BIOENGINEERED
2016, VOL. 7, NO. 2, 106–111
http://dx.doi.org/10.1080/21655979.2016.1148223

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ADDENDUM

Activation of futile cycles as an approach to increase ethanol yield during glucose fermentation in *Saccharomyces cerevisiae*

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ABSTRACT

An increase in ethanol yield by yeast from the fermentation of conventional sugars such as glucose and sucrose is possible by reducing the production of a key byproduct such as cellular biomass. Previously we have reported that overexpression of *PHO8* gene encoding non-specific ATP-hydrolyzing alkaline phosphatase can lead to a decrease in cellular ATP content and to an increase in ethanol yield during glucose fermentation by *Saccharomyces cerevisiae*. In this work we further report on 2 new successful approaches to reduce cellular levels of ATP that increase ethanol yield and productivity. The first approach is based on the overexpression of the heterologous *Escherichia coli apy* gene encoding apyrase or *SSB1* part of the chaperon that exhibit ATPase activity in yeast. In the second approach we constructed a futile cycle by the overexpression of *S. cerevisiae* genes encoding pyruvate carboxylase and phosphoenolpyruvate carboxykinase in *S. cerevisiae*. These genetically engineered strains accumulated more ethanol compared to the wild-type strain during alcoholic fermentation.

ARTICLE HISTORY
Received 28 December 2015
Revised 21 January 2016
Accepted 22 January 2016

KEYWORDS
ATPase; apyrase; ethanol; futile cycle; *Saccharomyces cerevisiae*

Introduction

Today, global industrial ethanol production mostly relies on the fermentation of conventional feedstocks such as glucose (derived from starch) and sucrose. The ethanol produced from these feedstocks is known as first generation ethanol.[1] In 2014, about 93 billion liters of first generation ethanol was produced worldwide (http://www.afdc.energy.gov/data/10331). Today, the production of first generation ethanol represents the largest industrial biotechnological application of yeast. For this reason, new scientific approaches to improve ethanol yield and productivity in yeast are of great importance. Ethanol is produced from glucose or sucrose by *Saccharomyces cerevisiae* under anaerobic conditions as the main end product of sugar catabolism. The pathway for the production of ethanol from glucose in yeast (known as glycolysis or Embden-Meyerhof-Parnas, EMP, pathway), yields 2 moles of ATP from one mole of glucose. Some of the ATP produced by glycolysis is subsequently used to produce cellular biomass. Under anaerobic condition, 93% of the glucose used is converted to ethanol and to other low-molecular weight products while the remaining 7% is primarily converted to cell biomass. Among the other minor products of alcoholic fermentation, glycerol is the most abundant. Theoretically, it is possible to increase ethanol yield and productivity by decreasing the accumulation of cellular biomass and glycerol. There are several metabolic engineering approaches to increase ethanol yield by reducing glycerol production by yeast.[1] In one approach, NADH accumulated in yeast during biomass production, is used for the reduction of acetate and to simultaneously lower the synthesis of the enzymes involved in glycerol synthesis from dihyroxyacetone-3-phosphate. By comparison to glycerol reduction, much less is known about ways to increase ethanol yield by decreasing biomass production.

There have been several attempts to reduce ATP production in yeast. One approach substituted genes of EMP pathway with *Zymomonas mobilis* genes encoding unique enzymes of Entner-Doudoroff (ED)

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Addendum to: Semkiv MV, Dmytruk KV, Abbas CA, and Sibirny AA. 2014. Increased ethanol accumulation from glucose via reduction of ATP level in a recombinant strain of *Saccharomyces cerevisiae* overexpressing alkaline phosphatase. BMC Biotechnology 14:42. http://www.biomedcentral.com/1472-6750/14/42; http://dx.doi.org/10.1186/1472-6750-14-42

pathway which is used for glucose alcoholic fermentation in this bacterium. The ED pathway in *Zymomonas* only yields 1 mol of ATP per mole of glucose.[2,3] As a result of the lower ATP yield from glucose, *Z. mobilis* converts 97% of glucose to ethanol with only 3% of the sugar converted to cell biomass. Attempts to express of *Z. mobilis* genes encoding specific aldolase and dehydratase in *S. cerevisiae* were only partially successful as transformants did not express dehydratase activity.[4] Other approaches to lower ATP production in yeast were based on ATP dissipation using futile cycle formed by phosphofructokinase and fructose-1,6-bisphosphatase[5] or by the activation of enzymes involved in ATP degradation, such as acid phosphatase Pho5[6] or F_o, the subunit of membrane ATPase.[7] Recently, we have found that the overexpression of the vacuolar alkaline phosphatase Pho8 leads to an increase in yield and productivity of ethanol synthesis from glucose in laboratory and in industrial strains of *S. cerevisiae* whereas the expression of the truncated cytosol located form is detrimental to the cells.[8] Since our earlier published work, we designed and tested several other approaches to increasing ethanol yield from glucose based on the construction of alternative futile cycles which could dissipate intracellular ATP level. In this work, we report on the successful engineering of yeast cells for the reduction of cellular ATP level with an increase in ethanol yield and productivity during glucose fermentation. These approaches entail the overexpression of ATP degrading enzymes apyrase and *SSB1* part of the chaperon or the construction of a futile cycle due to overexpression of *S. cerevisiae* genes encoding pyruvate carboxylase and phosphoenolpyruvate carboxykinase.

Results and discussion

NSSB1 *and apyrase*

S. cerevisiae SSB1 gene encodes ribosome associated molecular chaperon. Ssb1p is responsible for the correct folding of nascent polypeptide chain during its release from the ribosome. The energy from ATP hydrolysis is utilized to stabilize Ssb1p complex with nascent protein. The Ssb1p consists of 3 distinct domains. One of these is located in the N-terminal 44 kDa region of the protein and has ATPase activity that is repressed by 2 other C-terminal domains. The isolated 44 kDa ATPase domain was shown to have higher affinity to ATP with increased rate of reaction compared with the full length protein.[9] ATP-diphosphohydrolases (apyrases) ($_{EC}$ 3.6.1.5), are enzymes that hydrolyze both the γ- and β-phosphates of ATP and ADP. They are distinct from other phosphohydrolases with respect to their specific activity, nucleotide substrate specificity, divalent cation requirement, and sensitivity to inhibitors.[10,11] Apyrases are ubiquitously expressed in eukaryotes and have been found additionally in some prokaryotes, indicating a general role for these enzymes across these 2 major classes. In our experiments, the N-terminal region of Ssb1 protein (NSsb1) and bacterial apyrase apy from *E. coli* were used to decrease yeast intracellular ATP level.

The 1,233 bp fragment of *SSB1* ORF encoding the N-terminal 411 amino acid residues of the Ssb1p protein (we named it *NSSB1*) and the apyrase gene *apy* from *E. coli* lacking the N-terminal periplasmatic targeting sequence, were functionally expressed in *S. cerevisiae* under the control of the galactose-inducible *GAL1* promoter. The corresponding representative transformants had a slightly slower growth. Transformants that expressed ATPase activity had different growth rates with an increase of 21% for the transformant bearing *apy* gene (BY4742/pYES2-apy) with no increase in growth rate for the transformant bearing *NSSB1* gene (BY4742/pYES2-NSSB). Ethanol accumulation and yield of the constructed strains, was increased by 22% and 39% for BY4742/pYES2- NSSB strain and an increase of 17% and 28% for BY4742/pYES2-apy, when compared to the control BY4742/pYES2 during fermentation in the galactose containing media (1% glucose + 10% galactose)[12] (Table 1).

Futile cycles

Phosphofructokinase and fructose-1,6-bisphosphatase

Fructose-1,6-biphosphatase (FBPase) (EC 3.1.3.11) is one of the major gluconeogenesis enzymes. This enzyme hydrolyzes D-fructose-1,6-bisphosphate to D-fructose-6-phosphate in an ATP-dependent reaction. The simultaneous action of 2 enzymes – phosphofructokinase and fructose-1,6-biphosphatase –leads to the generation of a futile cycle between D-fructose-1,6-biphosphate and D-fructose-6-phosphate with ATP dissipation. In living cells, there are however multiple regulatory mechanisms of the *FBP1* gene and enzyme activity, such as catabolic repression, inactivation by ubiquitination, inhibition by AMP and fructose-2,6-biphosphate.[5,13] Therefore,

Table 1. Ethanol synthesis, yield and specific ATPase activity of *S. cerevisiae* transformants with overexpressed *apy* and *SSB1* genes and the wild-type strain on the third day of fermentation.

	1% glucose + 10% galactose		5% glucose + 10% galactose		
Strain	Ethanol, g L^{-1}	Ethanol, g g^{-1} of biomass	Ethanol, g L^{-1}	Ethanol, g g^{-1} of biomass	ATPase, U/mg
BY4742/pYES2	4.11 ± 0.3	5.73 ± 0.6	10.95 ± 0.9	14.26 ± 1.7	0.66 ± 0.06
BY4742/pYES2- apy	4.80 ± 0.4	7.34 ± 0.8	10.95 ± 1.1	14.10 ± 1.5	0.80 ± 0.10
BY4742/pYES2-NSSB	5.03 ± 0.5	7.94 ± 0.7	15.00 ± 1.3	16.70 ± 1.9	0.53 ± 0.04

The efficiency of galactose fermentation of the transformants with plasmid pYES2-apy, pYES2-NSSB and control plasmid pYES2 was evaluated on SD medium supplemented with mixture 1% or 5% glucose and 10% of galactose. Fermentation was carried out under semi-anaerobic (120 revolutions/min) condition for 3 d. The initial biomass concentration was 15 μg L^{-1}. The concentration of ethanol in fermentation media was determined using alcohol oxidase/peroxidase-based enzymatic kit "Alcotest."[19] ATPase activity was measured as described elsewhere.[12]

intracellular enzyme activity is maintained at basal level in cells grown in media containing fermentable carbon sources.

To overcome tight regulation of the yeast FBPase, the bacterial FBPase that is insensitive to fructose-2,6-biphosphate inhibition, was expressed in yeast.[13] The *E. coli* FBPase was expressed under the control of the constitutive yeast promoter of the *TPI1* gene encoding triose phosphate isomerase in the *S. cerevisiae* strain BY4742 using a replicative plasmid.

Fermentation experiments of the 2 independent transformants was performed using a YPD medium. Strains BY4742/fbp1_7 and BY4742/fbp1_13 produced 23.3 and 24.5 g L-1 of ethanol respectively, while control strain harboring basal plasmid pRS42H[14] produced only 21.3 g L-1 (Table 2). After re-calculation of the results taking into account the increase in yeast biomass, an increase of 5.2% and 8.8% in ethanol yield was demonstrated (Table 2). The ATP level of these strains was also measured. Total ATP level of analyzed transformants was decreased by 31-39% when compared to the control strain (Table 2).

The specific activity of fructose-1,6-bisphosphatase of the transformed strains was measured after 1 d of cultivation on a synthetic medium supplemented with 2% glucose and compared to FBPase activity of the wild type strain BY4742 grown on glucose or ethanol as a sole carbon source. The specific FBPase activity of the obtained recombinant strains was 1.5-3-folds higher than that of the parental strain BY4742. However, FBPase activity was around 2-folds lower than that of the wild-type strain cultivated on an ethanol containing medium as a sole carbon source (gluconeogenic substrates de-repress *FBP1* gene expression in yeast). These results are in agreement with previously published data.[5]

Pyruvate kinase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase

Another way to construct an alternate futile cycle is via the simultaneous activation of the enzymes, pyruvate carboxylase and phosphoenolpyruvate carboxykinase. Pyruvate carboxylase uses ATP energy to convert pyruvate into oxaloacetate, and phosphoenolpyruvate carboxykinase uses ATP energy to convert oxaloacetate into phosphoenolpyruvate. ATP is also synthesized when phosphoenolpyruvate is converted to pyruvate by pyruvate kinase. Therefore the resulting total loss in ATP is one molecule for each one turn of the cycle.

In order to increase pyruvate carboxylase activity, the promoter of the *PYC2* gene was substituted by the strong constitutive *TEF1* promoter. The selected recombinant strain showed a 3-5-fold increase in the

Table 2. Ethanol synthesis, biomass, yield and ATP of *S. cerevisiae* transformants with increased expression of *fbp1* gene of *E. coli* and the wild-type strain strain on the fourth day of fermentation.

Strain	Ethanol, g L^{-1}	Biomass, g L^{-1}	Ethanol, g g^{-1} of biomass	ATP, μM g^{-1}
BY4742/pRS42H	21.3 ± 0.9	3.7 ± 0.2	5.7 ± 0.15	7.75 ± 0.18
BY4742/fbp1_7	23.3 ± 1.1	3.9 ± 0.4	6.0 ± 0.12	4.76 ± 0.10
BY4742/fbp1_13	24.5 ± 1.2	3.9 ± 0.3	6.2 ± 0.20	5.31 ± 0.12

Alcoholic fermentation was performed using a 10% glucose containing medium at 30°C under semi-anaerobic condition (120 revolutions/min) for 4 d as described elsewhere.[8] The concentration of ethanol in fermentation media was determined using alcohol oxidase/peroxidase-based enzymatic kit "Alcotest."[19] ATP was measured by coupled enzymatic reactions with hexokinase and glucose-6-phosphate dehydrogenase as described elsewhere.[20]

specific activity of this target enzyme when compared to the initial strain.

As *S. cerevisiae* phosphoenolpyruvate carboxykinase activity is carefully regulated at the post-translational level, and in order to avoid this regulation, we decided to overexpress heterologous gene encoding the corresponding enzyme from *E. coli*. The expression module containing the *E. coli pckA* gene encoding phosphoenolpyruvate carboxykinase under the control of yeast *ADH1* gene promoter was integrated into the genome of the previously isolated strain overexpressing *PYC2* gene. The specific pyruvate carboxylase and phosphoenolpyruvate carboxykinase activities were elevated 3-4 and 6-7-fold, when compared to the parental strain BY4742, respectively. Ethanol production by the constructed recombinant strains showed a 2-fold increase over the parental strain (Fig. 1).

We have also considered the induction of an alternative futile cycle based on the interconversion between glucose and trehalose, which theoretically could lead to ATP dissipation in cells of *S. cerevisiae*. The disaccharide trehalose is an essential metabolite in *S. cerevisiae* cells. This disaccharide, serves in yeast as one of the major stress protectants. In yeast, trehalose is synthesized from glucose-1-phosphate and UDP-glucose in 2 sequential reactions catalyzed by the enzymes, trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase. Trehalose can be catabolized to glucose by the action of acid or neutral trehalase. In yeast, acid trehalase is mainly localized in the periplasmic space, with a small fraction of activity present in the cell wall.[15]. The cell wall bound enzyme fraction uses extracellular trehalose as a substrate. In yeast, neutral trehalase is a cytosolic enzyme that is required for the hydrolysis of intracellular trehalose.[16] In this metabolic pathway, ATP is used in the conversion of glucose into glucose-1-phosphate and UDP-glucose. To verify whether a perpetual conversion between glucose and trehalose will lead to ATP dissipation, we constructed several *S. cerevisiae* strains with simultaneous overexpression of the 2 genes: *TPS1*, encoding trehalose-6-phosphate synthase, and *NTH1*, encoding neutral trehalase. The over-expression of both genes was accomplished in both cases by using the strong constitutive promoter of the *ADH1* gene. Biochemical analysis of these strains showed that despite the increase in the activities of both trehalose-6-phosphate synthase and neutral trehalase in transformants, when compared to the wild type strain, biomass accumulation did not decrease (data not shown).

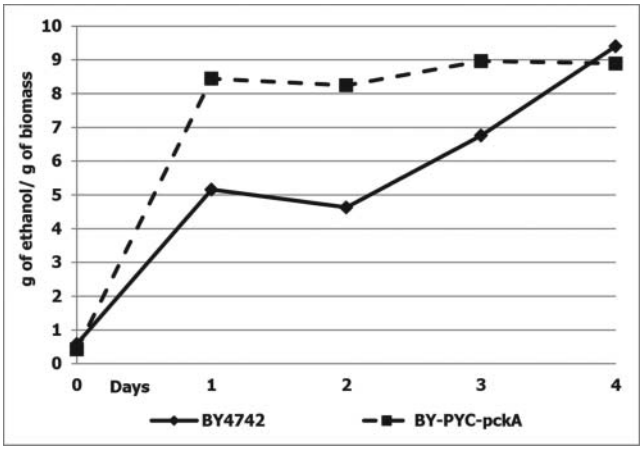

Figure 1. Ethanol production by strain overexpressing *PYC2* and *pckA* genes during glucose fermentation. Diamonds represent wild-type strain (BY4742); squares represent strain overexpressing *PYC2* and *pckA* genes. The data represent means of typical single cultivation. Alcoholic fermentation was performed in 10% glucose containing medium at 30°C under semi-anaerobic condition (120 revolutions/min) during 4 d as described elsewhere.[8]

We have also tried to induce the production of histatins in *S. cerevisiae* cells. Histatins constitute a group of small, cationic multifunctional proteins that are present in the saliva of human and in some other primates. It has been shown that histatin hst5 kills the fungal pathogen *Candida albicans* via a mechanism that involves the release of cellular ATP in the absence of cytolysis.[17] We tested if the expression of codon-optimized human histatin hst5 under the control of maltose-inducible promoter *MAL32* can induce ATP release and subsequent intracellular ATP depletion in *S. cerevisiae*. Unfortunately, the growth level of the WT and histatin-expressing strains on maltose was virtually the same, presumably due to inefficient expression of histatin gene in *S. cerevisiae*. Thus, the last 2 approaches turned out to be unsuccessful.

Our previous works[8,12] showed that it is possible to obtain an increase in the production of first generation ethanol following genetic manipulation of the ATP level in *S. cerevisiae*. The transformed strains accumulated more ethanol due to a reduction in yeast biomass. This was achieved by decreasing intracellular ATP content following the introduction of the ATP degrading enzymes or by the activation of the futile cycles that lead to the dissipation of ATP. We report here on the construction of *S. cerevisiae* strains which have over-expressed

genes encoding ATP degrading enzymes (*NSSB1*, an ATPase encoding part of the host chaperon, and the bacterial apyrase *apy*). Furthermore, we report on the introduction and activation of 2 futile cycles consisting of phosphofructokinase and fructose-1,6-bisphosphatase or pyruvate kinase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase. All these approaches appear to be in agreement with the results reported by other authors[5] on the introduction of an active futile cycle of phosphofructokinase and fructose-1,6-bisphosphatase into yeast. The new approaches that we describe are based on the overexpression of heterologous apyrase and futile cycle consisting of pyruvate kinase plus pyruvate carboxylase and phosphoenolpyruvate carboxykinase are original and have been developed and successfully applied by us for the first time. We hope these new modifications would lead to the successful improvement of yeast strains that can be used for the industrial production of first generation ethanol. The modifications we described here on strain improvements using gene cloning also complement other methods where we reported on the positive selection of ethanol overproducing mutants that are resistant to several antimetabolites, that are mostly known as glycolytic enzyme inhibitors.[18] By combining both approaches (i.e. classical selection for antimetabolite-resistant strains and the subsequent cloning and overexpression of ATP dissipating genes in antimetabolite-resistant strains), provide promising tools for the development of more efficient yeast strains with increased yield and productivity of ethanol synthesis from glucose.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

This work was supported in part by Archer Daniels Midland Company (Decatur, IL); Polish grant of National Scientific Center (NCN) DEC-2012/05/B/NZ1/01657 awarded to AAS and FEMS Fellowship grant # UA-SMU2012-2 awarded to MVS.

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