Co-Production of Hydrogen and Ethyl Acetate in E. Coli

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Research

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Posted Date: June 28th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-631018/v1

Co-production of hydrogen and ethyl acetate in *E. coli*

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Abstract

Background:

Ethyl acetate and hydrogen are industrially relevant compounds that preferably are produced via sustainable, non-petrochemical production processes. Both compounds are volatile and can be produced by *Escherichia coli* before. However, relatively low yields for hydrogen are obtained and a mix of by-products render the sole production of hydrogen by micro-organisms unfeasible. High yields for ethyl acetate have been achieved but accumulation of formate remained an undesired but inevitable obstacle. Coupling ethyl acetate production to the conversion of formate into H_2 may offer an interesting solution to both drawbacks. Ethyl acetate production requires equimolar amounts of ethanol and acetyl-CoA, which enables a redox neutral fermentation, without the need for production of by-products, other than hydrogen and CO_2.

Results:

We engineered *Escherichia coli*, towards improved conversion of formate into hydrogen and CO_2 by inactivating the formate hydrogen lyase repressor (*hycA*), both uptake hydrogenases (*hyaAB*, *hybBC*) and/or overexpressing the hydrogen formate lyase activator (*fhlA*). Initially 10 strains were evaluated in anaerobic serum bottles with respect to growth, after which four strains were further analyzed. Anaerobic co-production of hydrogen and ethyl acetate via heterologous ethanol acyltransferase (Eat1) was achieved in 1.5-L pH controlled bioreactors.

Conclusions:

We showed that the engineered strains co-produced ethyl acetate and hydrogen to yields exceeding 70 % of the pathway maximum for ethyl acetate and hydrogen, and propose *in-situ* product removal via gas stripping as efficient technique to isolate the products of interest.

Key words: ethyl acetate, hydrogen, co-production, fermentation, Escherichia coli, Eat1, formate hydrogen lyase

Background

Esters are a diverse group of compounds important not only for the food industry but also for various industrial purposes (Kruis *et al.*, 2019). Ethyl acetate is among the most relevant esters with respect to industrial use. It is considered relatively environmentally friendly and thus a popular solvent used in paints and adhesives, and other applications.

Yeasts are natural producers of a variety of esters, including ethyl acetate. Efforts have been made to understand and direct ester production and composition, focusing on bulk producers of ethyl acetate, including *Kluyveromyces marxianus (K. marxianus*) and *Wickerhamomyces anomalus (W. anomalus*) (Sabel *et al.*, 2014; Löser, Urit, Keil, *et al.*, 2015; Kruis *et al.*, 2017). Especially *K. marxianus* has been exploited and optimized with respect to efficient ethyl acetate production. In fermentations on whey based medium a yield of 0.265 $g_{ethyl\ acetate}/g_{sugar}$, corresponding to 50 % of the maximum yield, was reached in a 70-L reactor, demonstrating the scalability of the system (Löser *et al.*, 2013). Recently, we have shown that a heterologous expression system in *Escherichia coli* (*E. coli)* can compete with natural producers in terms of ethyl acetate yields (Bohnenkamp *et al.*, 2020). A streamlined *E. coli* strain harboring a truncated ethanol acetyltransferase (*eat1*) gene from *W. anomalus* reached 72 % of the maximum pathway yield on glucose under anoxic conditions. This is the highest reported yield to date.

In contrast to yeasts that use pyruvate decarboxylase to convert pyruvate to acetaldehyde, *E. coli* uses pyruvate formate lyase to produce acetyl-CoA during anaerobic conditions (Bohnenkamp *et al.*, 2020). This ultimately results in a redox and carbon balanced pathway under anoxic conditions, contributing to the overall efficiency of the process as less carbon is lost to biomass or respiration (Weusthuis 2011). However, as *E. coli* uses pyruvate formate lyase, one mole of formate is coproduced with every conversion of pyruvate into acetyl-CoA, coproducing two moles of formate per generated mole of ethyl acetate (Figure 1).

Formate is accumulating during the fermentation process, acidifying the medium and causing inhibiting effects on the *E. coli* cells. While the acidification of the medium can be prevented by pH-control of the reactor, buildup of formate to inhibiting concentrations may nevertheless negatively affect performance of the system. Formate concentrations below 100 mM already severely hamper *E. coli* growth, and concentrations of 50 mM have been reported to cause growth inhibition of 50 % (Zaldivar, Martinez and Ingram, 1999). One way *E. coli* counteracts these negative side-effects of formate, is by converting it to CO_2 and H_2 by a membrane bound formate hydrogen lyase (Fhl) after formate concentrations exceed a certain threshold (McDowall *et al.*, 2014).

Hydrogen is considered an attractive, environmentally friendly energy carrier, but 95 % of the current production is still derived from non-renewable resources (Balat and Kirtay, 2010; Nikolaidis and Poullikkas, 2017). In order to benefit from hydrogen as future fuel also its production needs to rely on sustainable methods paving the path for green or bio-hydrogen (Levin and Chahine, 2010; Dincer and Acar, 2014; Stephen *et al.*, 2017). Regarding microbial hydrogen production attention has been paid to increasing yields and productivity by means of genetic engineering, with a strong focus on *E. coli*. While *E. coli* primarily secretes formate and naturally is a poor hydrogen producer, the complexity and transcriptional regulation of the Fhl complex with the involvement of around 15 genes is well understood (Zinoni *et al.*, 1984; Birkmann *et al.*, 1987; Rossmann, Sawers and Böck, 1991). Due to its annotated genome and well established genetic engineering tools, several targets and strategies for improving hydrogen production have been identified (Maeda, Sanchez-Torres and Wood, 2012).

Several studies used formate as substrate for the production of bio-hydrogen from *E. coli* (Penfold, Forster and Macaskie, 2003; Yoshida *et al.*, 2005). Inactivating the Fhl repressor *hycA* was among the first modifications to promote Fhl activity, thus enhancing hydrogen production (Penfold, Forster and Macaskie, 2003). Combining *hycA* deactivation and overexpression of the formate hydrogen lyase transcriptional activator (FhlA) further improved strain performance (Yoshida *et al.*, 2005). In addition, Maeda and colleagues studied the effect

of various modifications concerning hydrogen production and uptake, extensively (Maeda, Sanchez-Torres and Wood, 2007). They found that besides inactivating *hycA* and overexpressing *fhlA*, inactivation of hydrogen uptake by knocking out hydrogenase 1 (*hyaB*) and 2 (*hybC*) further benefitted hydrogen production. Moreover, inactivating *hycA hyaB hybC* together with inactivating the formate transporter *focA* did not impact growth of *E. coli* under aerobic conditions, while leading to an almost 5-fold increased hydrogen production capacity with respect to wild-type *E. coli* (Maeda, Sanchez-Torres and Wood, 2008).

However, to date microbial hydrogen production with sole focus on generation of bio-hydrogen is considered rather unfeasible mainly due to the low conversion efficiency and low maximum yields obtained (Nikolaidis and Poullikkas, 2017). Therefore coupling it to the production of another relevant product may improve the overall feasibility of such process as shown with the example of ethanol (Sundara Sekar *et al.*, 2016; Stephen *et al.*, 2017; Lopez-Hidalgo, Balderas and de Leon-Rodriguez, 2021). However, ethanol and hydrogen are competing for electrons and maximum yields for one product will automatically decrease the achievable yield for the other product.

Here, we describe the efficient co-production of ethyl acetate and bio-hydrogen using an engineered *E. coli* strain, while restricting product accumulation by *in-situ* product removal.

Figure 1: Schematic representation of anaerobic ethyl acetate production from glucose in *E. coli* via the Embden-Meyerhof-Parnas (EMP) pathway with hydrogen co-production. Lactate and acetate formation is limited by ack and ldh inactivation. Heterologous alcohol acetyltransferase Eat1 generates ethyl acetate from ethanol and acetyl-CoA. Hydrogen co-production is achieved via formate hydrogen lyase (Fhl). Ack – acetate kinase, DHAP – dihydroxyacetone phosphate, eat1 – ethanol acetyltransferase, FBR – fructose 1,6-bisphosphate, F6P – fructose 6-phosphate, G6P – glucose 6-phosphate, PEP – phosphoenolpyruvate, GAP – glyceraldehyde 3-phosphate, pta – phosphate acetyltransferase

Results

Increasing hydrogen gas production

A series of modifications to a *BW25113 ΔldhA ΔackA* (BW25113 *ΔΔ)* background strain were applied in order to improve the conversion of formate into hydrogen. Sequential inactivation of

the Fhl repressor hycA, and the uptake hydrogenases *hyaAB* and *hybBC*, were combined with overexpression of the Fhl activator fhlA. A first evaluation of strains took place in anaerobic serum bottles with ethanol, pyruvate and formate as main fermentation outputs. Due to the *ackA* knockout in *BW25113 ΔΔ*, NADH requirements for ethanol formation cannot be balanced by co-production of acetate but are met by secretion of the intermediate metabolite pyruvate.

Neither the three individual knock-out events, nor a combination thereof, did have any effect on growth rates of the resulting strains when compared to their parental strain *BW25113 ΔΔ* (Figure 2). After 72 h of cultivation all strains reached an OD_{600} of around 0.64. Overexpression of fhlA was achieved by introduction of the *p3* promoter in front of the start codon of the native *fhlA*. This modification slightly affected growth of the double knockout strain *BW25113 ΔldhA ΔackA p3-fhlA (BW25113 ΔΔ p3-fhlA*) as well as in the quintuple knockout strain *BW25113 ΔldhA ΔackA ΔhycA ΔhybBC ΔhyaAB p3-fhlA* (*BW25113 ΔΔΔΔΔ p3-fhlA*) (Figure 2). Overexpression of fhlA led to a reduced OD_{600} after 72 h, 15 % lower compared to parental strains relying on native expression of fhlA.

Figure 2: OD_{600} after 72h of cultivation under anaerobic conditions with glucose as carbon source of a *BW25113 ΔldhA ΔackA* background strain containing additional KOs and/or overexpressing fhlA for improved hydrogen production. Initial OD_{600} was 0.2. Data and error bars indicate averages and standard deviations among duplicates

At the same time strains overexpressing fhlA consumed about 30 % less glucose, resulting in less ethanol, pyruvate and formate production (Figure 3abcd). Despite knocking out *ackA* some acetate production could not be avoided and reached levels around 6 mM for all strains tested (Figure 3e). Succinate titers reached 3.96 ± 0.2 mM for the parental strain *BW25113 ΔΔ*, but were increased by 10 % to 50 % by strains with additional modifications towards hydrogen production, likely due to increased CO_2 availability (Figure 3f).

It is difficult to determine hydrogen and carbon dioxide gas production accurately in serum bottles. The effect of the genetic modifications on the production of both gasses was therefore estimated indirectly, by subtracting the amount of formate produced from the amount of ethanol plus acetate formed to obtain a calculated hydrogen concentration (mM). For

estimating CO_2, fixation for succinate synthesis was included, but not CO_2 production associated with biosynthesis. This resulted in slightly negative calculated CO_2 concentrations (Figure 3g). While for the parental strain no H_2 could be calculated, the other strains generated between 2 and 8 mM (Figure 3h). However, variations in formate accumulation and conversion among duplicates led to large error bars in calculated concentrations.

Figure 3: Concentrations of glucose and products after 72 h of anaerobic cultivation for strains with a *ΔldhA ΔackA (ΔΔ*) background and further modifications for improved hydrogen production, from left to right: inactivation of *hycA, hyaAB* and *hybBC* (*ΔΔΔΔ*), overexpression of *fhlA* (*ΔΔ p3-fhlA*) and a combination of knockouts and overexpression (*ΔΔΔΔ p3-fhlA*). For CO_2 and H_2, data represent calculated concentrations. Data shows average values and standard deviations from biological duplicates.

Ethanol yields on glucose dropped by 12 % for strains overexpressing fhlA in respect to *BW25113 ΔΔ* and *BW25113 ΔΔΔΔΔ* for which yields of about 0.8 $mol_{ethanol}/mol_{glucose}$ were obtained (Figure 4). However, succinate yields significantly increased and doubled for *BW25113 ΔΔ p3-fhlA* and *BW25113 ΔΔΔΔΔ p3-fhlA* ($p<0.05$). For strain *BW25113 ΔΔΔΔΔ* the hydrogen yield on glucose was only 0.02 $mol_{hydrogen}/mol_{glucose}$. Both strains overexpressing fhlA reached a higher yield, around 0.1 and 0.25 $mol_{hydrogen}/mol_{glucose}$ respectively. However, due to variations in the replicas only *BW25113 ΔΔΔΔΔ* and *BW25113 ΔΔΔΔΔ* p3-fhlA showed significant increase in hydrogen yields ($p<0.05$).

Figure 4: Product yield on glucose on selected products after 72h of anaerobic fermentation for strains based on *ΔldhA ΔackA* (*ΔΔ*) with further modifications for improved hydrogen production, from left to right: inactivation of hycA, hyaAB and hybBC (*ΔΔΔΔΔ*), overexpression of fhlA (*ΔΔ p3-fhlA*) and a combination of knockouts and overexpression (*ΔΔΔΔΔ p3-fhlA*). Values are averages of two biological replicates and error bars represent standard deviations.

Concluding, the effect of the subsequent inactivation steps in strain *BW25113 ΔΔΔΔΔ* remains elusive while overexpression of fhlA supports hydrogen production. On the other hand, overexpression causes a reduction in biomass formation and slower glucose consumption.

Combining hydrogen gas and ethyl acetate production

After initial screening experiments and indirect performance assessments, three strains were generated with the purpose of co-producing hydrogen and ethyl acetate from glucose as carbon source. Strains *BW25113 Δldh Δack* p3-fhlA *(BW25113 ΔΔ p3-fhlA)*, *BW25113 Δldh*

Δack ΔhycA ΔhyaAB ΔhybBC (BW25113 ΔΔΔΔΔ) and *BW25113 Δldh Δack ΔhycA ΔhyaAB ΔhybBC p3-fhlA (BW25113 ΔΔΔΔΔ p3-fhl)* were equipped with the plasmid that encoded the ethanol acetyltransferase, pET26b:*T7/LacI-trEat1 Wan N13 (*trEat1*)* and gene expression was induced by 0.01 mM IPTG. Anaerobic ethyl acetate and hydrogen co-production were assessed in pH-controlled 1.5-L bioreactors with a continuous N_2 gas flow of 100 mL/min coupled to online MS measurements of the off gas. In this way stripped ethyl acetate, as well as produced CO_2 and H_2 could be measured and quantified.

Similar to observations during the serum bottle experiments, overexpression of fhlA led to a decrease in maximum OD_{600} and slower glucose conversion (Figure 5ab). In contrast, however, knocking out the formate hydrogen lyase repressor and both uptake hydrogenases improved overall fermentation performance of *BW25113 ΔΔΔΔΔ* trEat1 including a reduced total fermentation time by about 35 %. Expression of Eat1 and synthesis of ethyl acetate in a redox balanced way, apparently lifted the earlier observed NADH shortage and therefore prevented pyruvate excretion almost completely (Supplementary File 1). Gas stripping kept overall ethyl acetate levels in the fermentation broth well below 10 mmol and resulted in a cumulative amount of stripped ethyl acetate near to 20 mmol (Figure 5c). Formation of other by-products such as ethanol, acetate and succinate were mostly similar among all strains and did not exceed 10 mmol per compound (Figure 5def). However, *BW25113 ΔΔΔΔΔ* trEat1 did accumulate more than twice as much succinate as the remaining strains. Formate secretion was reduced for all engineered strains, while H_2 and CO_2 accumulated to 4-times higher levels than the control strain without modifications in Fhl regulation or hydrogenases (*BW25113 ΔΔ* trEat1) (Figure 5ghi).

Figure 5: Fermentation profile of four strains engineered for ethyl acetate and hydrogen co-production in pH-controlled bioreactors with continuous gas stripping. Strains based on *ΔldhA ΔackA* (*ΔΔ*) with further modifications for improved hydrogen production, from left to right: inactivation of hycA, hyaAB and hybBC (*ΔΔΔΔΔ*), overexpression of fhlA (*ΔΔ p3-fhlA*) and a combination of knockouts and overexpression (*ΔΔΔΔΔ p3-fhlA*) producing trEat1 Wan N-13 were induced by 0.01 mM IPTG and cultivated under anaerobic conditions in minimal medium with 55 mM glucose as carbon source.. Experiments were performed as biological duplicates; error bars represent the standard deviation. Circles – compounds in liquid broth, triangle – compounds in off-gas.

With respect to product yields no significant differences in ethyl acetate yields on glucose could be found. With yields ranging from 0.63 ± 0.03 to 0.71 ± 0.04 $mol_{ethyl\ acetate}/mol_{glucose}$ about 70% of the pathway maximum was reached (Figure 6a). The overall carbon yield Y_{Carbon} was 92 % or higher for all strains (Supplementary Table 1). Knocking out *hycA, hyaAB, hybBC*, as well as overexpressing fhlA significantly improved hydrogen yields, reaching 50 % and more of the pathway maximum. For the strain overexpressing fhlA (*BW25113 ΔΔ p3-fhlA* trEat1) the highest hydrogen yield was obtained with 1.47 ± 0.11 $mol_{hydrogen}/mol_{glucose}$, corresponding to 73% of the pathway maximum.

Figure 6: Effect of modifications towards improved hydrogen production on product yields and productivities for main fermentation products, with from left to right: inactivation of hycA, hyaAB and hybBC, overexpression of fhlA and a combination of knockouts and overexpression. Strains producing trEat1 *Wan* N-13 were induced by 0.01 mM IPTG and grown under anaerobic conditions in minimal medium containing 55 mM glucose using pH-controlled bioreactors with 0.5 L working volume. (a) Product yields for ethyl acetate, hydrogen and formate in $mol_{product}/mol_{glucose}$ after glucose depletion. The numbers above the bars represent the carbon recovery of the fermentations. (b) Volumetric productivities for ethyl acetate, hydrogen, and formate in mmol/L/h. Experiments were performed as biological duplicates or triplicates; error bars represent the standard deviation. Abbreviations: trEat1 – truncated Eat1 Wan N-13.

Despite that the product yield for ethyl acetate was rather similar, productivity of ethyl acetate did differ among the different strains. *BW25113 ΔΔΔΔΔ* trEat1 showed an improved ethyl acetate production by 41 % ($p=0.052$), while both fhlA overexpression strains showed a drop in productivity by 25-30 %, which was however, not statistically significant (Figure 6b). Regarding the co-production of hydrogen, all modifications led to a significant increase in conversion of formate into hydrogen and concomitantly CO_2 ($p<0.05$). The highest hydrogen productivity of 3.5 mmol/L/h was reached by *BW25113 ΔΔΔΔΔ* trEat1. Unexpectedly, overexpression of fhlA led to hydrogen production rates of only 2 mmol/L/h regardless whether only fhlA was overexpressed or additional knockouts were carried out.

Discussion

The current study demonstrates how anaerobic ethyl acetate production can be coupled to efficient hydrogen co-production thereby improving overall fermentation performance of the

system. With an ethyl acetate yield on glucose close to 70 % of the pathway yield *E. coli* can compete with natural producers, like *K. marxianus* (Löser *et al.*, 2013) and performs close to earlier reported values using a truncated version of *W. anomalus* Eat1 (Bohnenkamp *et al.*, 2020).

Inactivation of the uptake hydrogenases (*hyaAB* and *hybBC*) and the Fhl repressor (*hycA*) led to 4-times higher hydrogen production rates relative to the control strain. While other studies found that those modifications did not negatively affect growth rates, here, the strain performance was even slightly improved during batch reactor fermentations (Maeda, Sanchez-Torres and Wood, 2008). This is likely a consequence of reduced formate concentrations, that may impose inhibitory effects to the cells (Zaldivar, Martinez and Ingram, 1999).

Hydrogen yields realized by modified strains ranged from 1 – 1.47 $mol_{hydrogen}/mol_{glucose}$, thus the improvements are comparable to earlier reported values around 1.15-1.8 $mol_{hydrogen}/mol_{glucose}$ (Yoshida *et al.*, 2006; Maeda, Sanchez-Torres and Wood, 2007; Kim *et al.*, 2009; Mathews, Li and Wang, 2010; Sundara Sekar *et al.*, 2016). Overexpression of the Fhl activator fhlA using the *p3* promoter, led to the highest hydrogen yields on glucose in *BW25113 ΔΔ p3-fhlA* trEat1, with a product yield of 1.47 $mol_{hydrogen}/mol_{glucose}$ respectively. However, for this strain also reduced biomass formation and reduced production rates of hydrogen and ethyl acetate were observed. In previous research, overexpression of fhl from a low copy number plasmid improved growth rates and hydrogen production from formate (Yoshida *et al.*, 2005). Also on glucose no impact of overexpression was noted using an IPTG-inducible expression system while the plasmid insertion itself did reduce the growth rate of the strain and also impacted growth rates during aerobic cultivation on formate (Maeda, Sanchez-Torres and Wood, 2007, 2008). Therefore, finetuning the overexpression with different promoters or inducible expression systems, combined with adaptation seems necessary to keep the hydrogen overexpression strains competitive. While the applied modifications reportedly improve hydrogen (co-) production, there are still options to inactivate formate

exporters (focA) or other formate consuming enzymes including formate dehydrogenase-N (FdnG), dehydrogenase-O (FdoG), or nitrate reductase A (NarG) that positively impacted hydrogen production (Maeda, Sanchez-Torres and Wood, 2007).

In the mentioned studies, efficient hydrogen producing strains also carried an *frdAB* inactivation to eliminate succinate formation, which should be considered when optimizing further towards the maximum pathway yield of 2 $mol_{hydrogen}/mol_{glucose}$. Especially for strain *BW25113 ΔΔΔΔΔ* trEat1 the succinate yield was 2-times higher than the parental strain and may have masked the positive effects of hydrogen production as carbons were deviated from the intended co-product ethyl acetate.

Complete suppression of acetate formation is challenging and inactivation of *ackA* or *pta* often leads to a reduction in acetate accumulation only (Vuoristo *et al.*, 2015; Li *et al.*, 2017). Inactivation of the full *ackA-pta* operon, could help to lower acetate accumulation to negligible amounts (Vadali *et al.*, 2004; Seol *et al.*, 2014). Additionally, acetate may originate from Eat1 thiolysis or esterase side-activities converting ethyl acetate or acetyl-CoA into acetate (Bohnenkamp *et al.*, 2020; Patinios *et al.*, 2020). Eliminating side-activities by protein engineering may be one way to overcome this drawback of Eat1. Here, we applied gas stripping to remove ethyl acetate more efficiently and reduce the residence time in the fermentation broth. Next to product degradation, product toxicity is another factor tackled with this strategy (Löser, Urit, Gruner, *et al.*, 2015; Kruis *et al.*, 2019). Like most products, ethyl acetate can accumulate to toxic concentrations, thereby imposing inhibitory effects to the cells. For *E. coli* the threshold is estimated for ethyl acetate titers above 110 mM (Wilbanks and Trinh, 2017). While this concentration was not and could not be reached under the tested conditions, gas stripping will become more important once the process is further upscaled. Moreover, the production of H_2 and CO_2 instead of formate, also benefits from gas stripping and enables continuous removal of both products of interest.

Low hydrogen yields during fermentation in expression hosts like *E. coli* combined with a mix of other fermentation products is a major drawback in microbial hydrogen production (Maeda, Sanchez-Torres and Wood, 2012; Stephen *et al.*, 2017). Besides efficient production of hydrogen, production of only one other main fermentation product remains challenging Especially high yield production of ethanol is often limited by NAD(P)H availability. Since NAD(P)H is only produced during the EMP pathway (GAP oxidation), ethanol formation can only amount to 1 $mol_{ethanol}/mol_{glucose}$, with the concomitant formation of 1 $mol_{acetate}/mol_{glucose}$. Higher ethanol yields requires additional NAD(P)H. Various engineering approaches have been used to generate extra NAD(P)H; Sundara Sekar et al. (2016) employed a partial pentose phosphate pathway, which resulted in co-production of ethanol and hydrogen, with limited by-products formation or loss of growth, reaching yields for ethanol and hydrogen on glucose of 1.4 $mol_{ethanol}/mol_{glucose}$ and 1.88 $mol_{hydrogen}/mol_{glucose}$ respectively. Others made use of a pyruvate dehydrogenase instead of the pyruvate formate lyase yielding more NAD(P)H and reaching ethanol yields of 1.8 $mol_{ethanol}/mol_{glucose}$ (Zhou, Iverson and Grayburn, 2008). The latter obviously occurs at the expense of formate or hydrogen. Thus, optimal co-production of hydrogen and one other product requires a redox balanced acetyl-CoA conversion. The production of ethyl acetate as demonstrated here enables such redox neutral acetyl-CoA conversion and simultaneously co-production of hydrogen at its theoretical maximum of 2 $mol_{hydrogen}/mol_{glucose}$. With the co-production of ethyl acetate and hydrogen from glucose of 0.71 $mol_{ethyl\ acetate}/mol_{glucose}$ and 1.47 $mol_{hydrogen}/mol_{glucose}$ for strain *BW25113 Δldh Δack p3-fhlA* pET26b:Eat Wan N13 we successfully provide a first outlook on the applicability of this strategy towards another industrially relevant compound.

Conclusion

Modification of the Fhl regulation system is an effective way to improve hydrogen production in *E. coli*. Overexpression of the Fhl activator *fhlA*, but also the inactivation of the Fhl repressor *hycA* and hydrogenases 1 and 2 by knocking out *hyaAB* and *hybBC* improved hydrogen production 4-fold. During anaerobic fermentation of *BW25113 Δldh Δack p3-fhlA*

pET26b:*T7/LacI-trEat1 Wan* N-13 on glucose 70 % of the pathway yields for ethyl acetate and hydrogen, 0.695 $mol_{ethyl\ acetate}/mol_{glucose}$ and 1.44 $mol_{hydrogen}/mol_{glucose}$ respectively, were obtained. Cultivation of *BW25113 ΔldhΔackΔhycAΔhyaABΔhybBC* pET26b: *T7/LacI-trEat1 Wan* N-13 led to highest ethyl acetate and hydrogen production rates, being 1.41- and 4-fold higher than the parental strain that mainly accumulated formate. Coupled to *in-situ* product removal by gas stripping both products can efficiently be produced and recovered, offering attractive downstream processing opportunities for co-production of bio-based ethyl acetate and green hydrogen by *E. coli*.

Methods

Strain and Plasmid construction

All strains and plasmids used can be found in Tables 1 and 2. Generation of genomic knockouts and insertion of *p3*-promoter (Mutalik *et al.*, 2013) was achieved by CRISPR-Cas9 (Jiang *et al.*, 2015). To generate the corresponding pTarget plasmid, a sequence containing gRNA module and the homologous sequences of 50 bp immediately upstream the start codon and downstream the stop codon were ordered as synthetic gBlocks (IDT). For insertion of the p3-promoter sequence, the homologous sequences were located 35 bp upstream and beginning with the start codon for the downstream sequence. Using 2X HiFi assembly master mix (NEB) according to manufacturer's instructions plasmids were assembled and propagated in competent NEB® 5-alpha cells. The pET26b:*T7/LacI-trEat1 Wan N-13* plasmid was inserted by following instructions from the Mix&Go *E. coli* Transformation Kit (ZYMO Research). PCR amplification was performed using Q5 polymerase (NEB).

Table 1: Strains used in this study

Strain	**Characteristics**	**Source**
Escherichia coli BW25113 (DE3)	Wild type with integrated DE3 lysogen	(Vuoristo *et al.*, 2015)
Escherichia coli BW25113 Δ*ackA*Δ*ldhA*	Disruption of lactate and acetate production (via *ackA*)	Kruis, 2020

Escherichia coli BW25113 Δ*ackA*Δ*ldhA p3-fhlA*	Disruption of lactate and acetate production (via *ackA*) and overexpression of formate hydrogen lyase transcriptional activator (fhlA)	This study
Escherichia coli BW25113 Δ*ackA*Δ*ldhA*Δ*hycA*	Disruption of lactate and acetate production (via *ackA*) and inactivation of Fhl repressor (*hycA*)	This study
Escherichia coli BW25113 Δ*ackA*Δ*ldhA*Δ*hyaAB*	Disruption of lactate and acetate production (via *ackA*) and inactivation of uptake hydrogenase (hyaAB)	This study
Escherichia coli BW25113 Δ*ackA*Δ*ldhA*Δ*hybBC*	Disruption of lactate and acetate production (via *ackA*) and inactivation of uptake hydrogenase (hybBC)	This study
Escherichia coli BW25113 Δ*ackA*Δ*ldhA*Δ*hycA*Δ*hyaAB*	Disruption of lactate and acetate production (via *ackA*) and inactivation of Fhl repressor (hycA) and uptake hydrogenase (hyaAB)	This study
Escherichia coli BW25113 Δ*ackA*Δ*ldhA*Δ*hycA*Δ*hybBC*	Disruption of lactate and acetate production (via *ackA*) and inactivation of Fhl repressor (hycA) and uptake hydrogenase (hybBC)	This study
Escherichia coli BW25113 Δ*ackA*Δ*ldhA*Δ*hyaAB*Δ*hybBC*	Disruption of lactate and acetate production (via *ackA*) and inactivation of uptake hydrogenases (hyaAB, hybBC)	This study
Escherichia coli BW25113 Δ*ackA*Δ*ldhA*Δ*hycA*Δ*hyaAB*Δ*hybBC*	Disruption of lactate and acetate production (via *ackA*) and inactivation of Fhl repressor (hycA) and uptake hydrogenases (hyaAB and hybBC)	This study
Escherichia coli BW25113 Δ*ackA*Δ*ldhA*Δ*hycA*Δ*hyaAB*Δ*hybBC p3-fhlA*	Disruption of lactate and acetate production (via *ackA*) and inactivation of Fhl repressor (hycA) and uptake hydrogenases (hyaAB and hybBC) with overexpression of Fhl activator (fhlA)	This study
Escherichia coli T7 Express	fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(LacI::PlacUV5::T7 gene1) i21 Δnin5	NEB
Escherichia coli NEB® 5-alpha	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	NEB

Table 2: Plasmids used in this study

Plasmid	**Promoter**	**Gene/Protein**	**Source**
pET26b	LacI/*T7*	/	This study
pET26b:hWan trEat1 N-13	LacI/*T7*	Codon harmonised *eat1* from *Wickerhamomyces anomalus* DSM 6766	Kruis, 2020
pCas9	/		(Jiang *et al.*, 2015)
pTarget	/		(Jiang *et al.*, 2015)
pTarget-*hycA*	/		This study
pTarget-*hyaAB*	/		This study
pTarget-*hybBC*	/		This study
pTarget-p3	/		This study

Cultivation

Strains were routinely cultured on LB medium with supplementation of spectinomycin (50 µg/mL) and/or kanamycin (50 µg/mL) when appropriate. Starting from single colonies, overnight cultures for transformations or experiments were inoculated into 10 mL LB medium in a 50-mL tube and grown at 30°C and 250 rpm. For pre-cultures and anaerobic experiments, 250-mL Erlenmeyer flasks or serum bottles were filled with 50 mL modified M9 medium consisting of M9 salts (Difco, 1X), glucose (55 mM), $MgSO_4$ (2 mM), $CaCl_2$ * 2 H_2O (0.1 mM), MOPS (100 mM) and 1X trace elements and vitamin solutions based on Verduyn *et al.*, 1992. The serum bottles were capped and flushed with nitrogen gas for anoxic conditions. From overnight cultures 1-2 mL were transferred to 50 mL modified M9 medium in 250-mL Erlenmeyer flasks and grown at 30°C and 250 rpm. Strains for anaerobic experiments were inoculated as biological duplicates at an initial OD_{600} of 0.2 and incubated at 30°C and 150 rpm.

Batch reactor fermentation

Batch fermentations were performed in 1.5-L bioreactors (Applikon) with a working volume of 0.5 L as described before (Bohnenkamp *et al.*, 2020). Defined medium contained glucose (55 mM), $(NH_4)_2SO_4$ (37.8 mM), KH_2PO_4 (22 mM), NaCl (171 mM), kanamycin (100 µg/mL), Na_2SeO_3 (0.3 mg/L) and 1X trace elements and vitamin solutions Verduyn *et al.*, 1992. Eat1 gene induction was achieved by addition of 0.01 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Stirring at 400 rpm with a Rushton turbine was controlled by a ADI 1012 Motor Controller (Applikon), the target pH of 7 was maintained by automated addition of 3 M KOH solution and a temperature of 30°C was achieved by a Thermo Circulator ADI 1018 (Applikon). Oxygen impermeable Marprene tubing (Watson-Marlow) and a gas flow of 6 L/h N_2 set the framework for anaerobic conditions. Pre-cultures were prepared as stated above and used to inoculate the reactors to a starting OD_{600} of 0.4. Liquid samples were taken regularly via a sampling port to assess optical density and composition of the fermentation broth. Metabolites were analyzed by high performance liquid chromatography (HPLC) and gas chromatography coupled to a flame ionization detector (GC). The off-gas composition was determined by online measurements of a δB Process Mass Spectrometer (MS, Thermo Scientific™).

Calculations

During anaerobic serum bottle experiments, H_2 and CO_2 production was estimated indirectly. Calculated H_2 and CO_2 concentrations (C in mol/L) were derived by assuming that significant production of either compound is solely attributed to Fhl activity, thus following the stoichiometric relation as shown in Equation 1:

$$n\,CH_2O_2 \rightarrow n\,CO_2 + n\,H_2 \qquad \text{(Eq. 1)}$$

The deficit in formate measured and formate expected due to acetate and ethanol formation, combined with Equation 1 leads to Equation 2 with C_C (mol/L):

$$C_{H_2} = \left(C_{C_2H_5OH} + C_{CH_3COOH}\right) - C_{CH_2O_2} \qquad \text{(Eq. 2)}$$

For CO_2 calculations also the incorporation of CO_2 during the synthesis of succinate needs to be accounted for. Therefore Eq. 2 is expanded to Eq. 3 for calculated CO_2 concentrations (mol/L):

$$C_{CO_2} = \left(C_{C_2H_5OH} + C_{CH_3COOH}\right) - C_{CH_2O_2} - C_{C_4H_6O_4} \quad \text{(Eq. 3)}$$

In batch reactor fermentations the off-gas composition was analyzed via online measurements via MS. Nitrogen, carbon dioxide, hydrogen, oxygen, ethanol and ethyl acetate fractions in the gas phase were considered and the cumulative amounts calculated as described in earlier research (Bohnenkamp *et al.*, 2020).

Carbon yields were estimated for all experiments according to Eq. 4 including glucose as substrate; ethyl acetate, ethanol, pyruvate, lactate, acetate, succinate, formate and CO_2 as products and biomass based on a conversion factor of 0.3232 from OD_{600} to g/L dry weight (Bohnenkamp *et al.*, 2020) and assuming a biomass composition of $CH_2O_{0.5}N_{0.2}$

$$Y_{Carbon} = \frac{\text{C-mol products formed}}{\text{C-mol glucose consumed}} \quad \text{(Eq. 4)}$$

Volumetric productivities (Q_P) were calculated in mmol/L/h by taking the slope of a linear trendline including at least four data points. For ethyl acetate productivity only three data points could be included (Supplementary Figure 2 and Table 2).

Statistical significance was assessed by using a two-sided students t-test assuming equal variance and $p<0.05$.

Analytics

Liquid samples, including 50 mM propionic acid as internal standard, were analyzed with respect to glucose and organic acids using an Agilent 1290 LC II system, with an Agilent 1290 Infinity Binary Pump, Agilent 1290 Infinity Autosampler, Agilent 1290 Infinity diode array detector operated at 210 nm, and an Agilent 1260 Infinity RI detector operated at 45 °C. The

406 HPLC was operated with an Aminex HPX-97H (Bio-Rad) column at 60°C and 0.008 mM
407 H_2SO_4 as mobile phase at 0.8 mL/min as flow rate.

408 Analysis of ethanol and ethyl acetate in the liquid phase was carried out by an Agilent 7890B
409 gas chromatograph equipped with a flame ionization detector (GC-FID) and an Agilent 7693
410 autosampler. Samples were injected into a Nukol™ column (30 m x 0.53 mm, 1.0 µm coating,
411 Supelco). Column temperature was maintained at 50 ˚C for 2 min, then increased to 200 ˚C
412 at the rate of 50 ˚C/min, with a split ratio of 10. As internal standard 2 mM 1-butanol was
413 added.

414 Online measurements of volatile compounds and gases removed from the reactor vessel by
415 gas stripping were performed with an δB Process Mass Spectrometer (MS, Thermo
416 Scientific™).

417

List of abbreviations

AAT	alcohol acetyltransferase
AckA	acetate kinase
C_C	concentration of compound C
DHAP	dihydroxyacetone phosphate
Eat1	ethanol acetyltransferase 1
EMP	Embden-Meyerhof-Parnas Pathway
F6P	fructose 6-phosphate
FBR	fructose 1,6-bisphosphate
FocA	formate transporter
FdoG	α-subunit of formate dehydrogenase-O
FdnG	α-subunit of formate dehydrogenase-N
Fhl	formate hydrogen lyase
FhlA	formate hydrogen lyase activator
G6P	glucose 6-phosphate
GAP	glyceraldehyde 3-phosphate
hyaAB	subunits hyaA and hyaB of uptake hydrogenase 1
hybBC	subunits hybB and hybC of uptake hydrogenase 2
hycA	formate hydrogen lyase repressor
IPTG	isopropyl β-D-1-thiogalactopyranoside

Kma	Kluyveromyces marxianus
Ldh	lactate dehydrogenase
narG	α-subunit of nitrate reductase A
pdc	pyruvate decarboxylase
pfl	pyruvate formate lyase
pta	phosphate acetyltransferase
Q_P	volumetric productivity of product P (mmol/L/h)
trEat1	N-terminally truncated ethanol acetyltransferase 1
Wan	Wickerhamomyces anomalus
Y_{Carbon}	Carbon yield (C-mol/C-mol)

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its additional information files.

Competing interests

The authors declare that they have no competing interests.

Funding

We would like to acknowledge Nouryon for funding the research.

Authors' contribution

AB, SK, RW, and RAW designed the work. AB conducted, analysed and interpreted the experiments. AB drafted and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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579

Appendix

Supplementary Figure 1: Fermentation profile for pyruvate in pH-controlled bioreactors with continuous gas stripping. Strains based on *ΔldhA ΔackA* (*ΔΔ*) with further modifications for improved hydrogen production, from left to right: inactivation of hycA, hyaAB and hybBC (*ΔΔΔΔΔ*), overexpression of fhlA (*ΔΔ p3-fhlA*) and a combination of knockouts and overexpression (*ΔΔΔΔΔ p3-fhlA*) producing trEat1 Wan N-13 were induced by 0.01 mM IPTG and cultivated under anaerobic conditions in minimal medium with 55 mM glucose as carbon source. Experiments were performed as biological duplicates; error bars represent the standard deviation. Circles – compounds in liquid broth, triangle – compounds in off-gas.

Supplementary Table 1: Product and carbon yield in C-mol$_{product}$/C-mol$_{glucose}$ for strains cultivated in pH-controlled bioreactors with constant gas stripping after glucose depletion. Strains based on *ΔldhA ΔackA* (*ΔΔ*) with further modifications for improved hydrogen production, from left to right: inactivation of hycA, hyaAB and hybBC (*ΔΔΔΔΔ*), overexpression of fhlA (*ΔΔ p3-fhlA*) and a combination of knockouts and overexpression (*ΔΔΔΔΔ p3-fhlA*) producing trEat1 Wan N-13 were induced by 0.01 mM IPTG and cultivated under anaerobic conditions in minimal medium with 55 mM glucose as carbon source.

	ΔΔ trEat1	ΔΔΔΔΔ trEat1	ΔΔ p3-fhlA trEat1	ΔΔΔΔΔ p3-fhlA trEat1
Ethyl acetate	0.47 ± 0.02	0.42 ± 0.02	0.47 ± 0.04	0.43 ± 0.01
Ethanol	0.10 ± 0.01	0.06 ± 0.01	0.10 ± 0.00	0.07 ± 0.00
Pyruvate	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Acetate	0.08 ± 0.00	0.09 ± 0.00	0.08 ± 0.00	0.08 ± 0.00
Lactate	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Succinate	0.05 ± 0.01	0.10 ± 0.01	0.04 ± 0.00	0.05 ± 0.00
Formate	0.22 ± 0.01	0.10 ± 0.02	0.05 ± 0.01	0.09 ± 0.03
CO_2	0.07 ± 0.01	0.16 ± 0.04	0.24 ± 0.02	0.19 ± 0.02
Y_{Carbon}	1.00 ± 0.04	0.93 ± 0.05	0.98 ± 0.05	0.92 ± 0.01

Supplementary Figure 2: Product formation rates for strains co-producing ethyl acetate and hydrogen in pH-controlled reactors under anaerobic conditions. Rates are estimated by the slope of a linear trendline for cumulated product (mmol) per reactor volume (0.5 L) vs. time (h) to obtain rates in mmol/L/h. The rates and its corresponding R^2 value per replicate is listed by compound in Supplementary Table 2.

605 Supplementary Table 2: Product formation rates and R^2 values of generated trendlines.

Strain		Ethyl acetate		Formate		Hydrogen	
		Rate (mmol/L/h)	R^2	Rate (mmol/L/h)	R^2	Rate (mmol/L/h)	R^2
ΔΔ trEat1	A	1.3031	0.999	2.1632	0.9862	0.4527	0.9995
	B	1.0721	0.9961	1.8179	0.9876	0.5875	0.9980
ΔΔ p3-fhl trEat1	A	0.9592	0.9955	0.6044	0.9994	2.3493	0.9996
	B	0.714	0.9996	0.8691	0.9983	1.9801	0.9988
ΔΔΔΔΔ trEat1	A	1.6875	0.9658	1.385	0.9971	4.1158	0.9975
	B	1.6687	0.9802	1.4792	0.9897	2.8801	0.9915
ΔΔΔΔΔ p3-fhl trEat1	A	0.8703	0.9996	1.4444	0.9947	1.7516	0.9935
	B	0.9112	0.9993	1.1814	0.9890	2.3546	0.9964

606

Figures

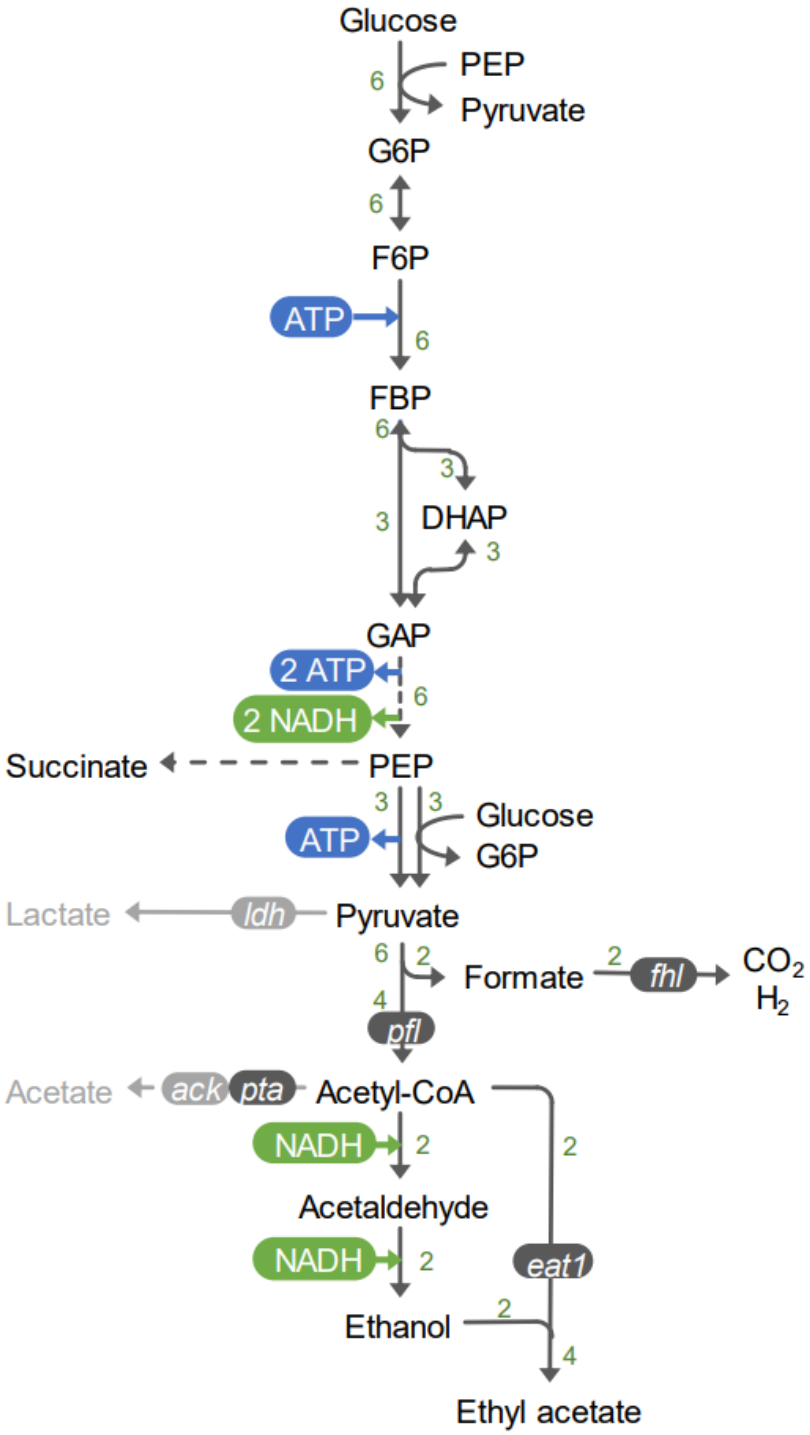

Figure 1

Schematic representation of anaerobic ethyl acetate production from glucose in E. coli via the Embden-Meyerhof-Parnas (EMP) pathway with hydrogen co-production. Lactate and acetate formation is limited by ack and ldh inactivation. Heterologous alcohol acetyltransferase Eat1 generates ethyl acetate from

ethanol and acetyl-CoA. Hydrogen co-production is achieved via formate hydrogen lyase (Fhl). Ack – acetate kinase, DHAP – dihydroxyacetone phosphate, eat1 – ethanol acetyltransferase, FBR – fructose 1,6-bisphosphate, F6P – fructose 6-phosphate, G6P – glucose 6-phosphate, PEP – phosphoenolpyruvate, GAP – glyceraldehyde 3-phosphate, pta – phosphate acetyltransferase

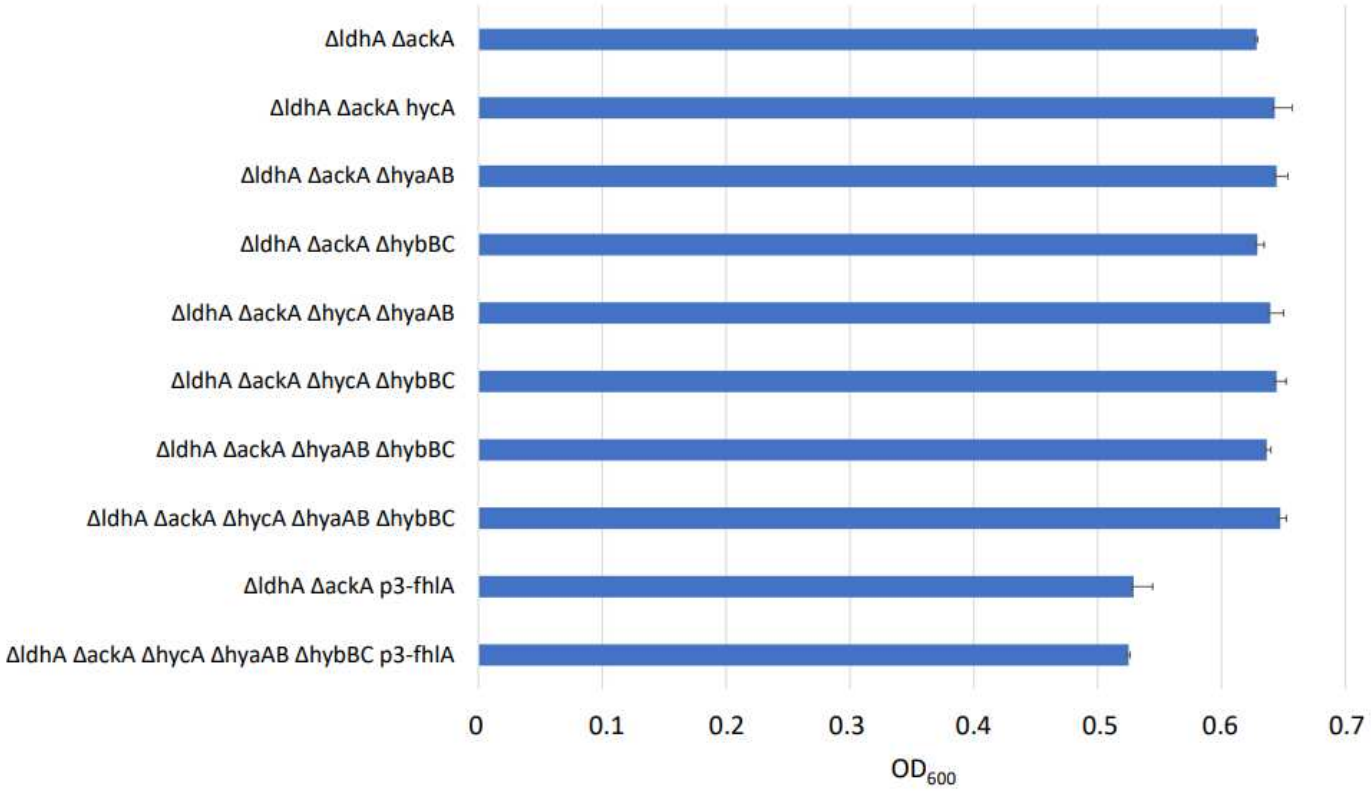

Figure 2

OD600 after 72h of cultivation under anaerobic conditions with glucose as carbon source of a BW25113 ΔldhA ΔackA background strain containing additional KOs and/or overexpressing fhlA for improved hydrogen production. Initial OD600 was 0.2. Data and error bars indicate averages and standard deviations among duplicates

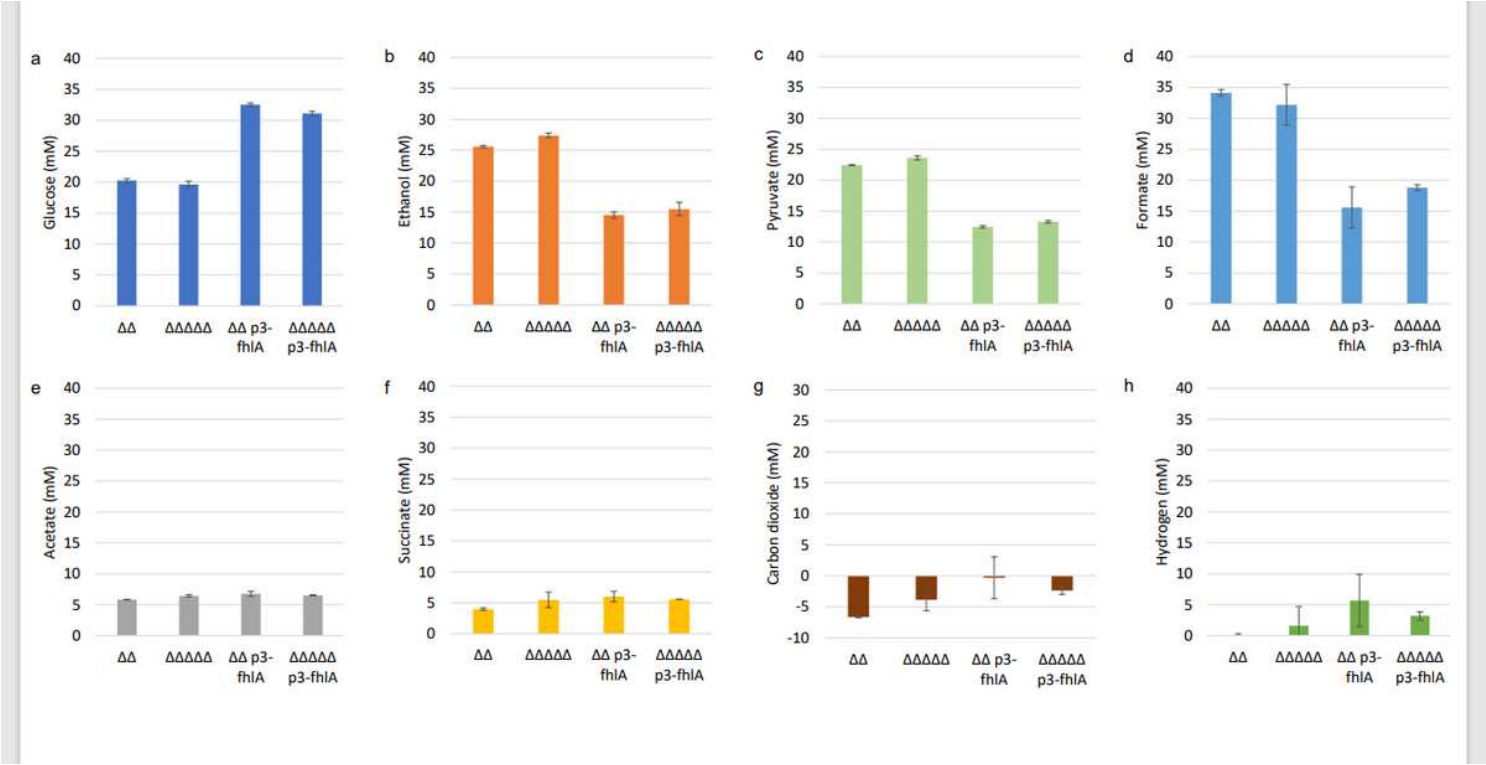

Figure 3

Concentrations of glucose and products after 72 h of anaerobic cultivation for strains with a ΔldhA ΔackA (ΔΔ) background and further modifications for improved hydrogen production, from left to right: inactivation of hycA, hyaAB and hybBC (ΔΔΔΔ), overexpression of fhlA (ΔΔ p3-fhlA) and a combination of knockouts and overexpression (ΔΔΔΔ p3-fhlA). For CO2 and H2, data represent calculated concentrations. Data shows average values and standard deviations from biological duplicates.

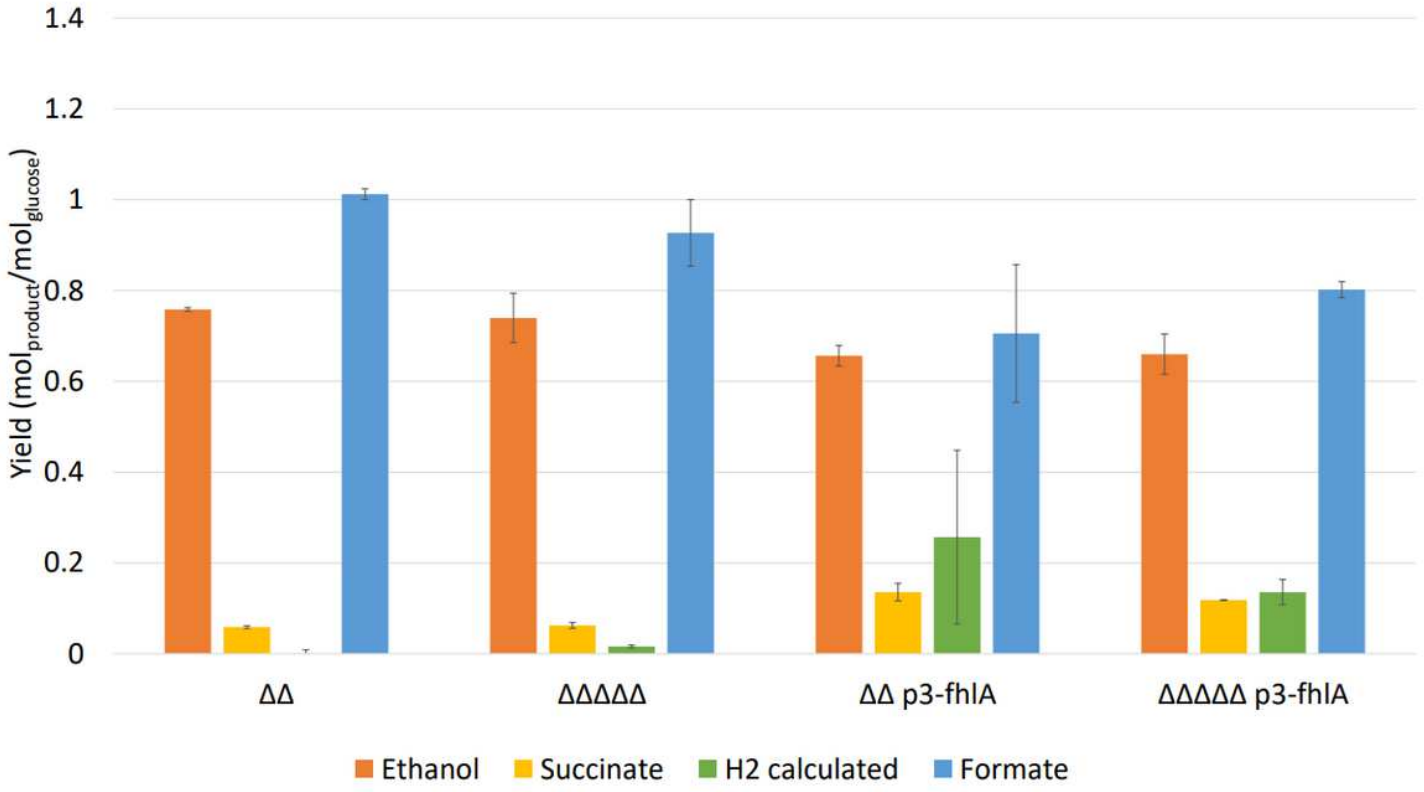

Figure 4

Product yield on glucose on selected products after 72h of anaerobic fermentation for strains based on ΔldhA ΔackA (ΔΔ) with further modifications for improved hydrogen production, from left to right: inactivation of hycA, hyaAB and hybBC (ΔΔΔΔΔ), overexpression of fhlA (ΔΔ p3-fhlA) and a combination of knockouts and overexpression (ΔΔΔΔΔ p3-fhlA). Values are averages of two biological replicates and error bars represent standard deviations.

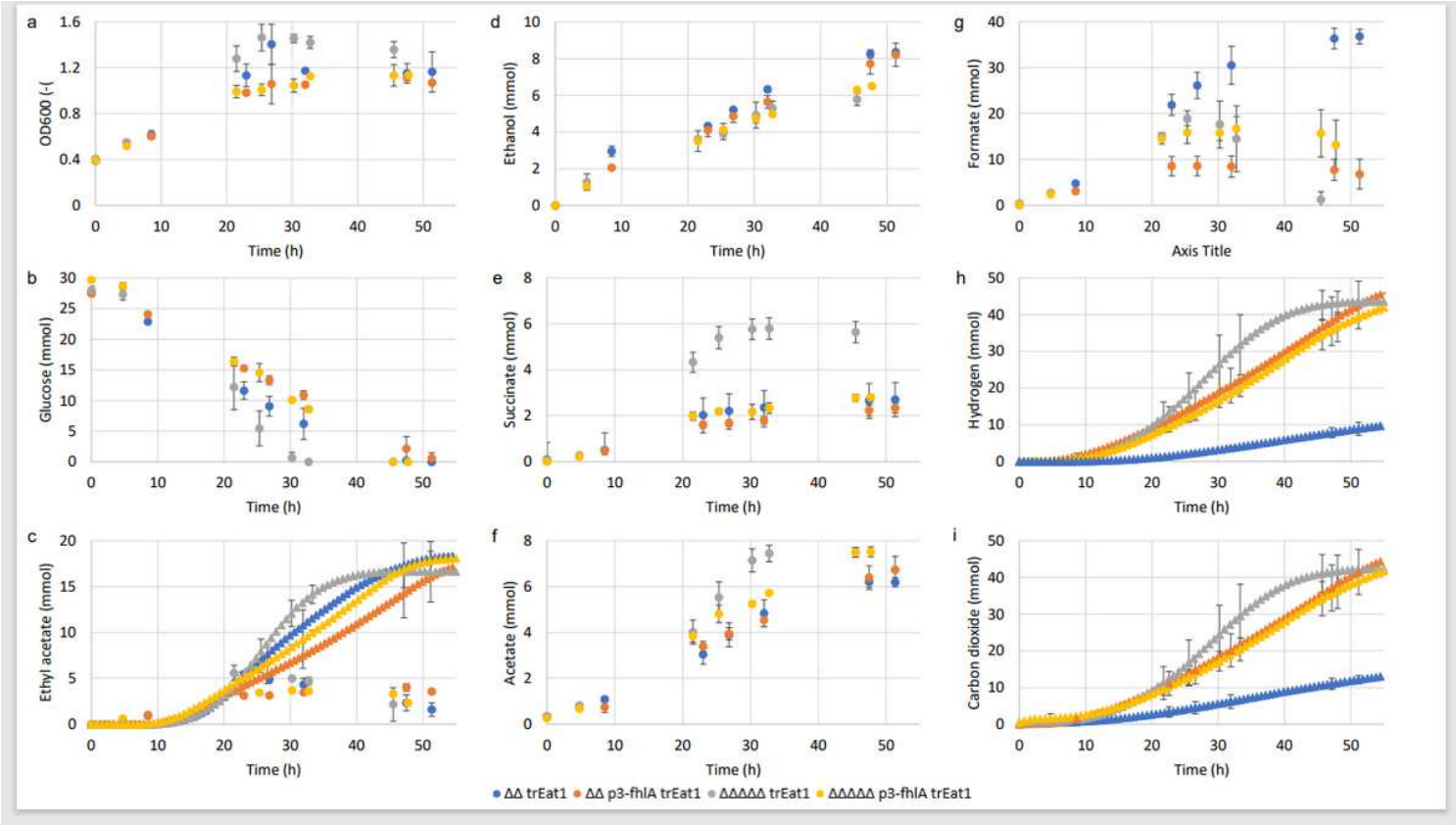

Figure 5

Fermentation profile of four strains engineered for ethyl acetate and hydrogen co-production in pH-controlled bioreactors with continuous gas stripping. Strains based on ΔldhA ΔackA (ΔΔ) with further modifications for improved hydrogen production, from left to right: inactivation of hycA, hyaAB and hybBC (ΔΔΔΔΔ), overexpression of fhlA (ΔΔ p3-fhlA) and a combination of knockouts and overexpression (ΔΔΔΔΔ p3-fhlA) producing trEat1 Wan N-13 were induced by 0.01 mM IPTG and cultivated under anaerobic conditions in minimal medium with 55 mM glucose as carbon source.. Experiments were performed as biological duplicates; error bars represent the standard deviation. Circles – compounds in liquid broth, triangle – compounds in off-gas.

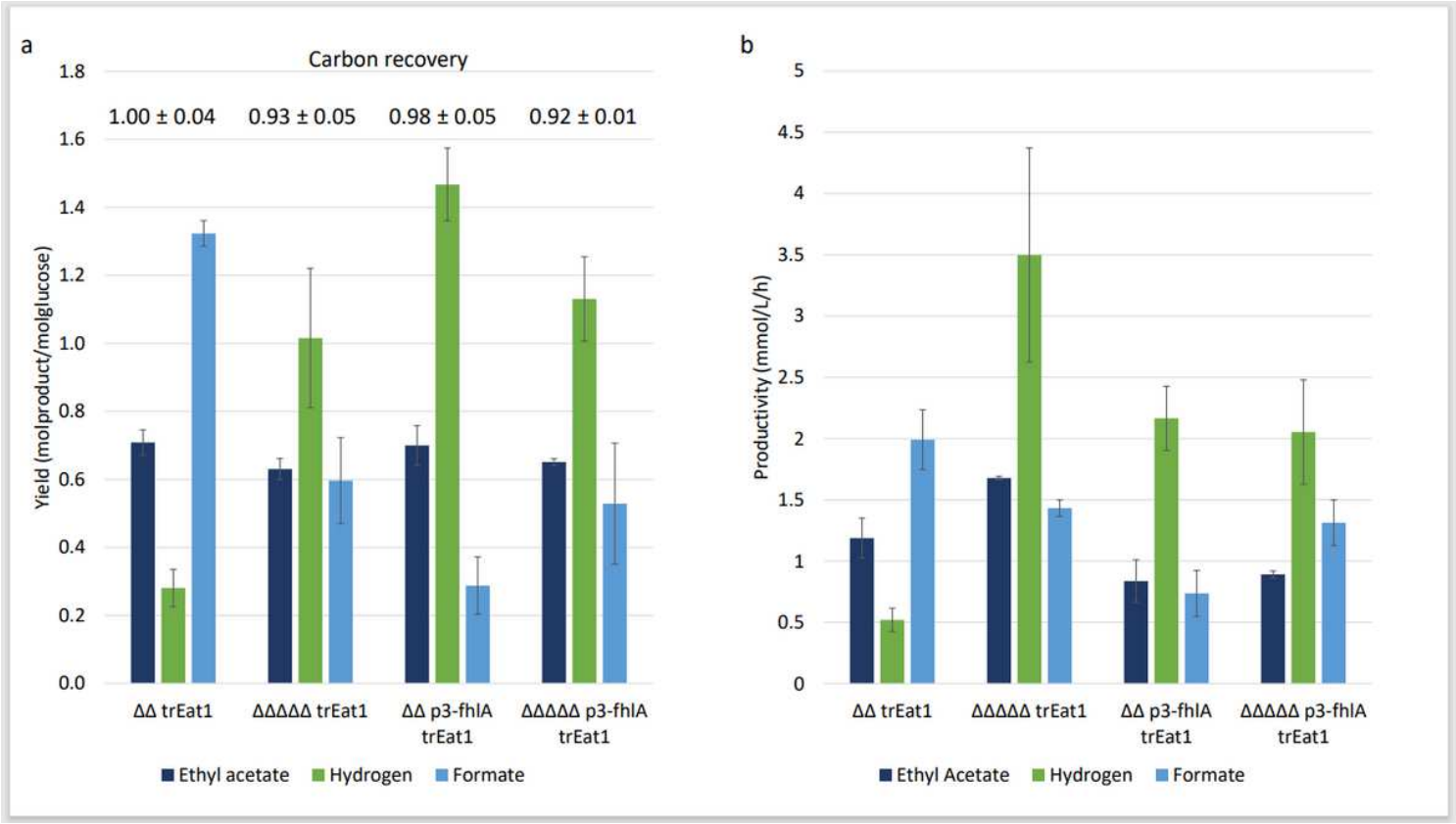

Figure 6

Effect of modifications towards improved hydrogen production on product yields and productivities for main fermentation products, with from left to right: inactivation of hycA, hyaAB and hybBC, overexpression of fhlA and a combination of knockouts and overexpression. Strains producing trEat1 Wan N-13 were induced by 0.01 mM IPTG and grown under anaerobic conditions in minimal medium containing 55 mM glucose using pH-controlled bioreactors with 0.5 L working volume. (a) Product yields for ethyl acetate, hydrogen and formate in molproduct/molglucose after glucose depletion. The numbers above the bars represent the carbon recovery of the fermentations. (b) Volumetric productivities for ethyl acetate, hydrogen, and formate in mmol/L/h. Experiments were performed as biological duplicates or triplicates; error bars represent the standard deviation. Abbreviations: trEat1 – truncated Eat1 Wan N-13.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure1.pdf
- SupplementaryFigure2Rates.pdf