ÅNGPANNEFÖRENINGENS FORSKNINGSSTIFTELSE

Design and Assembly of Novel Pathways for Advanced Biofuel Synthesis

Final Report

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Summary

Microbial production of fuels and chemicals from renewable resources offers sustainable alternatives to the current petroleum-based production. However, many challenges still remain in realizing the commercialization and to compete with more conventional processes. The key factor to commercialization is engineering of the microorganism's metabolism, to reach the titers, yields and productivities (rates) required to meet economic targets. The yeast *Saccharomyces cerevisiae* is an attractive cell factory for production of many industrially relevant products. Especially in the field of biofuel production, much interest has centered on this organism for advanced biofuel production, as it is well adapted to industrial conditions and thus allows flexibilities of production facilities, which are very capital intensive. The objective of this project is to develop yeast platform cell factories for efficient production of advanced biofuels, e.g., fatty acid derived esters (fatty acid ethyl esters, FAEEs) and alkanes. More specifically, the primary aim of this project is to design and assemble a synthetic pathway in yeast, providing an alternative route for efficient provision of acetyl-CoA and malonyl-CoA as precursor metabolites for advanced biofuels production, and combining this feature with elimination of pyruvate decarboxylase activity and thus avoiding by-product ethanol formation. Through the investigations in this project we have firstly delimited the rate limiting step in conversion of acetyl-CoA to malonyl-CoA, via removing the reversible phosphorylation sites in acetyl-CoA carboxylase Acc1. By doing so the enzyme activity of Acc1 increased by three fold, and in turn the production of FAEEs enhanced by three fold, which indicates the increased supply of malonyl-CoA. Secondly we have constructed a Pdc minus yeast strain in order to abolish ethanol formation, via knocking out the main enzymes responsible for pyruvate decarboxylase activity – *PDC1*, *PDC5* and *PDC6*. The resulted strain cannot grow on glucose as the sole carbon source due to the lack of cytosolic acetyl-CoA. Therefore, adaptive evolution has been performed to get better growth of Pdc minus strains. In parallel, we have identified a mechanism, in which Ach1 is involved in shuttling mitochondrial acetyl units for cytosolic C2 provision in *Saccharomyces cerevisiae* lacking pyruvate decarboxylase. This new finding significantly contribute to our knowledge on yeast carbon metabolism and further to better strain design. Thirdly, In order to reconstruct the phosphoketolase pathway in yeast that allows the shunt of carbon from the glycolysis/pentose phosphate pathway intermediates frucose-6-phosphate (F6P)/xylulose-5-phosphate (X5p) to acetyl-CoA, offering an alternative route for acetyl-CoA synthesis. We have screened nine phosphoketolase enzymes from Bifidobacterium genus and, the Firmucute phyla (includes lactic acid bacteria and Clostridia). Characterizing the strains with phosphoketolase expression reveals additional C2 compounds (acetate) formation, a potential direct substrate for acetyl-CoA biosynthesis.

Introduction

Microbial production of transportation fuels from plant-derived raw materials offers sustainable and economically attractive alternatives to their petroleum-based production. Currently, the most widely used biofuels are ethanol produced from sugar cane or corn, and biodiesel derived from vegetable oil or animal fats. However, there is often raised criticism against these biofuels owing to, for example, a food versus fuel issue. The development of second generation bioethanol which is derived from biomass (lignocellulose) will overcome these problems. Nevertheless, ethanol is not the sole or optimal fuel to be produced from lignocellulosic biomass. Other higher alcohols, alkanes, and various types of products represent potential biochemically derived biofuels. Many of these products are commonly formed through an acetyl-CoA intermediate. So far the baker yeast *Saccharomyces cerevisiae* has been widely used for production of many different products. Compare to *E.coli*, yeast's robustness, its low medium pH, its lack of susceptibility to bacteriophage, it is already applied on a large scale for bioethanol production, its genetic accessibility and an extensive technology platform in terms of system biology and fermentation technology available for this organism, make it the preferred organism as the key biofuel cell factory. The industry is therefore keen to further exploit this cell factory as a platform for production of more advanced biofuels. Therefore, in this project we

aimed to design a synthetic pathway that can be used for efficient provision of acetyl-CoA and malonyl-CoA as precursor metabolites for advanced biofuels production.

Results

1. Enhanced activity of the key enzyme Acc1 led to improvements of FAEEs production

We have developed a strategy for improving the activity of Acc1 in *S. cerevisiae* by abolishing Snf1-dependent posttranslational regulation of Acc1 via site-directed mutagenesis. It was found that introduction of two site mutation in Acc1, Sre659 and Ser1157, resulted in an enhanced activity of Acc1 and increased total fatty acid content. As can be seen in Table 1, CB0 holds the empty plasmid pSP-GM2 and was used as control. Acc1 activities in all strains were determined in stationary phase. Overexpression of both wild-type and point mutated *ACC1* enhanced the ACCase activity compared to control strain CB0. In strain CAW, which harbors *ACC1*^{wt} on plasmid, ACCase activity was increased by 20%. While with the single-mutated version of *ACC1* (*ACC1*^{ser1157ala}) in strain CAS, the enzyme activity was 80% higher than for the control strain, overexpression of *ACC1*$^{ser659ala,\ ser1157ala}$ resulted in a 3-fold increase of Acc1 activity, which was the highest increase.

TABLE 1 Activities of acetyl coenzyme A carboxylase and total fatty acid content in strains holding different versions of *ACC1*[a]

	CB0	CAW	CAS	CAD
Total fatty acid content (% of CDW)	7.1 ± 1.5	7.4 ± 1.5	8.1 ± 1.5	11.7 ± 2.0
ACCase activity (pmol/min/mg of cell-free protein extract)	24 ± 3	29 ± 3	43 ± 4	74 ± 9

[a] CB0: control strain with empty plasmid; CAW: strain with overexpressed *ACC1*^{wt}; CAS: strain with overexpressed *ACC1*^{ser1157ala}; CAD: strain with overexpressed *ACC1*$^{ser659ala,\ ser1157ala}$. The total fatty acid contents are expressed as percentage of dry-weight biomass. Strains were sampled at stationary phase. The reported results are the average of three replicate experiments ± standard deviation.

Overexpression of wild-type or point mutated Acc1 clearly led to an increase in the enzyme activity, and hereby supposedly increased the formation of malonyl-CoA, a crucial precursor for fatty acid biosynthesis. Therefore we also measured the concentration of total fatty acids in strains CB0, CAW, CAS, and CAD (Table 1). It was found no significant increase in the total fatty acid content when overexpressing *ACC1*^{wt} compared with the control strain. Overexpression of *ACC1*^{ser1157ala}, however, resulted in a 14 ± 0.2 % improvement in the total fatty acid content and overexpression of *ACC1*$^{ser659ala,\ ser1157ala}$ resulted in a 65 ± 0.3 % increase in the total fatty acid content (strain CAD).

As Snf1 regulation of Acc1 is particularly active under glucose-limited conditions, we evaluated the effect of the two site mutations in chemostat cultures. The effect of increased activity of Acc1 shown in Table 1 could be explained by the introduced site mutations, Ser659 and Ser1157, which are suggested to function as phosphorylation sites recognized by Snf1 protein kinase. To evaluate this further we expressed the two mutated as well as the wild-type form of the enzyme in a snf1 strain. *SNF1* was deleted in strain CEN.PK 113-5D resulting in strain SC00. Empty plasmid and plasmids

holding three different versions of *ACC1* were introduced into SC00 resulting in strains SC01, SC02, SC03, and SC04. These strains were cultured in glucose-limited chemostat cultures (growth rate at 0.1 h^{-1}), where Snf1 is normally active, and analyzed for their Acc1 activity. No any apparent physiological difference was found in chemostat cultures when different versions of Acc1 were overexpressed. Compared to the higher enzyme activity for the mutated versions of Acc1 in the presence of *SNF1* (Table 1), all the strains with over-expression of different versions of *ACC1*, i.e. strains SC02, SC03, and SC04, had a similar Acc1 activity in the absence of *SNF1* (Table 2). These results clearly indicate that Ser1157 and Ser659 play a role in regulation of Acc1 activity, most likely as phosphorylation target sites of Snf1.

TABLE 2 Activities of acetyl coenzyme A carboxylase in *snf1* deletion strains holding different versions of *ACC1*[a]

Strain	Enzyme activity (pmol/min/mg of cell-free protein extract)
SC01	33 ± 8
SC02	57 ± 5
SC03	62 ± 9
SC04	63± 6

[a] SC01: control *snf1* deletion strain with empty plasmid; SC02: *snf1* deletion strain with overexpressed *ACC1*^{wt}; SC03: *snf1* deletion strain with overexpressed *ACC1*^{ser1157ala}; SC04: *snf1* deletion strain with overexpressed *ACC1*$^{ser659ala,\ ser1157ala}$. Samples were taken from chemostat cultures after a steady state was maintained for about 50 h. The reported results are the average of duplicate experiments ± standard deviation.

From the above it is clear that site-directed mutagenesis of ACC1 results in increased activity leading to higher total lipid content (Table 1), indicating increased supply of malonyl-CoA. We therefore evaluated if this phenomenon could be applied for improving the production of malonyl-CoA derived products, e.g., FAEEs.

From our results, overexpression of the different mutant *ACC1* genes increased FAEEs production (Table 3). Compared to the FAEE producing reference strain CB2H1 that only holds the *ws2* gene with a FAEE production of 4.8 ± 0.7 mg/l, strain CB2H2 that overexpresses *ACC1*^{wt} has a slightly increased FAEE production (around 20%), while strain CB2H3 that overexpresses *ACC1*^{ser659ala} showed a much higher increase (around two-fold). Furthermore, overexpression of *ACC1*$^{ser659ala,\ ser1157ala}$ in strain CB2H4 resulted in the highest FAEE production, corresponding to a 3-fold improvement.

TABLE 3 Production of FAEEs and 3-HP in different strains holding different versions of *ACC1*

Strains	(Over)expressed genes (*HIS3*) & (*URA3*)[a]	Total FAEE production (mg/l)
CB2H1	*ws2 &* ---[b]	4.7 ± 0.7

CB2H2	*ws2 & ACC1*^{wt}	5.6 ± 0.8
CB2H3	*ws2 & ACC1*^{ser1157ala}	9.5 ± 0.9
CB2H4	*ws2 & ACC1*$^{ser659ala, ser1157ala}$	15.8 ±2.5

[a]Gene (over)expressed in HIS3 and URA3 based plasmid.
[b]No gene was inserted for (over)expression.

Overexpression of *ACC1* has previously been applied in many studies for redirecting flux towards malonyl-CoA. However, these approaches are still limited by the inherent low enzyme activity. Here, it is interesting to note that a more efficient Acc1 was obtained by the introduction of two site mutations at Ser1157 and Ser659, which are suggested to be related to Snf1 mediated phosphorylation. The strains with higher activity of Acc1 showed a notable advantage for the production of malonyl-CoA derived products, as demonstrated for the production of FAEEs.

2. Identification of Ach1 as a key component to compensate cytosolic acetyl-CoA in Pdc minus strain

One important feature of this project is to establish a yeast platform cell factory that can efficiently provide acetyl-CoA and combine this feature with elimination of ethanol formation through abolishing pyruvate decarboxylase activity. Here we have constructed a Pdc minus yeast strain via knocking out the main enzymes responsible for pyruvate decarboxylase activity – *PDC1*, *PDC5* and *PDC6*. The resulted strain cannot grow on glucose as the sole carbon source due to the lack of cytosolic acetyl-CoA. It has been reported that a *MTH1* internal deletion (*MTH1-ΔT*) that resulted in reduced glucose uptake could restore the growth of Pdc minus strain on glucose. However, the source of cytosolic acetyl-CoA is still a mystery in a Pdc negative strain with *MTH1-ΔT* allele. Understanding the underlying mechanism would be very important for this project. It is also a very interesting question for the yeast research community.

Using the latest Genome Scale Metabolic Model of *S. cerevisiae*, 57 reactions were found related with C_2 compound metabolism, including 34 reactions directly related with acetyl-CoA metabolism and transport, and 23 more reactions involved in other C_2 compounds metabolism such as ethanol, acetaldehyde and acetate. Through analysis of all these reactions, we identified a putative route for acetyl-CoA biosynthesis, namely formation of acetate in the mitochondrial matrix followed by transport of acetate to the cytosol where acetyl-CoA synthetase can convert it to acetyl-CoA. In this route, Ach1 play a key role of conversion of acetyl-CoA into acetate in the mitochondria. Ach1 has originally been recognized as a hydrolase catalyzing the hydrolysis of acetyl-CoA to acetate and CoA, like many other acetyl-CoA hydrolases found in mammalian tissues. The exact catalytic role of this enzyme was later questioned by the observations of its role in acetate but not ethanol utilization. It was also proposed that this enzyme may have a novel function concerning acetyl-CoA metabolism, but it was not until recently, when Fleck and Brock characterized Ach1 as a CoA transferase not just wasting energy by hydrolyzing acetyl-CoA that new insight was provided on this enzyme.

Based on these analysis we proposed that this enzyme could transfer CoA unit from acetyl-CoA to succinate, forming acetate and succinyl-CoA. In order to test our hypothesis, we deleted the *ACH1* gene in a background of Pdc minus and *MTH1* internal deletion strain. The growth assay of strain in

absence of Ach1 was performed firstly on synthetic medium agarose plates. The growth of the *ACH1* deletion strain YACH01, however, was slightly impaired as shown in **Fig. 1A**. This could be an effect of the medium used for the solid growth assays, for example, it contains threonine in the drop-out medium used, or any other potential C_2-contamination as speculated previously. Therefore, the growth was also investigated in liquid cultures using defined minimal medium. Cells of IMI076 and the ach1 mutant YACH01 were washed twice after pre-growth in synthetic ethanol media, and then transferred to a minimal glucose media. Strain IMI076 grew as described before, with a specific growth rate of 0.066 ± 0.001 h^{-1}, whereas the ach1 mutant YACH01 could not grow on glucose as the sole carbon source (**Fig. 1B**), clearly showing that *ACH1* is essential for growth of the IMI076 strain, which has a background of Pdc minus and *MTH1* internal deletion.

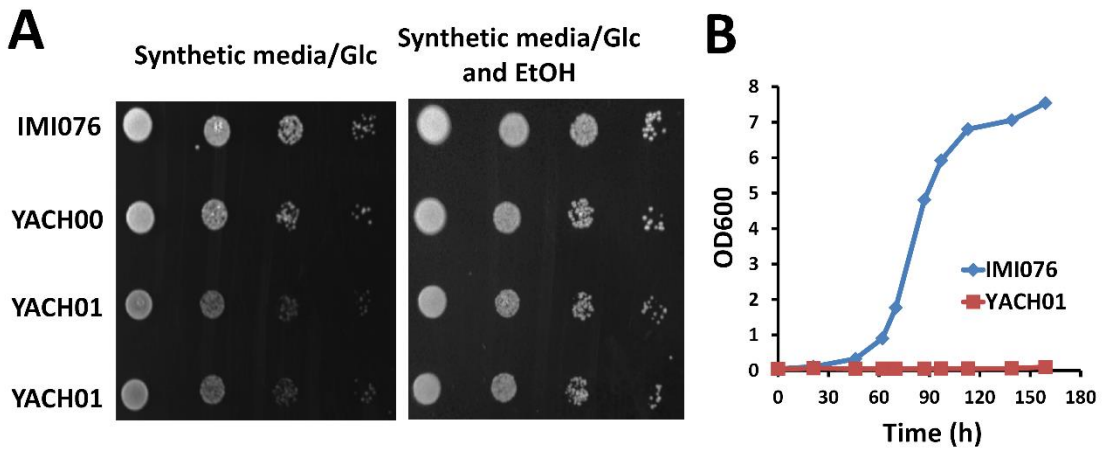

Fig. 1 The growth of strain IMI076 (Pdc- *MTH1-ΔT*) relies on Ach1. A) Growth assays on solid synthetic medium with 20 g L^{-1} glucose or 20 g L^{-1} glucose plus 0.3% (v/v) ethanol. The plates were incubated at 30 °C and recorded photographically 4 days after inoculation. B) Growth assays in defined liquid minimal media with 20 g L^{-1} glucose as the sole carbon source.

To further confirm our hypothesis we performed complementation of the ach1 deletion strain with both the wild-type gene and a truncated version of *ACH1*, where the N-terminus of Ach1 was removed which is reported to result in redirection of this protein to the cytoplasm. *ACH1* with its N-terminal region being deleted or the entire protein coding gene was reintroduced into the ach1 mutant by chromosomal integration. Growth assays of the resulting strains showed that complementation with the complete *ACH1* gene could restore growth of the deletion mutant, whereas complementation with the truncated version could not restore growth of the ach1 mutant (**Fig. 2**). The maximum specific growth rate of the *ACH1* complemented strain (YACH02) was increased by 51% compared with the control strain IMI076, which may be ascribed to increased expression in the complementation strain, where *ACH1* was expressed under control of the strong *TEF1* promoter.

Furthermore, to validate if the cytosolic acetyl-CoA in IMI076 is likely coming from mitochondrial acetyl-CoA we cultivated this strain in absence or presence of UK-5099, which is known as a specific and potent inhibitor of the mitochondrial pyruvate carrier. When the cells entered the exponential growth phase 0.2 mM UK-5099 was added to the culture. This resulted in a significant decrease in growth compared with the control experiment where no inhibitor was added. While there is no significant effect on the growth of the control strain (CEN.PK 113-5D) when supplemented with 0.2

mM UK-5099, the maximum specific growth rate of strain IMI076 decreased in presence of UK-5099, from 0.066 ± 0.001 h^{-1} to 0.018 ± 0.002 h^{-1}. These observations clearly indicate that the flux of mitochondrial pyruvate uptake is limiting cell growth, which again supports the hypothesis that cytosolic acetyl-CoA is derived from mitochondrial acetyl-CoA.

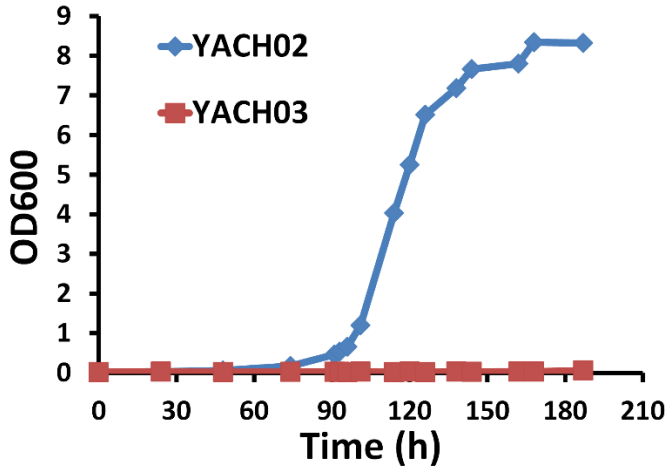

Fig.2 Complementation of ACH1 but not its truncated version restores the growth of YACH01 (ach1 mutant). Cells were first cultured in defined minimal medium with 2% (v/v) ethanol, and then the cells were washed twice with sterile water before inoculation of 20 g L-1 glucose medium.

Based on these results we have proposed the model of acetyl-CoA metabolism for the Pdc deficient strain. We conclude that a Pdc minus S. cerevisiae strain under glucose derepressed conditions uses acetate instead of citrate to transfer acetyl units from the mitochondria to the cytosol. In this transfer system acetate is produced in the mitochondrial matrix from acetyl-CoA by Ach1. This newly identified functionality of Ach1 could be potentially useful for designing yeast as a microbial cell factory to produce acetyl-CoA derived products.

3. Establishment of a synthetic phosphoketolase pathway

In order to select phosphoketolase enzymes that are functionally expressed in yeast and display high enzymatic activity, an in vitro enzyme assay was performed on crude cell extracts from phosphoketolase expressing strains based on the established ferric hydroxamate method. The enzymatic activity towards ribose-5-phosphae (R5P) is assumed to resemble to the enzymes activity towards X5P due to endogenous enzymatic activity of R5P isomerase and ribulose-5-phosphate 3-epimerase.

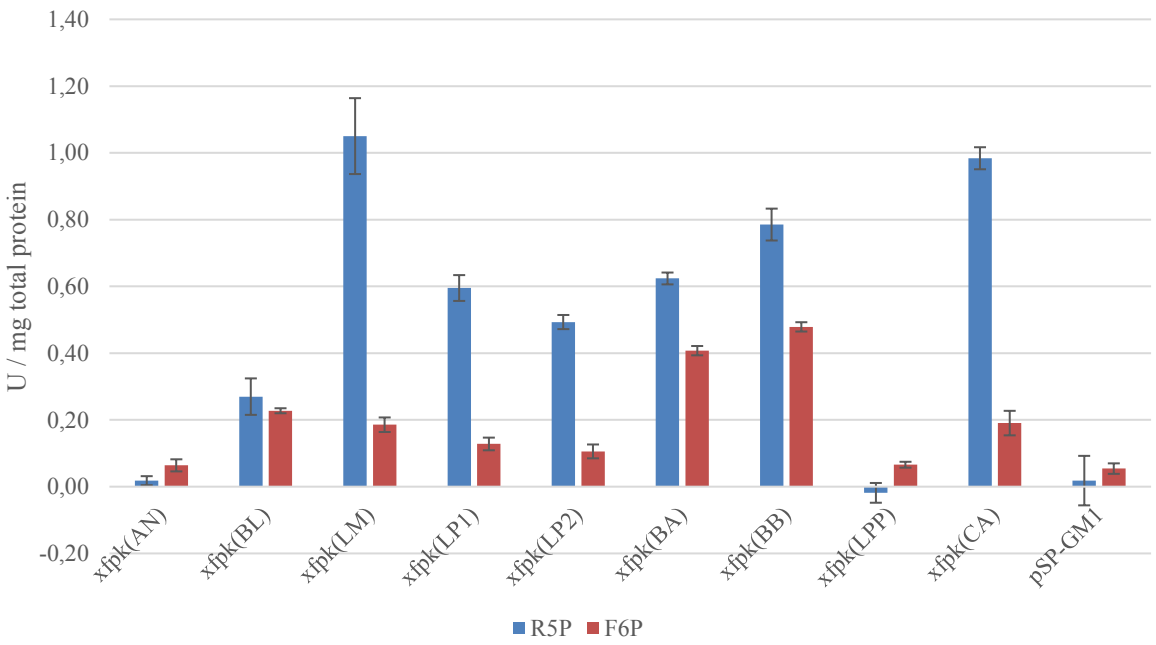

Fig.3. Specific activities (U) of the 9 tested xfpk enzymes tested in this study with respect to the substrates D-ribose-5-phosphate (R5P) and D-fructose-6-phosphate (F6P). In figure, legends correspond to xfpk used, expressed in strains AB1-AB9. pSP-GM1 serves as negative control, and corresponds to strain AB10. All xfpk:s except for xfpk(AN) and xfpk(LPP) displayed significantly higher activity ($p<0,005$) when compared to the negative control with respect to both substrates. The results shown are averages from two biological replicates each performed in duplicates; error bars indicate the standard deviation.

From the spectrophotometric results obtained, the specific activities of the enzyme candidates (based on the total protein amount in the crude extracts) were calculated, which are shown in **Fig.3**. Seven out of the nine evaluated phosphoketolase enzymes show significant activity towards both substrates (R5P and F6P) compared to the strain AB10 harboring the empty plasmid pSP-GM1 ($p<0,005$), the exceptions being phosphoketolase candidates from *A. nidulans* and *L. paraplantarum*.

Furthermore, all active enzyme candidates show dual substrate specificity, where activity towards X5P appears to be most pronounced for all candidates examined. However, the activity profile for the enzymes originating from Bifidobacterium all shows a higher relative affinity towards F6P when compared results obtained from the Firmicute phyla (F6P breakdown corresponds to 38-46% of total activity compared to15-18%). Also yeast appears to form AcP (or other compound that can react with the enzymatic assay components to yield absorbance at 505 nm) from F6P, as the wild type as well as xfpk(AN) and xfpk(LPP) strains show a low degree of activity. Thus, the true activity of statistically significant F6P-phosphoketolases might be lower than reported here.

The three candidates with the highest obtained specific activities upon X5P – xfpk(LM) from L. mesentorides (1.05±0.11 U/mg and 0.19±0.02 for X5P and F6P, respectively), xfpk(CA) from C. acetobutylicum (0.98±0.03 U/mg and 0.19±0.04 for X5P and F6P, respectively), and xfpk(BB) from B. breve (0.79±0.05 U/mg and 0.48±0.02 for X5P and F6P, respectively) – were selected for further studies. All of the enzymes showed a cumulative specific activity close to 1.2-1.3 U/mg total protein.

The relative affinities of xfpk(LM) and xfpk(CA) appears to be very similar, while the specificity of xfpk(BB) towards F6P was considerably higher than for the other two candidates, making it an interesting candidate to evaluate in vivo where intracellular concentrations of the substrates in questions differ greatly.

During the setup of the phosphoketolase enzyme assay, it appeared as if *S. cerevisiae* possessed a natural ability to break down the enzymatically produced acetyl-phosphate. Examining literature on the topic led us to believe that this breakdown led to a production of acetate [1], and to a large extent was catalyzed by two endogenous phosphatases involved in glycerol biosynthesis (genes GPP1 and GPP2) [2]. In order to confirm these findings, these genes were deleted (either alone or in combination) and crude cell free extracts prepared thereof were used in an in vitro assay to measure the strains ability to break down acetyl-phosphate over time, results of which is shown in **Fig.4**.

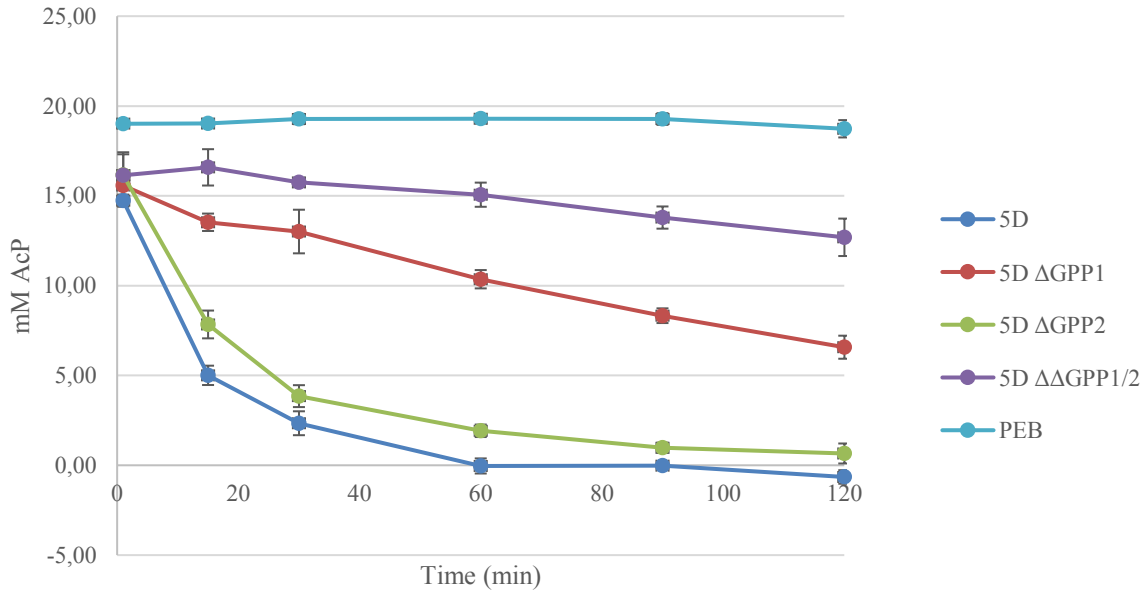

Fig.4. Acetyl-phosphate (AcP) breakdown in crude cell lysates of CEN.PK 113-5D and corresponding GPP1 and/or GPP2 deletions strains. The results show that AcP is effectively degraded enzymatically in wild type yeast, a process that largely can be prevented by deleting the two native glycerol-phosphatases with an additive effect. The results shown are averages from two biological replicates each performed in duplicates; error bars indicate the standard deviation.

GPP1 appears to contribute most to this breakdown, as the ΔGPP1 deletion strain retained about 66% of the initial AcP concentration after 60 min incubation, while the corresponding number was 12% for the ΔGPP2 deletion strain. The double deletion strain reduced the endogenous breakdown activity drastically – 93% of initial AcP content was retained after the 60 min incubation (as compared to 0% of the wild type yeast), and 79% after 120 minutes. Based on these results and the literature support, we assumed that in vivo phosphoketolase activity could be assessed by cultivating the wild type xfpk expressing strains and monitoring acetate levels.

In order to confirm the in vitro findings, the three candidates with the highest specific activities upon R5P – xfpk(LM), xfpk(CA) and xfpk(BB) – and the previously examined phosphoketolase xfpk(AN) which failed to show activity in the in vitro assay conditions, were selected for a more detailed

characterization in vivo. Based on the hypothesis that enzymatically produced AcP quickly is converted endogenously to acetate in wild type yeast, we quantified the acetate accumulation in strains carrying the selected xfpk-plasmids and the control plasmid pSP-GM1 over time in small scale batch cultures.

Fig.5 shows that expression of the three phosphoketolases with high activity reported from the in vitro assay significantly ($p<0.05$) increased acetate accumulation in vivo compared to the control. The greatest increase was seen for strains expressing xfpk(BB), with a maximal level at 1.21±0.04 g/L as compared to the 0.47±0.02 g/L by the wild type yeast. The strains expressing xfpk(LM) and xfpk(CA), the xfpk:s showing similar activity patterns in the in vitro screen, both showed a similar accumulation pattern with maximal accumulation at 0.66±0.06 and 0.75±0.06 g/L respectively, whereas xfpk(AN) did not show any significant difference in acetate accumulation compared to the control. In all strains, the acetate was consumed when cultivations reached stationary phase.

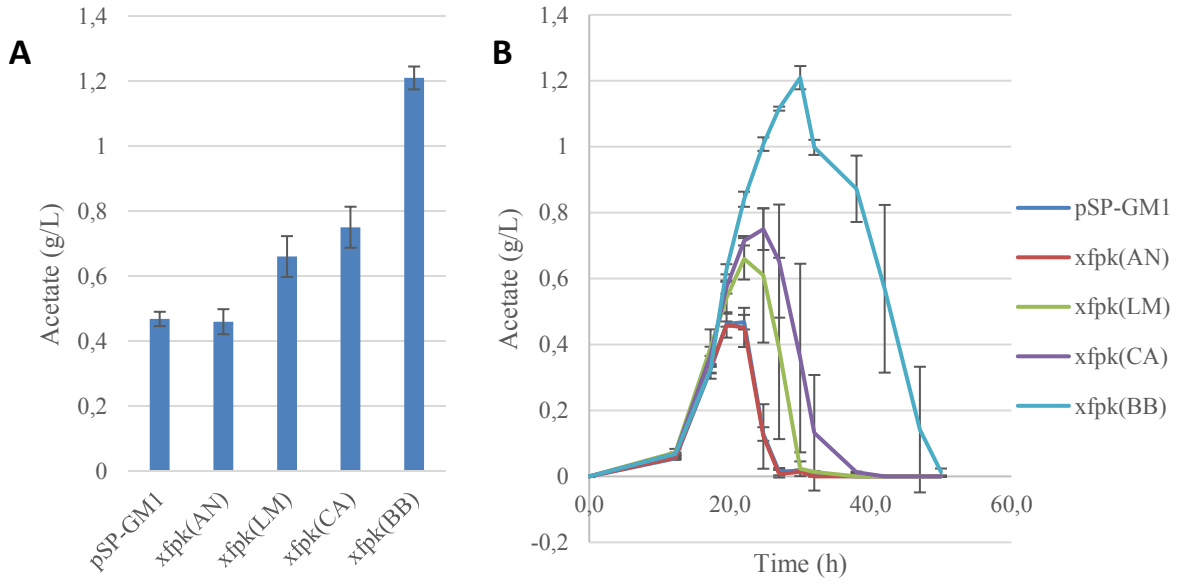

Fig.5. Acetate measurements in xfpk-expressing yeast strains shows that the high activity phosphoketolases from the in vitro screen positively correlates with acetate accumulation. A) Maximal acetate accumulation measured in cultivations of xfpk expressing strains. B) Acetate accumulation during whole growth phase show an increased time period for the accumulation and consumption of acetate in xfpk expressing strains. In figure, legends correspond to xfpk used, expressed in strains (AB1, AB3. AB7, AB9). pSP-GM1 serves as negative control, and corresponds to strain AB10. Strains were grown in shake flasks in defined medium, 2% glucose. The results shown are averages from three biological; error bars indicate the standard deviation.

Xfpk-expression also correlates to a decreased growth rate (**Fig.6B**), decreased biomass formation and an increased diauxic shift (figure 5A). Growth rate was significantly reduced in all xfpk-expressing strains ($p<0.01$). This decrease correlated negatively to the increased acetate accumulation, which however does not indicate that acetate production per se brought about this decrease. The biggest decrease was seen for xfpk(BB), where both growth rate and final biomass decreased to approximately 75% of the wild type.

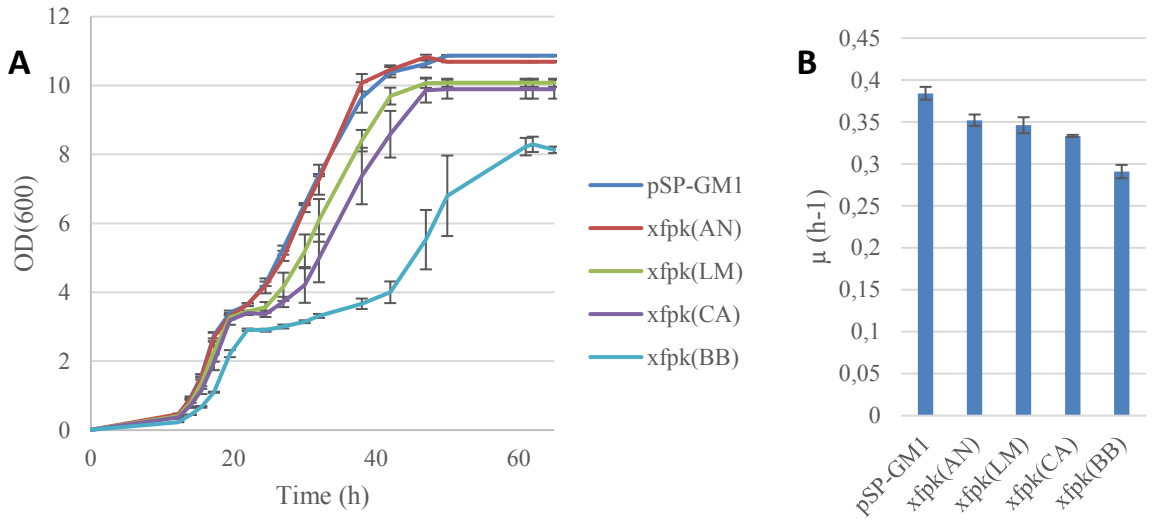

Fig.6. Growth of xfpk-expressing strains shows that expression of a more highly active phosphoketolase. A) Growth curves obtained from shake flask cultivations. B) Calculated maximum specific growth rates (in glucose phase). In figure, legends correspond to xfpk used, expressed in strains AB1-AB9. pSP-GM1 serves as negative control, and corresponds to strain AB10. Strains were grown in shake flasks in defined medium, 2% glucose. The results shown are averages from three biological; error bars indicate the standard deviation.

When **Fig 5B** and **6A** is compared, it can be seen that the time point of the maximum acetate peak was reached almost coincidental with glucose depletion for the wild type as well as the strain expressing xfpk(AN). For the strains expressing the active xfpk versions, however, this time point shifted to 2.5, 5.5 and 8 hours after glucose depletion for xfpk(LM), xfpk(CA) and xfpk(BB), respectively.

With regards to other metabolites (data not shown), glycerol accumulation during glucose phase was slightly reduced in phosphoketolase expressing strains, from 0.82 g/L in the wild type strain to 0.73 g/L for xfpk(BB) and 0.63 g/L for xfpk(LM) and xfpk(CA). Ethanol accumulation was not significantly affected by phosphoketolase expression in any of the strains tested, and maximally reached at 6.3-6.6 g/L.

Conclusions

In this project we have demonstrated that engineering the key steps for more efficient provision of acetyl-CoA and malonyl-CoA intermediates, which are precursor metabolites for production of biofuels. The results are promising and form a solid basis for development more efficient yeast cell factories for production of biofuels and chemicals, though further optimization is needed.

- Abolishing the phosphorylation sites of Acc1 could increase its enzyme activity and in turn improve fatty acid derived products such as FAEEs
- Ach1 can potentially shuttle mitochondrial acetyl units for cytosolic C2 provision in *S. cerevisiae* lacking pyruvate decarboxylase

- Several bacterial x(f)pk candidates have been newly identified for expression in *S. cerevisiae*, displaying different substrate specificities, efficiently can divert intracellular carbon flux towards C2-synthesis.

Outcomes through the project

1. Shi S[#], **Chen Y**[#], Siewers V., Nielsen J. (2014) Improving production of malonyl coenzyme A-derived metabolites by abolishing Snf1-dependent regulation of Acc1. *mBio*. **5**(3):e01130-14. (# contributed equally)
2. **Chen Y**, Zhang YM, Siewers V & Nielsen J. (2015) Ach1 is involved in shuttling mitochondrial acetyl units for cytosolic C2 provision in Saccharomyces cerevisiae lacking pyruvate decarboxylase. *FEMS Yeast Research* **15**: fov015.
3. Krivoruchko A, Zhang Y, Siewers V, **Chen Y** & Nielsen J. (2015) Microbial acetyl-CoA metabolism and metabolic engineering. *Metabolic engineering* **28**: 28-42.
4. **Chen Y**, Nielsen J. (2016) Biobased organic acids production by metabolically engineered microorganisms. *Current Opinion in Biotechnology* **37**:165-172.
5. Bergman A, **Chen Y**, Siewers V, Nielsen J. Functional expression and evaluation of heterologous phosphoketolases in *Saccharomyces cerevisiae*. Manuscript in preparation.