolicus* but it is not able to support growth. [35,63] More importantly, we found that the xp promoter was tightly controlled as almost no acetoin accumulation was observed when no xylose was added (Table 3). This is a beneficial feature of the expression system that can be used in cultivation schemes where growth is decoupled from production of target compounds and high cell densities are reached before induction of gene expression, avoiding the toxic effects and metabolic burden of acetoin production during biomass accumulation. Thus, concerns about acetoin toxicity at higher concentrations can be circumvented by use of the inducible promoter.

We investigated acetoin accumulation in the growth medium over the course of the cultivation for the best producing strain MGA3 acet^{Bs}. Supernatants were collected every 2 hours for the first 8 hours after induction, followed a final sample 21.5 hours after inoculation. As shown in Figure 4, the acetoin was accumulated in a manner proportional to the growth pattern over the course of first 6 hours after induction, which indicates that acetoin production is coupled to biomass formation, and that its production starts soon after induction with 10 g/L xylose.

Furthermore, we have analyzed the formation of by-products in MGA3 acet^{Bs} in order to investigate putative targets for pathway optimization (Table 4). We have focused on accumulation of amino acids in the cultivation medium, because L-glutamate is known to be one of the main products of the overflow metabolism in *B. methanolicus* and L-alanine is synthetized directly from pyruvate, which means that its production directly competes with acetoin biosynthesis. [64,65] The L-alanine titer was not affected in the acetoin producing strain MGA3 acet^{Bs}. Surprisingly the L-glutamate titer increased from 0.01 ± 0.00 g/L in the empty vector control to 0.02 ± 0.00 g/L in the MGA3 acet^{Bs} strain, although the acetoin titer was more than tenfold higher (Table 4). For this reason, we focused our next step on replenishing the pyruvate pool from the tricarboxylic acid (TCA) cycle.

Influence of overproduction of pyruvate replenishing enzymes on acetoin accumulation

Pyruvate, being one of the metabolites which belong to the central carbon metabolism, is formed from methanol via the ribulose monophosphate (RuMP) cycle (Figure 1). It can either enter the TCA cycle for oxidation to CO_2 or be used as biosynthetic precursor to f. ex. L-alanine or for the product acetoin. For this reason, to increase the titers of acetoin in *B. methanolicus* MGA3 from methanol, we decided to increase the pyruvate availability in *B. methanolicus* MGA3 acet^{Bs} by overexpressing genes coding for enzymes involved in pyruvate generating reactions. Using similar criteria to those previously described, five candidates were selected for testing; malic enzyme (encoded by *mae*^{Bl}) from *Bacillus licheniformis,* malic enzyme (encoded by *mae*^{Gs}) from *Geobacillus stearothermophilus,* pyruvate kinase (encoded by *pyk*^{Bm}) and phosphoenolpyruvate carboxykinase (encoded by *pckA*^{Bm}) from *B. methanolicus* and oxaloacetate decarboxylase (encoded by *odx*^{Cg}) from *Corynebacterium glutamicum, and* oxaloacetate decarboxylase (encoded by *citM*^{Ll}) from *Lactococcus lactis*. It was shown before that the optimum temperature for malic enzyme from *G. stearothermophilus* is 55 °C, which makes it a suitable target for use in the thermophilic *B. methanolicus*; furthermore it shares 48% identity with the YtsJ from *B. subtilis* which performs the major physiological role of the four paralogous malic enzyme isoforms in *Bacillus subtilis.* [66,67] Additionally, a *B. licheniformis*-derived malic enzyme was chosen as a suitable target because of its high similarity (80% identity) to *B. subtilis*-derived, *ywkA*- encoded malic enzyme which belongs to other subgroup of malic enzymes in *B. subtilis* than YtsJ. [68] Both *C. glutamicum*-derived *odx* and *L. lactis*-derived *citM* were characterized to code for functional oxaloacetate decarboxylases (OAD). The *L. lactis*-derived oxaloacetate decarboxylase exhibits optimal activity at 50 °C and the one derived from *C. glutamicum* was only assayed at 22°C, however no test for the optimum temperature has been performed. [69,70] Lastly, the transcripts of *pyk*^{Bm} coding for pyruvate kinase and *pckA*^{Bm} coding for phosphoenolpyruvate carboxykinase were found in the whole transcriptome study of *B. methanolicus* MGA3 indicating that these two genes are transcribed. [36] Additionally, Pyk was present in the proteome of *B. methanolicus* signifying that it is produced in this bacterium. [71]

The genes were cloned into a rolling circle plasmid, pTH1mp (or, in the case of *citM*^{Ll}, pMI2; the only difference between pTH1mp and pMI2mp being copy number in *E. coli)*, under the control of the methanol dehydrogenase promoter (mp) from *B. methanolicus* MGA3. Competent *B. methanolicus* MGA3 acet^{Bs} cells were transformed with these plasmids, and cultured for 25 hours, after which

culture broth samples were collected. Cleared samples were analyzed by RP-HPLC to quantify acetoin concentrations.

Table 5 presents the acetoin titers of *B. methanolicus* MGA3 acet^{Bs} strains producing pyruvate replenishing enzymes. The acetoin titer for the control strain MGA3 acet^{Bs} + pTH1mp was 0.31 ± 0.01 g/L, which was 20 % higher in comparison to the parent strain acet^{Bs}, despite the expected metabolic burden of carrying the second plasmid. As shown in Table 5, acetoin titers were only improved for MGA3 acet^{Bs} + pyr^{Cg} in comparison to the control strain MGA3 acet^{Bs} + pTH1mp, resulting in an increase of acetoin concentration by approximately 13 %, from 0.31 ± 0.01 g/L to 0.35 ± 0.02 g/L. The fact that overproduction of *C. glutamicum*-derived OAD led to an increase of acetoin accumulation while overproduction of the *L. lactis*-derived OAD did not may be caused by the low pH optimum of the latter. [69] It has been shown before that enzymes with an optimal pH below 5 are functional in *B. methanolicus* only to a limited extent. [33]

Comparison of growth curves of the *B. methanolicus* MGA3 strains during acetoin production revealed that the best producer, MGA3 acet^{Bs} + pyr^{Cg}, had a prolonged lag-phase during the main cultivation (Figure S2, supplementary information). However, it is unlikely that this influenced the final acetoin titers. The rest of the strains seemed to follow very similar growth patterns with exponential and stationary growth phases, followed by cell lysis.

Effect of glyoxylate shunt overexpression on acetoin production

To further improve the acetoin titer in *B. methanolicus* MGA3 we investigated the effect of overexpressing genes coding for enzymes involved in the native glyoxylate shunt from *B. methanolicus* MGA3. It is shown in Table 5 that overexpression of some of the genes coding for pyruvate producing enzymes had a positive effect on acetoin titers, and we wanted to investigate if coupling one of these reactions to an overexpressed glyoxylate shunt pathway would further improve acetoin titers. The glyoxylate shunt genes in MGA3 have been annotated, but their functionality has not yet been tested. Genetic complementation of *C. glutamicum* Δ*aceAB* deletion mutants by heterologous overexpression of *aceBA*^{Bm} demonstrated the functionality of the *B. methanolicus* MGA3 gloxylate shunt genes *in vivo* (Figure 5). In cultures with potassium acetate as the sole carbon source the complemented strain reached a higher OD_{600} (Figure 5B, 7.4 ± 0.2) than the empty vector control (Figure 5C, 1.5 ± 0.2) indicating more biomass formation and improved acetate utilization.

Moreover, functional overproduction of endogenous AceA^{Bm} was confirmed by an *in vitro* enzyme assay (Figure 6). Specific activities of isocitrate lyase in crude extracts of overexpression strains were significantly higher compared to the native crude extract background activity. The specific activities of both empty vector and full vector samples increased when temperature was increased from 30 °C to 50 °C as expected from Arrhenius law. Thus, homologous overexpression of the glyoxylate shunt genes should aid in precursor supply for acetoin production.

Even though no difference was observed in acetoin accumulation between the control strain MGA3 acet^{Bs} + pTH1mp and MGA3 acet^{Bs} + pyr^{Gs}, we chose the latter as the target for further genetic modifications because we wanted to use a strain which utilized malate directly for pyruvate generation, instead of oxaloacetate. The isocitrate lyase (*aceA*) gene from *B. methanolicus* was cloned into pTH1mp-*mae*^{Gs} and used for transformation of competent MGA3 acet^{Bs} cells. The resulting strain, MGA3 acet^{Bs} + glyox^{Bm} was cultivated for acetoin production as described before alongside MGA3 acet^{Bs} + pTH1mp and MGA3 acet^{Bs} + pyr^{Gs}. The acetoin concentration is depicted in Figure 7 with growth patterns of the strains shown in Figure S2 (supplementary information).

As shown in Figure 7 the additional overexpression of the *aceA* gene from *B. methanolicus* MGA3 together with *mae*^{Gs} on the pTH1mp plasmid (glyox^{Bm}) had a significant impact on acetoin titers with an increase of 33 % in comparison to MGA3 acet^{Bs} + pTH1mp. Surprisingly, despite increased acetoin accumulation by the MGA3 acet^{Bs} + glyox^{Bm} strain in comparison to MGA3 acet^{Bs} + pyr^{Gs} and MGA3 acet^{Bs} + pTH1mp, no major difference in growth patterns between the strains was observed (Figure S3, supplementary information).

We showed that metabolic engineering aimed at increasing pyruvate availability for conversion to acetoin in MGA3 improved acetoin titers, from 0.31 ± 0.01 g/L (MGA3 acet^{Bs} + pTH1mp) to 0.35 ± 0.02 g/L (MGA3 acet^{Bs} + pyr^{Cg}) and to 0.42 g/L (MGA3 acet^{Bs} + glyox^{Bm}). Increasing pyruvate availability should exert a metabolic "push" effect on the acetolactate synthase reaction, thereby increasing the rate of conversion. At the same time, previous studies have shown that in MGA3 the activity of pyruvate carboxylase encoded by *pyc*, converting pyruvate to oxaloacetate, is in a much lower range (nM/min) in comparison to our heterologously produced AlsSD enzymes (µM/min). [42] The combined overexpression of the gene coding for malic enzyme from *G. stearothermophilus* and the *aceA* gene from MGA3 resulted in significantly increased acetoin titers in comparison to MGA3

acet^{Bs} + pTH1mp. Together, these two enzymes provide a path from isocitrate to pyruvate, bypassing large parts of the citric acid cycle.

Manipulation of carbon flux through precursor supply reactions has received attention due to its potential to significantly change precursor pools in metabolite overproduction processes. [72] In particular, the "phosphoenolpyruvate-pyruvate-oxaloacetate node" has been highlighted as a critical target for metabolic engineering, and was extensively covered by Sauer and Eikmanns (2005). [73] Evidence for the impact of increased pyruvate availability on acetoin production was shown when Nielsen et al. (2010) improved acetoin titers in *E. coli* by increasing intracellular pyruvate availability through extensive metabolic engineering. [74]

The titers achieved in this study are lower than in sugar-based acetoin production bioprocesses. It has to be noted that production of acetoin from methanol requires C-C bond formation (here in the RuMP cycle) whereas its production from glucose or other sugars does not. The titer of 0.42 g/L of acetoin produced solely from the one carbon feedstock methanol is comparable to titers reached for other products when one carbon raw materials were used. *Methylomicrobium buryatense* was engineered for microbial conversion of methane to lactate with initial titers of 0.06 g/L in 0.5 L bioreactors, improved to 0.8 g/L of lactate in a 5 L tank bioreactor (0.05 g/g yield). [75] Recombinant *Clostridium ljungdahlii* produced 0.07 g/L of butyrate from syngas, and metabolically engineered *Methylobacterium extorquens* produced 0.08 g/L α-humulene from methanol in shake flasks, which was further increased to 1.6 g/L in a fed-batch bioreactor (0.031 g/g yield). [76,77] The recombinant *Acetobacterium woodii* produced 0.87 g/L of acetone from CO_2 in bottle fermentation (0.07 g/g yield), which was enhanced to 3.02 g/L in continuous fermentation (0.03 g/g yield). [78] Metabolic engineering of *Methylobacterium rhodesianum* lead to production of 0.36 g/L of (R)-3-hydroxybutyrate in batch cultures (0.036 g/g yield) and 2.8 g/L in fed-batch cultures (0.009 g/g yield) from methanol. [79] In all these examples, the titers in small scale batch cultivations were either one order of magnitude lower or in the same range as those achieved in our study. Recently, production of acetoin from an H_2/CO_2 gas mixture was achieved for engineered *Cupriavidus necator* with a titer of 1.2 g/L in a 55-hour 1L-Schott bottle cultivation and 3.9 g/L in 14-day continuous gas flow fermentation. [80] These titers were achieved in a fermentation over a longer time period and in a bigger scale than our bioprocess which demonstrated a potential for future enhancements of C1-based production of acetoin.

With future optimizations in strain genetics and fermentation conditions it is plausible that methanol-based acetoin production can be cost competitive with sucrose-based acetoin production. It is worth noting that

the pathway yield (Y^P) for action production from methanol and glucose are very similar, 0.46 g/g and 0.49 g/g, respectively, and with our current yield of 0.07 g acetoin/g methanol there is a great potential for future developments. [81] As a comparison, the highest yield (*R*)-acetoin bioprocess from glucose reported a yield of 0.47 g/g, while the highest (*R*)-acetoin titers recorded from glucose reached a yield of 0.36 g/g. [82,83] It seems that one of the most limiting factors for acetoin production is NADH accumulation. The majority of reduction potential in MGA3 is formed during methanol assimilation and formaldehyde oxidation, resulting in 1 mol NADH for every mol methanol assimilated, and 1 additional mol of both NADH and NADPH for every mol of formaldehyde oxidized. [42] During growth on methanol the citric acid cycle is therefore not critical for the generation of reduction potential, but in combination with anaplerotic reactions provides intermediates for amino acid synthesis and other coupled processes. Wildtype MGA3 has previously been shown to overproduce and secrete glutamate, which likely explains why redirection of the metabolic flux from the TCA cycle to acetoin production could result in significantly improved acetoin titers. [84] In the reaction catalyzed by glutamate dehydrogenase, which in *B. methanolicus* has a key role in L-glutamate synthesis, one mol of NADH is oxidized for every mol of glutamate synthesized, making it an important player in maintaining the redox balance in *B. methanolicus* MGA3. [65] It is therefore likely that strategies where acetoin titers are increased at the expense of glutamate would need to involve metabolic engineering to maintain redox balance, similar to that shown by Liu et al. (2017) for increased acetoin titers from glucose in *Lactococcus lactis.* [85]

Feasibility of methanol-based acetoin production

There are several points to be made that speak in favor of methanol as a replacement of sugar as feedstock, even though the current titers in our methanol-based approach are lower than titers achieved in sugar-based systems. The main advantage of methanol-based acetoin production is the decoupling of production costs from the global sugar market, which can be influenced by external environmental factors such as heat waves and other extreme weather events, an issue which is forecasted to increase in severity in the coming decades. [86–88] Methanol can be produced from natural gas or fermentation of waste biomass by steam reformation of methane, making its production less cost vulnerable to these factors. [89] In the last reported quarter of 2019, the average methanol spot price for Europe as announced by the Methanol Institute, a trade association for methanol producers , was €288/metric ton. [90] With the best acetoin yield presented in this paper, this puts the methanol cost per kg acetoin at €4.38 for our method compared to feedstock costs at

€0.81-0.86/kg acetoin using sugar. [7,8,91] This means that our methanol-based bioprocess is currently more expensive with regards to feedstock costs, but a 5-fold improvement to titers of 2 g/L would abolish this discrepancy. Additionally, it is worth mentioning that methanol-based bioprocesses have several advantages over sugar-based processes, such as a reduced demand for complex nutrients in methanol-based processes compared to sugar-based ones resulting in decreased costs of the production medium and a decrease of the downstream processing cost, which can contribute to more than 50 % of the production costs in conventional bioprocesses. [17]

Conclusions

Here, a method for production of acetoin from methanol with the use of the thermophilic *Bacillus methanolicus* MGA3 has been developed for the first time. Until now, only plant-biomass based carbon sources such as glucose, xylose, cellulose, hemicellulose and seaweed have been used to produce acetoin, a valuable platform chemical. Here, a natural methylotroph, *Bacillus methanolicus* MGA3, was used to overexpress *B. subtilis* - derived genes coding for an acetoin production pathway, yielding a strain producing up to 0.26 g/L acetoin from 6.4 g/L methanol. The engineered acetoin producing MGA3 strain was further improved through bypassing parts of the TCA cycle by overproduction of isocitrate lyase together with malic enzyme. Employment of this strategy led to development of a strain producing 0.42 g/L of acetoin in batch experiments (0.07 g/g methanol). This work helps pave an unprecedented path for production of valuable platform chemicals from methanol.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

We thank professors Cuiqing Ma and Ping Xu from State Key Laboratory of Microbial Technology, Shandong University, Jinan, People's Republic of China for providing us with the pET-RABC plasmid; prof. dr. Friedhelm Meinhardt from University of Münster for providing us with the strain *B. licheniformis* MW3, and dr. Pierre Renault from Micalis Institute in the National Institute for Agronomical Research (INRA) for providing us with *Lactococcus lactis* ssp. *lactis* IL1403.

573 This work was funded by the Faculty of Natural Sciences, Norwegian University of Science and
574 Technology, Norway. CH and VFW gratefully acknowledge support by ERACoBiotech via grant C1Pro
575 (BMEL 22023617),

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728

729 # 1 TABLES

730 Table 1 Bacterial strains, plasmids and used in this study.

Strain name		Relevant characteristics	Reference
E. coli DH5α		General cloning host, F-*thi*-1 *endA*1 *hsdR*17(r-,m-) *supE*44 _*lacU*169 (_80*lacZ*_M15) *recA*1 *gyrA*96 *relA*1	Stratagene
B. methanolicus MGA3		Wild type strain	ATCC 53907
B. subtilis 168		Wild type strain	ATCC 23857
B. licheniformis MW3		*Bacillus licheniformis* DSM13 (*ΔhsdR1*, *ΔhsdR2*)	Waschkau et al. (2008) [92]
C. glutamicum ATCC 13032		Wild type strain	ATCC 13032
C. glutamicum MH20-22b		Lysine-producing Leu⁻ derivative of ATCC13032	Schrumpf et al. (1992) [93]
C. glutamicum MH20-22b Δ*aceAB*		MH20-22b derivative with an *aceAB* deletion	Wendisch (1997) [94]
G. stearothermophilus 10		Wild type strain	ATCC 12980
Lactococcus lactis ssp. *lactis* IL1403		Wild type strain	Bolotin et al. (2001) [95]
Abbreviation	**Plasmid name**	**Relevant characteristics**	**Reference**
pTH1mp	pTH1mp	Cm^R; derivative of pTH1mp-*lysC* for gene expression under control of the *mdh* promoter. The *lysC* gene was replaced with multiple cloning site.	Irla et al. (2016) [35]
pTH1xp	pTH1xp	Cm^R; *E. coli/Bacillus* spp. shuttle vector for gene expression under control of the inducible xylose promoter from *B. megaterium*.	Irla et al. (2016) [35]
pMI2mp	pMI2mp	Cm^R; low copy derivative (in *E. coli*) of pTH1mp	This study
pBV2mp	pBV2mp	Kan^R; pHCMC04 derivative, gene expression under control of the *mdh* promoter, theta replicating	Irla et al. (2016) [35]
pBV2xp	pBV2xp	Kan^R; pHCMC04 derivative, gene expression under the control of the inducible xylose promoter from *B. megaterium*, theta replicating	This study

pEC-XT99A	pEC-XT99A	Tet^R; *C. glutamicum* / *E. coli* shuttle vector under control of the *trc* promoter. Based on the medium copy number plasmid pGA1.	Kirchner and Tauch (2003) [96]
pET-RABC	pET-RABC	Kan^R; pET28a carrying 2,3-BD gene cluster with its operon originated from *Enterobacter cloacae* subsp. *dissolvens* SDM	Xu et al. (2014) [97]
$acet^{Bs}$	pBV2xp-*alsSD*^{Bs}	Kan^R; pBV2xp derivative for expression of *alsSD* operon from *B. subtilis* under control of xylose inducible promoter xp	This study
$acet^{Bl}$	pBV2xp-*alsSD*^{Bl}	Kan^R; pBV2xp derivative for expression of *budA-alsD* operon from *B. licheniformis* under control of xylose inducible promoter xp	This study
$acet^{Ec}$	pBV2xp-*budAB*^{Ec}	Kan^R; pBV2xp derivative for expression of *budAB* operon from *Enterobacter cloacae* under control of xylose inducible promoter xp	This study
pyr^{Cg}	pTH1mp-*odx*^{Cg}	Cm^R; pTH1mp derivative for expression of *odx* from *C. glutamicum* ATCC 13032 under control of the *mdh* promoter	This study
pyr^{Ll}	pMI2mp-*citM*^{Ll}	Cm^R; pTH1mp derivative for expression of *citM* from *Lactococcus lactis* IL1403under control of the *mdh* promoter	This study
pyr^{Gs}	pTH1mp-*mae*^{Gs}	Cm^R; pTH1mp derivative for expression of *mae*^{Gs} from *Geobacillus stearothermophilus* 10 under control of the *mdh* promoter	This study
pyr^{Bl}	pTH1mp-*mae*^{Bl}	Cm^R; pTH1mp derivative for expression of *mae*^{Bl} from *Bacillus licheniformis* MW3 under control of the *mdh* promoter	This study
pyr^{Bm}	pTH1mp-*pckA-pyk*	Cm^R; pTH1mp derivative for expression of *B. methanolicus*-derived *pckA* and *pyk* under control of the *mdh* promoter	This study
$glyox^{Bm}$	pTH1mp-*mae*^{Gs}-*aceA*	Cm^R; pTH1mp derivative for expression of *mae*^{Gs} from *Geobacillus stearothermophilus* 10 and *B. methanolicus*-derived *aceA* under control of the *mdh* promoter	This study
pBV2xp-*aceBA*	pBV2xp-*aceBA*	Kan^R; pBV2xp derivative for expression of *aceBA* operon from *B. methanolicus* under control of xylose inducible promoter xp	This study
pEC-XT99A-*aceBA*	pEC-XT99A-*aceBA*	Tet^R; pEC-XT99A derivative for expression of *aceBA* operon from *B. methanolicus* under control of IPTG inducible promoter trc	This study

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Table 2 Specific enzyme activity, acetoin final titers and specific growth rates for MGA3 acetoin production strains. The means of triplicates with standard deviations are shown.

Strain name	Coupled AlsSD activity [U/mg protein]	Acetoin titer [g/L]	Specific growth rate, μ [h^{-1}]
MGA3 pBV2xp	0.56 ± 0.32	0.01 ± 0.00	0.27
MGA3 $acet^{Bs}$	11.28 ± 2.28	0.26 ± 0.04	0.22
MGA3 $acet^{Bl}$	4.75 ± 1.68	0.09 ± 0.03	0.29
MGA3 $acet^{Ec}$	0.39 ± 0.17	0.03 ± 0.00	0.30

Table 3 Effect of different inducer concentrations on final acetoin titers in MGA3 $acet^{Bs}$. The strain was grown in minimal medium with 200 mM methanol and induced with different concentrations of xylose added to the growth medium two hours after inoculation. The means of triplicates with standard deviations are shown.

Xylose concentration [g/L]	0.0	0.5	1.0	5.0	10.0	20.0
Acetoin titer [g/L]	0.00 ± 0.00	0.12 ± 0.00	0.14 ± 0.00	0.15 ± 0.01	0.15 ± 0.01	0.14 ± 0.01

Table 4 Final titers of acetoin, L-glutamate and L-alanine for MGA3 $acet^{Bs}$. The means of triplicates with standard deviations are shown.

Strain name	Acetoin titer [g/L]	L-glutamate titer [g/L]	L-alanine titer [g/L]
MGA3 pBV2xp	0.01 ± 0.00	0.01 ± 0.00	0.04 ± 0.00
MGA3 $acet^{Bs}$	0.26 ± 0.04	0.02 ± 0.00	0.04 ± 0.00

Table 5 Acetoin titers in *B. methanolicus* MGA3 $acet^{Bs}$ strains with a second plasmid overexpressing pyruvate replenishing genes. The strains were grown in minimal medium with 200 mM methanol and induced with 10 g/L of xylose added to the growth medium two hours after inoculation. The concentration of acetoin was measured 25 h after inoculation. The means of triplicates with standard deviations are shown.

Strain	Acetoin concentration [g/L]
MGA3 $acet^{Bs}$ + pTH1mp	0.31 ± 0.01
MGA3 $acet^{Bs}$ + pyr^{Ll}	0.30 ± 0.02
MGA3 $acet^{Bs}$ + pyr^{Gs}	0.29 ± 0.03
MGA3 $acet^{Bs}$ + pyr^{Bl}	0.32 ± 0.01
MGA3 $acet^{Bs}$ + pyr^{Cg}	0.35 ± 0.02
MGA3 $acet^{Bs}$ + pyr^{Bm}	0.33 ± 0.02

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2 FIGURES

Figure 1 a) Overview of heterologously expressed genes and their metabolic functions in this study. The dotted arrows represent chain of more than one enzymatic reaction, solid lines represent one enzymatic reaction. Gene names coding for enzymes catalyzing relevant reactions are depicted in the graph. Abbreviations: *mae* - malic enzyme gene, *pyk* - *pyruvate kinase gene, pckA* - phosphoenolpyruvate carboxykinase gene, *odx* - oxaloacetate decarboxylase gene, *alsS* - acetolactate synthase gene, *alsD* - acetolactate decarboxylase gene, *aceA* - isocitrate lyase gene, RuMP cycle - ribulose monophosphate (RuMP) cycle. b) Metabolic pathway from pyruvate to acetoin showing stereochemistry for intermediate and end products.

Figure 2 Growth rates of *B. methanolicus* MGA3 cultivated in minimal medium supplemented with 200 mM methanol and with increasing concentrations of acetoin. IC_{50} of acetoin in *B. methanolicus* was determined to be 26 g/L (293 mM). The means of triplicates with standard deviations are shown.

Figure 3 Coupled AlsSD specific enzyme activities with and without induction of the xp promoter by 10 g/L xylose. MGA3 $acet^{Bs}$ was selected for this experiment because it displayed the highest activities previously. MGA3 pBV2xp was used as a negative control. The means of triplicates with standard deviations are shown.

Figure 4 Acetoin accumulation in the growth medium and OD_{600} over time for MGA3 $acet^{Bs}$. The strain was grown in minimal medium with 200 mM methanol and induced with 10 g/L of xylose added to the growth medium two hours after inoculation. The concentration of acetoin and OD_{600} were monitored every 2 hours over a period of 8 hours and at time 21.5 h after inoculation. The means of triplicates with standard deviations are shown.

Figure 5 Complementation of *C. glutamicum* Δ*aceAB* with *B. methanolicus* MGA3 genes. ΔOD_{600} of strains MH20-22b(pEC-XT99A) a) MH20-22B Δ*aceAB*(pEC-XT99A; b) MH20-22B Δ*aceAB*(pEC-XT99A-$aceBA^{Bm}$); c) after growth with glucose (gluc) and/or potassium acetate (KAc). The means of triplicates with standard deviations are shown.

Figure 6 *In vitro* isocitrate lyase specific activity in *B. methanolicus* MGA3 crude extracts at 30°C and 50°C. The means of triplicates with standard deviations are shown.

Figure 7 Acetoin production in MGA3 $acet^{Bs}$ strain with overexpressed genes coding for the glyoxylate shunt pathway compared to control strains. The strains were grown in minimal medium with 200 mM methanol and induced with 10 g/L of xylose added to the growth medium two hours after inoculation. The concentration of acetoin was measured at time 25 h after inoculation. The means of triplicates with standard deviations are shown.

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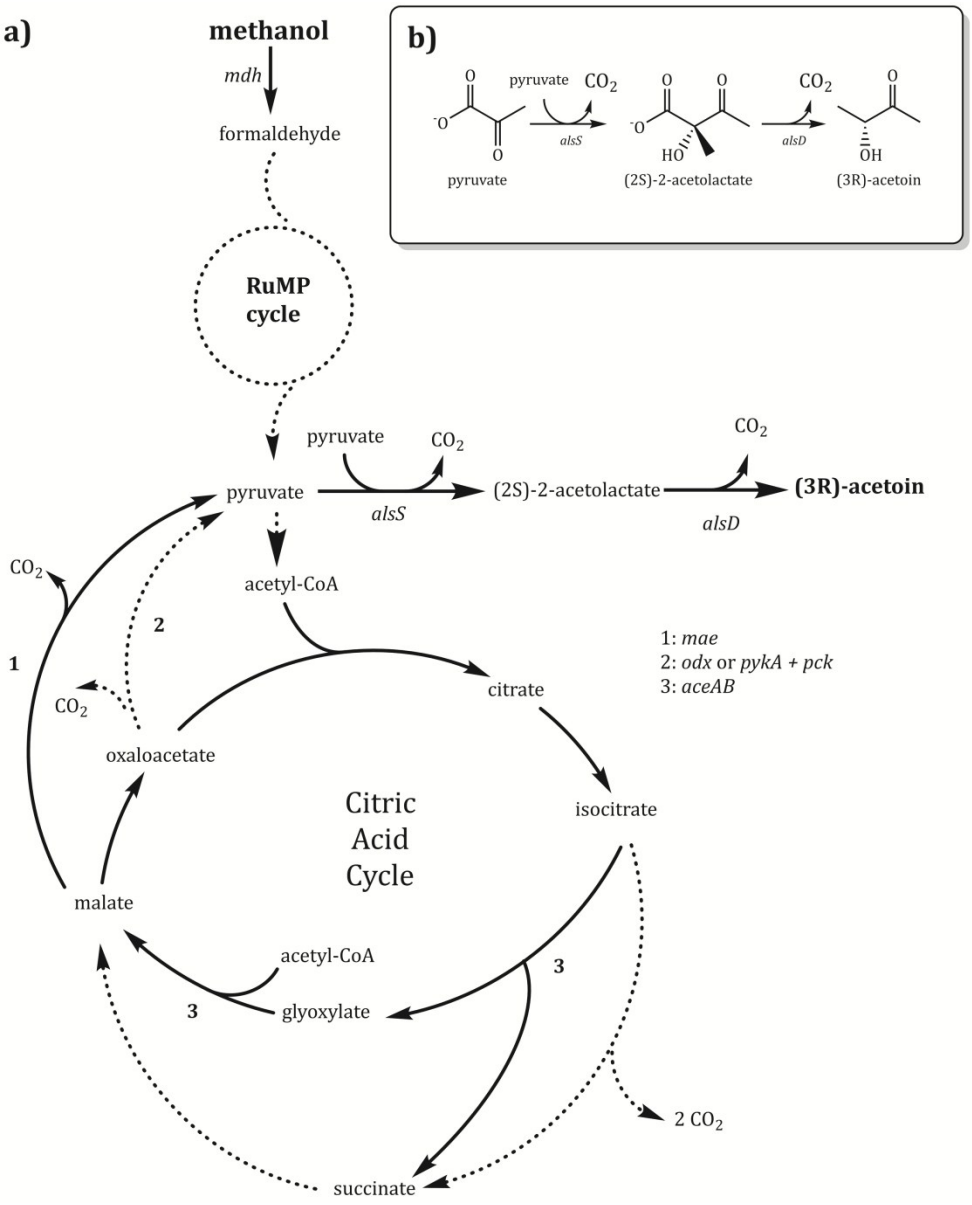

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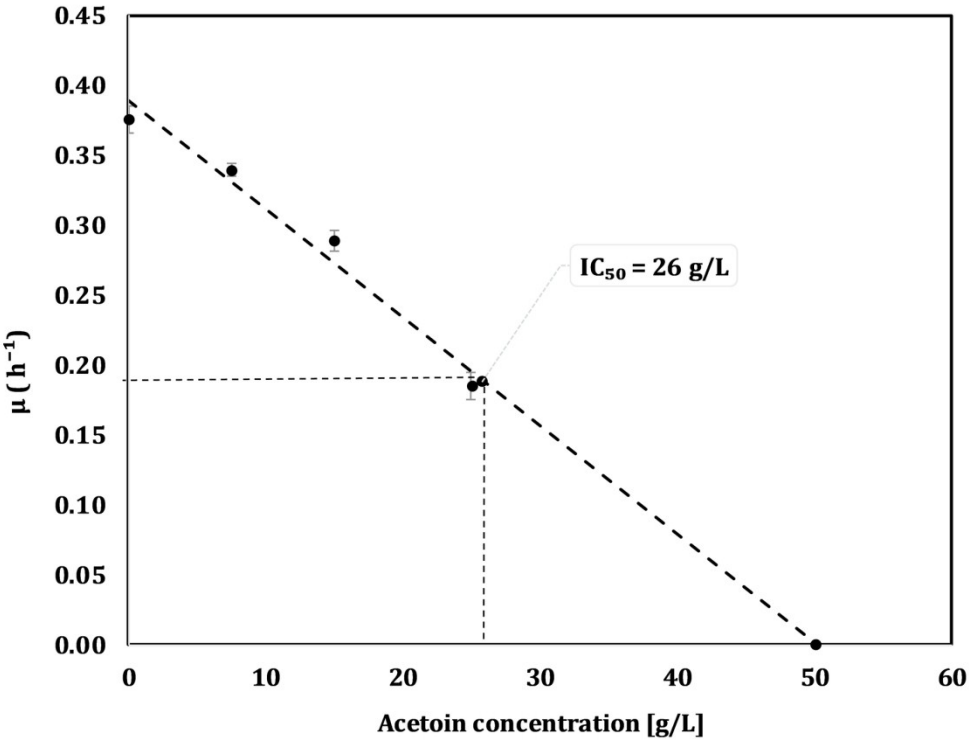

Figure 2 Growth rates of *B. methanolicus* MGA3 cultivated in minimal medium supplemented with 200 mM methanol and with increasing concentrations of acetoin. IC_{50} of acetoin in *B. methanolicus* was determined to be 26 g/L (293 mM). The means of triplicates with standard deviations are shown.

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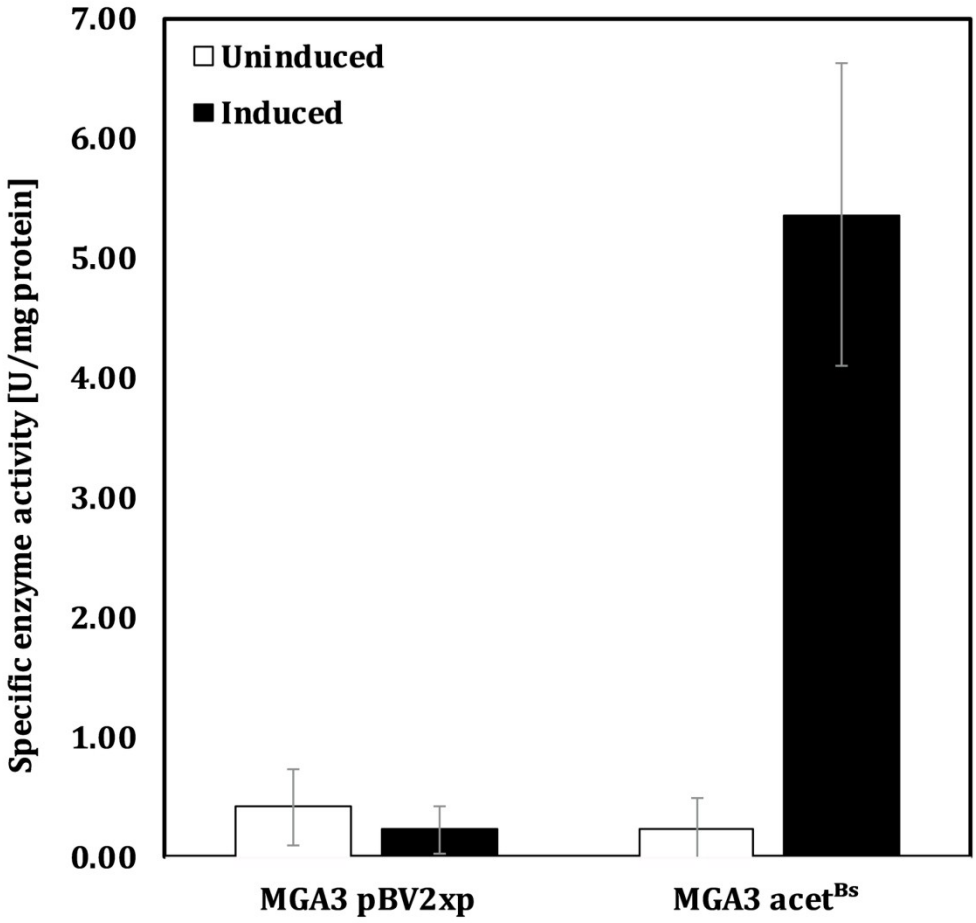

Figure 3 Coupled AlsSD specific enzyme activities with and without induction of the xp promoter by 10 g/L xylose. MGA3 acetBs was selected for this experiment because it displayed the highest activities previously. MGA3 pBV2xp was used as a negative control. The means of triplicates with standard deviations are shown.

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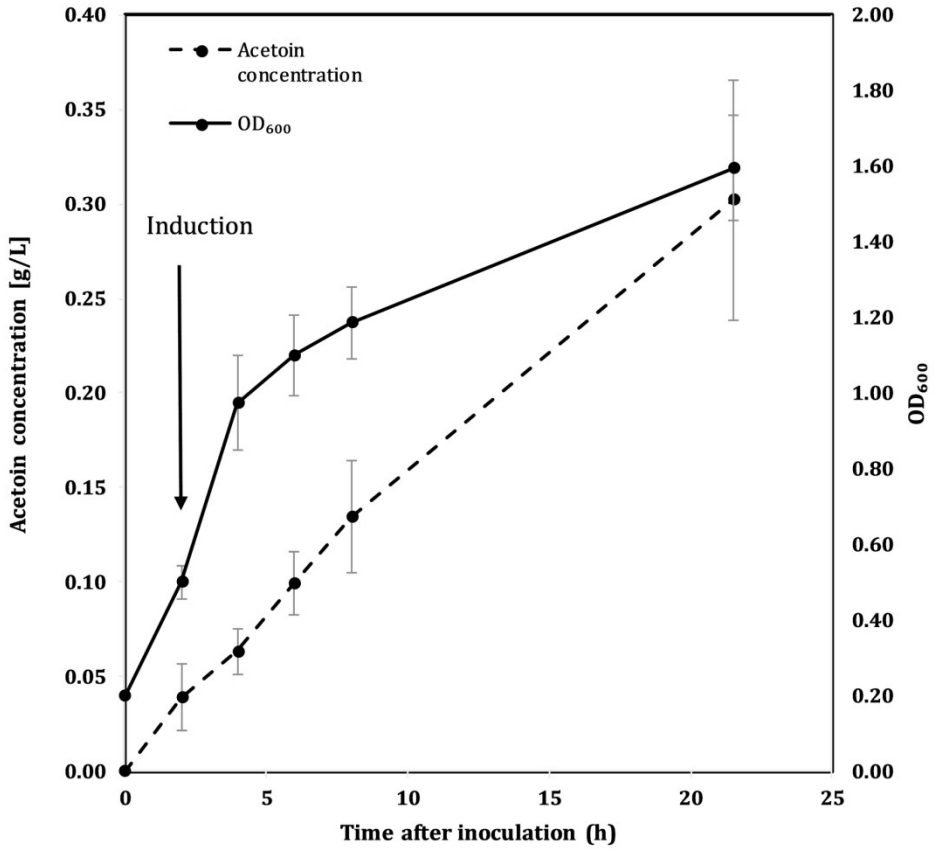

Figure 4 Acetoin accumulation in the growth medium and OD_{600} over time for MGA3 acetBs. The strain was grown in minimal medium with 200 mM methanol and induced with 10 g/L of xylose added to the growth medium two hours after inoculation. The concentration of acetoin and OD_{600} were monitored every 2 hours over a period of 8 hours and at time 21.5 h after inoculation. The means of triplicates with standard deviations are shown.

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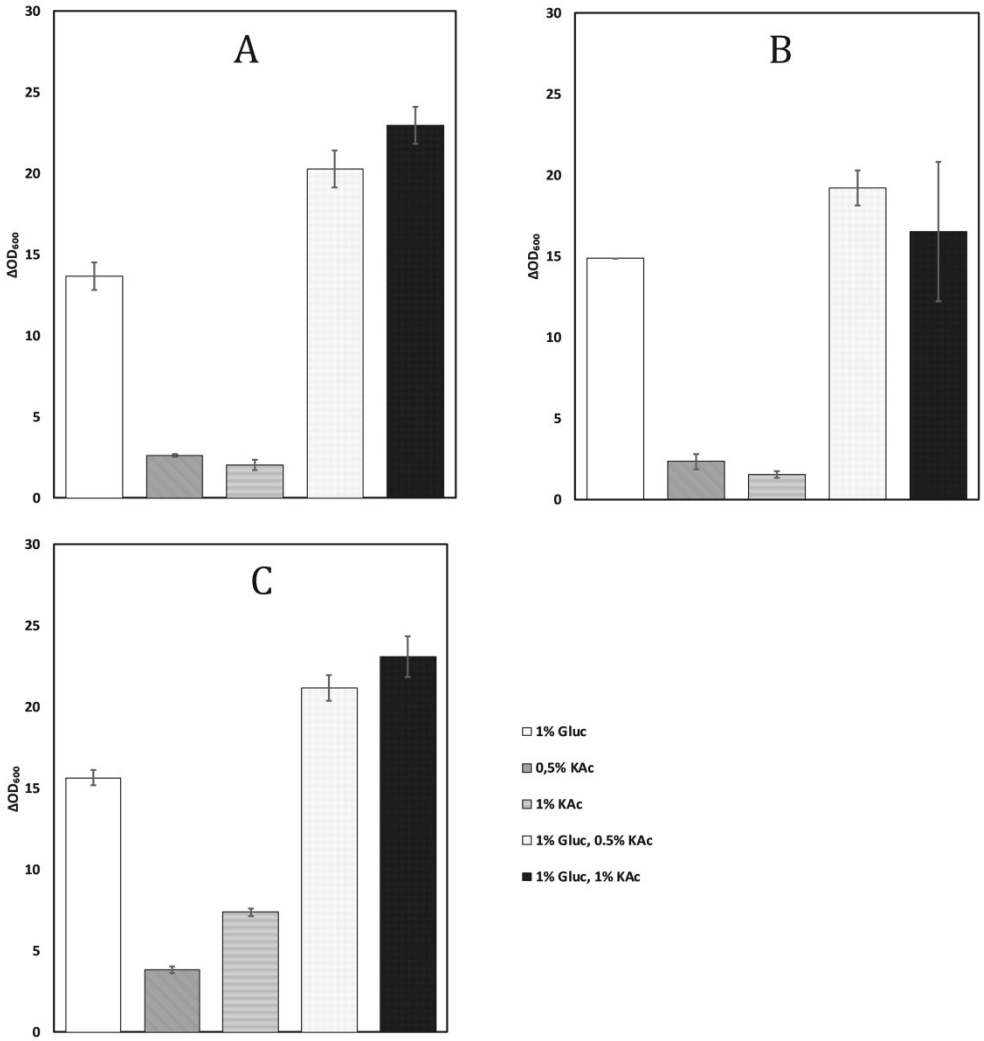

Figure 5 Complementation of *C. glutamicum* Δ*aceAB* with *B. methanolicus* MGA3 genes. ΔOD_{600} of strains MH20-22b(pEC-XT99A) a) MH20-22B Δ*aceAB*(pEC-XT99A); b) MH20-22B Δ*aceAB*(pEC-XT99A-*aceBA*^{Bm}); c) after growth with glucose (gluc) and/or potassium acetate (KAc). The means of triplicates with standard deviations are shown.

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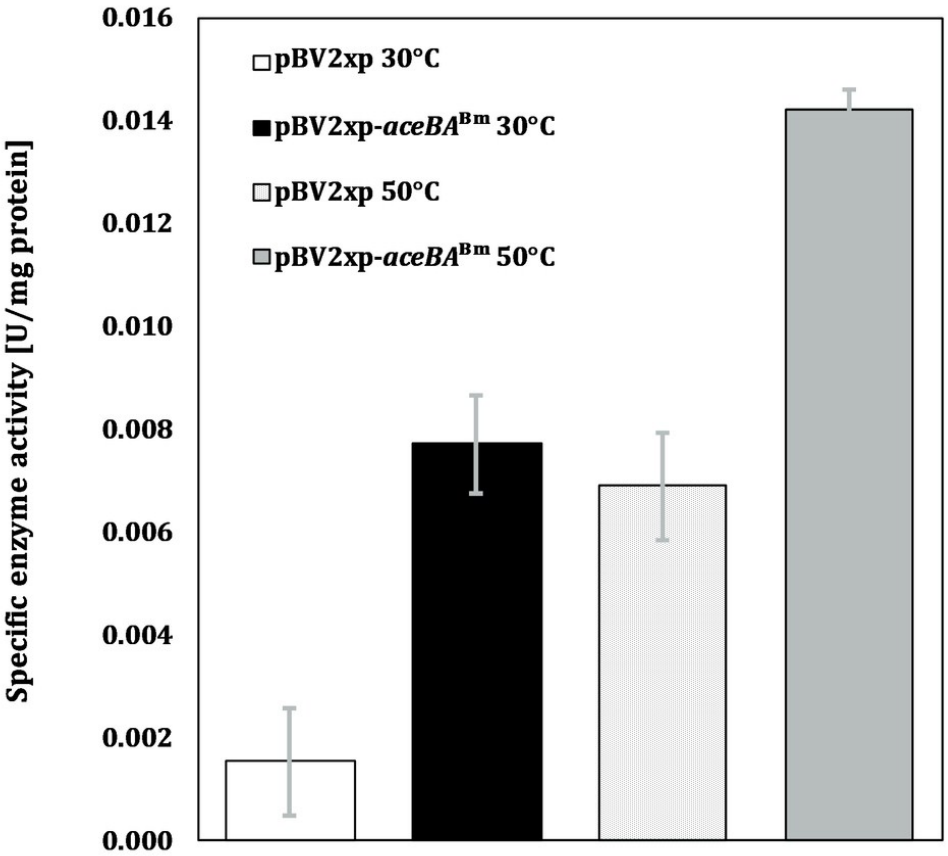

Figure 6 *In vitro* isocitrate lyase specific activity in *B. methanolicus* MGA3 crude extracts at 30°C and 50°C. The means of triplicates with standard deviations are shown.

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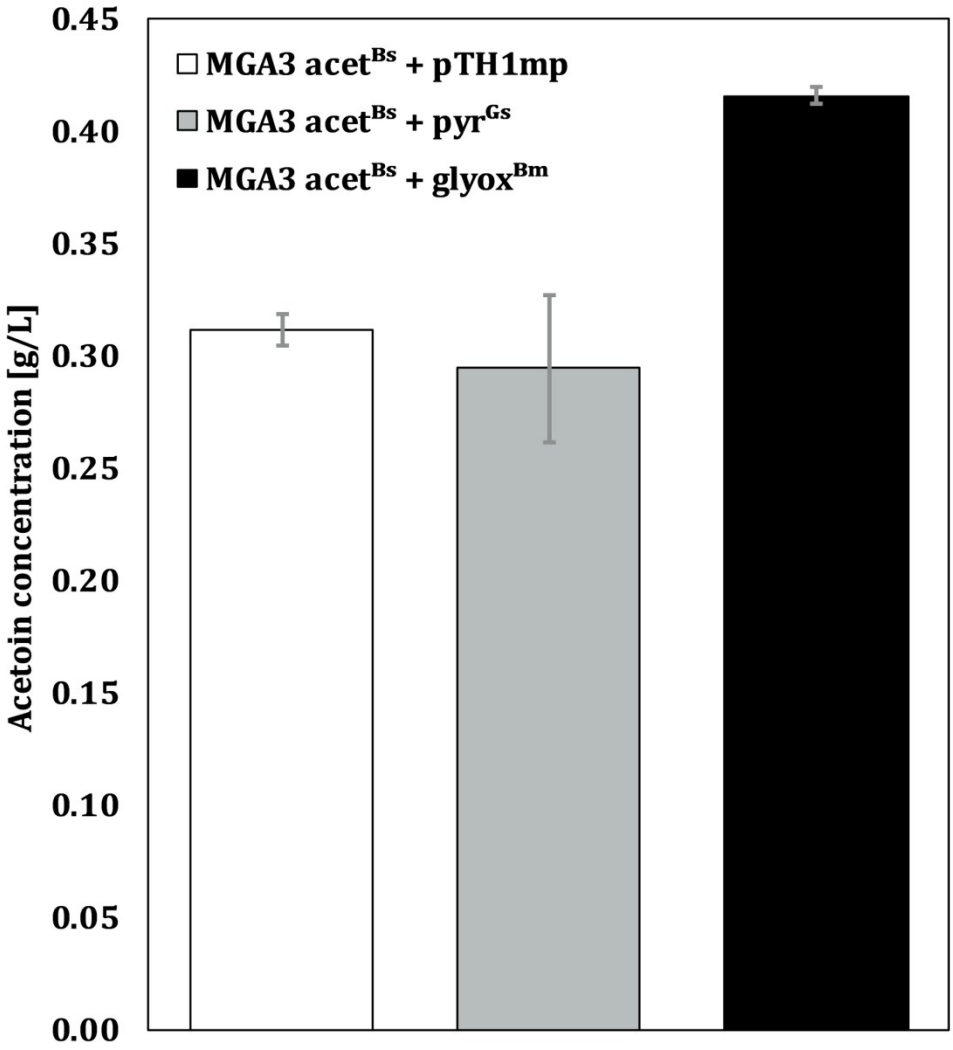

Figure 7 Acetoin production in MGA3 acetBs strain with overexpressed genes coding for the glyoxylate shunt pathway compared to control strains. The strains were grown in minimal medium with 200 mM methanol and induced with 10 g/L of xylose added to the growth medium two hours after inoculation. The concentration of acetoin was measured at time 25 h after inoculation. The means of triplicates with standard deviations are shown.

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