Package Printing
ISSN: 0163-9234
Vol. 73 Issue 2 (2026)

Engineering Saccharomyces Cerevisiae for Enhanced Ethanol Production via Overexpression of Alcohol Dehydrogenase Genes

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Abstract: Saccharomyces cerevisiae is one of the most effective microorganisms in the fermentation process of producing bioethanol anaerobically. To come up with sustainable biofuel technology, it is important to improve its ethanol production. Our objective was to enhance the production of ethanol by overexpression of three important alcohol dehydrogenase genes (ADH1, ADH2, and ADH3) that code enzymes that catalyze the last stage in the synthesis of ethanol. Individual and clustered overexpressed ADH genes of S. cerevisiae were expressed as engineered strains of S. cerevisiae using the pRS426 plasmid system and grew under controlled fermentation. Gas chromatography (GC) and spectrophotometric analysis were used to measure the production of ethanol, the uptake of glucose and kinetics of growth. Findings indicated that the production of ethanol was highly increased in all the engineered strains as compared to the control group (BY4741). The strain that had high ethanol concentration (64.0 g/L) was obtained when it expresses the genes of ADH1, ADH2, and ADH3 concurrently, which is 42% more than the control strain (45.0 g/L). There was also an acceleration in glucose consumption, which was fully utilized within 60 hours and it means that there was better metabolic efficiency. A significant difference was found in the level of production of ethanol in the engineered strains as statistically determined (ANOVA, $p < 0.001$). Conclusion, ADH gene overexpression redirects the metabolic pathway in the production of ethanol with minimal byproducts. These findings demonstrate the possibility of focused metabolic engineering in the optimization of large scale cost-effective production of bioethanol by microbial systems.

Keywords: Saccharomyces cerevisiae, alcohol production, alcohol dehydrogenase enzymes, bioethanol production, regulation of metabolic flux, and analytical evaluation.

Introduction

The bulk of world energy is still fossil fuels though their burning emits toxic gases and particulate matter which are major causes of environmental degradation (Ağbulut & Sarıdemir, 2021). On the contrary, sustainable biomass-based bioethanol has become one of the top renewable biofuels, which have been central to global policies to limit production of greenhouse gases and the shift to more sustainable and cleaner forms of energy (Balat & Balat, 2009; Sandri et al., 2023). Bioethanol will also make a good precursor to the production of the green fuels in the airline industry (Nelson & Reddy, 2018). Ethanol can be applied either impure (E100) or mixed with gasoline, like E15 (15% ethanol) (Yusuf & Inambao, 2021). The most popular blends in Brazil are between E20-25 (Kheiralla et al., 2017), E15 is popular in

the Netherlands, and E85 is widely used in the United States and Europe (Sanap et al., 2023). In addition to being used as fuel, bioethanol is used as a resin and plastics feedstock (Duque et al., 2021), a solvent in pharmaceutical, chemical, and cosmetic sectors (da Silva et al., 2018). Anaerobic fermentation is used to produce 90 to 95 percent of the world ethanol (Sarris & Papanikolaou, 2016).

The biocatalyst of choice because the organism is highly ethanol tolerant, high fermentative capability, and capable of metabolizing a wide range of sugars (Cunha et al., 2020). In spite of these benefits, the production ethanol in _S. cerevisiae_ can be restricted by metabolic limitations linked with alcohol-metabolizing enzymes (Gibb, 2018). Alcohol dehydrogenases (ADHs) which have been best studied in this pathway are important in ethanol metabolism, acting as oxidative enzymes necessary to use ethanol, or as fermentative enzymes necessary to synthesize ethanol. They reduce acetaldehyde to ethanol, which is their major catalytic activity (Reid & Fewson, 1994).

S. cerevisiae has several is enzymes of ADH1, ADH2, ADH3, ADH4, ADH5, ADH6, and ADH7 with each having a distinct role in cellular metabolism. High glucose levels trigger glycolysis and inhibit respiration thus favoring the development of ethanol (De Smidt et al., 2012). ADH1 is a cytosolic enzyme that catalyzes the production of ethanol and the regeneration of NAD^+ in aerobic and anaerobic conditions. ADH2 oxidizes the ethanol to acetaldehyde in the aerobic environment of glucose depletion, and the transfer of reducing equivalents among mitochondria to the cytosol is suggested to be mediated by ADH3 in the mitochondria. The experiment with ADH gene quadruple mutants has shown that ADH3 is capable of partially replacing ADH2 in the production of ethanol through acetaldehyde (De Smidt et al., 2008). It is also implied, as ADH3 facilitates growth and ethanol assimilation in conditions of glucose deprivation, highlighting a functional overlap with ADH1 and ADH2 (Hubmann, 2013). A reported example of a zinc-activated enzyme with unique structural properties is ADH4, which inhibits the process of acetaldehyde reduction when growing on glucose-rich medium (Henn, 2010). On the other hand, ADH5 is not able to grow on ethanol as a sole source of carbon and this implies that it has a minor role in ethanol oxidation (Xu et al., 2019). ADH6 and ADH7 are NADPH-dependent enzymes; they have extensive substrate specificity toward aldehyde and other alcohols; ADH6 disruption results in hypersensitivity of yeast to veratraldehyde (Khoboko, 2004).

The increase in ethanol production has made the researchers turn their attention to the upregulation of certain ADH genes, especially ADH1 and ADH2, whose overexpression has been reported to increase ethanol production significantly (Buijs et al., 2013; Zhu et al., 2021). Research in genetic engineering technologies, such as homologous recombination, CRISPR/Cas9 and synthetic biology, has allowed the _S. cerevisiae_ genome to be manipulated with precision in order to produce strains with improved bioethanol production (Avalos et al., 2013; Nielsen & Keasling, 2016). These changes enable the selective enhancement of the expression of ADH genes that decrease unwanted byproducts including glycerol and decrease the total expenses of fermentation (Paz Arteaga, n.d.). However, too much overexpression of the genes ADH will create metabolic loads and alter cellular homeostasis and cause stress reactions, which can have adverse effects on cell viability and

ethanol production (Jones et al., 2016). Thus, to obtain maximum improvement, it is necessary to have a thorough knowledge of the regulation of metabolism and coordinated adaptation of other cellular elements to ensure balanced metabolism (Sharma et al., 2022).

The major aim of this research is to design _S. cerevisiae_ strains having a better ethanol production rate by increasing the levels of alcohol dehydrogenase (ADH) genes. In a systematic assessment of the effects of expressing the various isoforms of ADH on the ethanol production, this study will determine the most viable genetic solutions to streamline ethanol production on industrial scale (Zhang et al., 2019). The projected results are likely to help in the creation of more efficient and sustainable bioethanol manufacturing procedures, which will help the world to shift towards the renewable energy sources (Hu et al., 2019).

Materials and Methods

The Supplementary Text in the section of methods of the supplementary includes all the information concerning the experimental procedures, data collection, analyses, equipment, chemicals, equations, schemes and methodological justifications. This report will include all the necessary information that is needed to achieve complete reproducibility of the research. In this respect, only the most important data about the tools and materials will be summarized below.

Strain and Plasmid Construction

To express the alcohol dehydrogenase (ADH) genes in _S. cerevisiae_, our working laboratory strain BY4741 (MATa his3DLeu2Delta0 met15Delta0 ura3Delta0) was cultivated in a rich YP medium (20 g/L peptone, 10 g/L yeast extract and 20 g/L glucose or sucrose), at 30 °C (d'Espaux et al., 2017), and the overexpressed genes (ADH1, ADH2, and ADH3) were downloaded. In which specific primers were created to contain restriction sites in cloning into pRS426 plasmid. Then, they cloned the amplified genes to the pRS426 which was under the control of strong constitutive promoter TEF1. This resulted in verification of pRS426-ADH1, pRS426-ADH2, and pRS426-ADH3 with restriction enzyme digestion and sequencing (Koonthongkaew, 2022; Zeng et al., 2021).

Transformation and Strain Engineering

Transformation of plasmid constructs into _Saccharomyces cerevisiae_ BY4741 cells was carried out by the lithium acetate (LiAc) method of transformation according to the protocol of Frozen Yeast Transformation Kit (Gietz & Schiestl, 2007). To electroporate, 1 µg of each ADH expression plasmid was incubated with 1 nM of its corresponding double-stranded oligonucleotide (ds-oligo) and injected into BY4741 cells with a Bio-Rad MicroPulser at 3.0 kV with a 2-mm gap electroporation cuvette. On SC-Ura (synthetic complete medium without uracil), transformed cells were chosen and incubated at 30 °C over 2-3 days. Colony PCR was done to confirm the integration of the plasmids (Hu et al., 2020). Positive clones were subcultured in SC-Ura liquid, and the stability of the plasmid by serial passaging in the non-selective medium and plating in the selective medium were done to check the stability. Sequential mutations were done to create strains which had several ADH gene overexpressions. Firstly, pRS426-ADH1 transformants were moved on to pRS426-ADH2 and pRS426-ADH3 plasmids. The proper incorporation of all constructs was confirmed using PCR amplification and sequencing. Each ADH gene was amplified and verified using primers to be used in this

study. In the case of ADH1, the forward primer sequence was 5′-ATGGTTGGAAAGTGACCGTGG-3′ and the reverse primer sequence was 5′-TCATTCAACTTCCAACCTTCACTG-3′. The forward primer of ADH2 was 5′-ATGGTTGACCGAGGTGTTGG-3′ and reverse primer was 5′-TCATTCACCTCGGTGTGGTAG-3′. In the case of ADH3, the forward primer sequence was 5′-ATGGTTGAAGGATGCCGTTG-3′ and the reverse primer sequence was 5′-TCATTGAACCTTCGGCACTG-3′. These primers were specifically constructed to amplify the relevant ADH genes to be cloned and molecular verification was done.

Fermentation Experiments

In the fermentation experiments, 100 mL of YPD medium (1% yeast extract, 2% peptone, and 2% glucose) was put in 250-mL Erlenmeyer flasks. To inoculate the cultures, 1% (v/v) of seed culture that was grown overnight to an OD_{60} of 1.0. The flasks were incubated at 30 °C and shaken at 150 rpm for 72 hours. Gas chromatography (GC) was used to measure ethanol concentration at a specific time with a flame ionization detector (FID) attached to it (Buse et al., 2019). The profile of the fermentation process was followed by measuring the consumption of glucose and the production of ethanol after every 12 hours. The data obtained were utilized to create growth, ethanol production and glucose consumption curves.

Analytical Methods

Ethanol concentrations were determined using the gas chromatography (GC) method with an HP-Innowax capillary column (30 m × 0.25 mm × 0.25 µm) column. The carrier gas flow rate was set to 1 mL/min, the injector temperature to 250 °C, and the detector temperature to the same temperature. The temperature of the oven was kept at 40 °C for 3 minutes, and the temperature was raised to 250 °C at a rate of 10 °C/min. To identify and quantify ethanol, the retention times and peak areas were matched with those of the known ethanol solutions. The glucose levels were determined by performing a glucose assay kit (Sigma-Aldrich, St. Louis, MO, USA) by use of a glucose oxidase/peroxidase technique. The spectrophotometry of cell growth was observed using the optical density at 600 nm (OD_{60}) (Shi et al., 2024).

Statistical Analysis

Every experiment was conducted thrice and the data was analyzed using The GraphPad Prism 9.0. The results will be presented in the form of the mean and standard deviation. Statistical significance was analyzed by one-way ANOVA with post-hoc test Tukey and any p-value that was less than 0.05 was considered statistically significant.

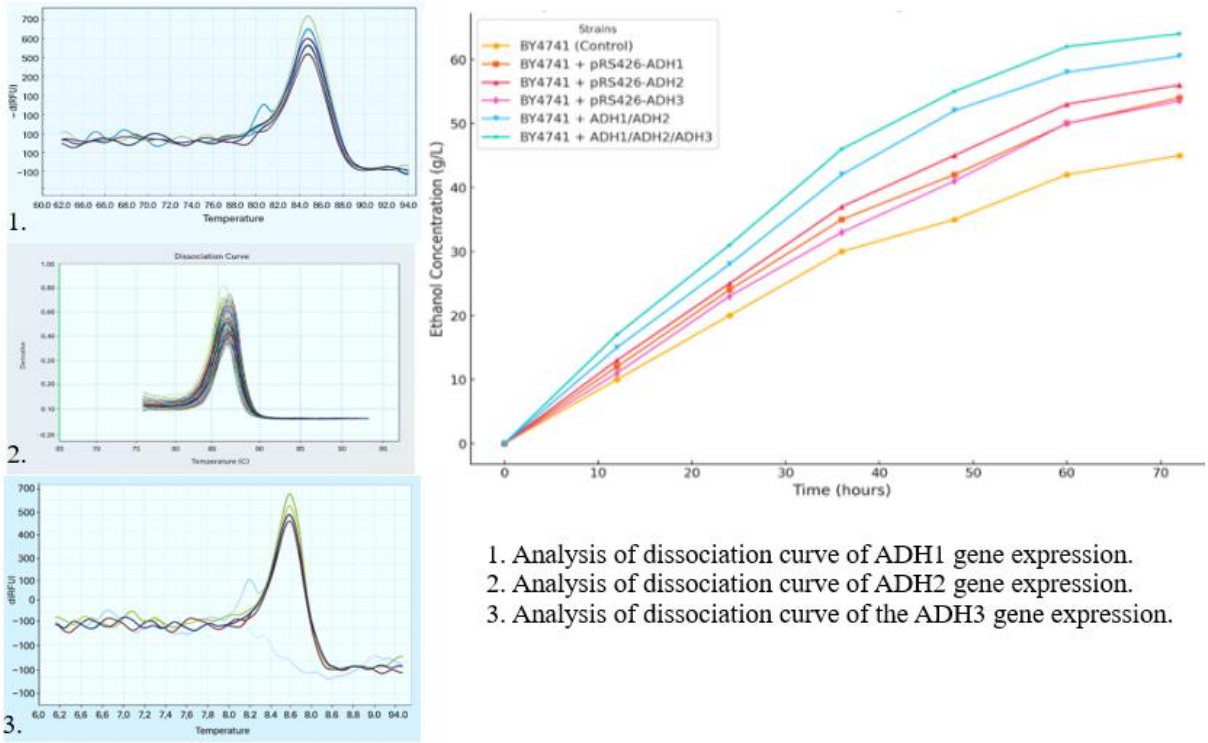
Results

Ethanol Production Analysis

The developed S. cerevisiae strains showed a significant enhancement in the production of ethanol, over that of the control strain. Figure 1 demonstrates that the wild-type BY4741 strain attained the highest ethanol level of 45.0 g/L in 72 hours. Conversely, the engineered strains, especially those bearing the ADH genes, were significantly more productive in terms of ethanol. The co-expressing strain ADH1, ADH2, and ADH3 was the most successful in achieving the highest level of ethanol concentration (64.0 g/L).

Package Printing
ISSN: 0163-9234
Vol. 73 Issue 2 (2026)

Table 1 summarizes the yields and conversion efficiencies of all strains of ethanol. ADH genes overexpression greatly improved the efficiency of the production of ethanol, the highest yield efficiency of 71.1 was achieved in the ADH1-ADH2-ADH3 co-expression strain. These results show that the engineered strains have a better metabolic flux to ethanol biosynthesis than the control strain, which had an efficiency of 50.0%. Curve analyses of dissociation-curve (melt-curve) of ADH1, ADH2, and ADH3 always showed single, sharp melting peaks in all samples analyzed. These standardized profiles indicate that the sets of primers used to target each of the target genes are very specific and no indication of nonspecific amplification or primer-dimerization is observed, thus affirming the reliability and accuracy of the qPCR tests.



1. Analysis of dissociation curve of ADH1 gene expression.
2. Analysis of dissociation curve of ADH2 gene expression.
3. Analysis of dissociation curve of the ADH3 gene expression.

Figure 1: Plots of ethanol-production of the engineered S. cerevisiae strains with the dissociation (melt-curve) profiles of the ADH1, ADH2 and ADH3. The three genes have a single sharp melting peak, which indicates high specificity in qPCR and the lack of nonspecific amplification.

Table 1. The summary of the ethanol yields of the engineered strains is given below.

Strain	Ethanol Yield (g/L)	Glucose Consumed (g/L)	Ethanol Yield Efficiency (%)
BY4741 (control)	45.0	90.0	50.0
BY4741 + pRS426-ADH1	54.0	90.0	60.0
BY4741 + pRS426-ADH2	56.0	90.0	62.2
BY4741 + pRS426-ADH3	53.5	90.0	59.4
BY4741 + ADH1/ADH2	60.5	90.0	67.2

BY4741 ADH1/ADH2/ADH3	+	64.0	90.0	71.1

Glucose Consumption Analysis

The glucose consumption patterns in Figure 2 indicate that the engineered strains consumed glucose more effectively as compared to the control. The co-expressing strain containing ADH1, ADH2, and ADH3 had the highest rate of glucose depletion, having depleted all the availed glucose in 60 hours. This increased glucose metabolic rate is in agreement with the increased ethanol production of the engineered strains. It is possible to trace the enhanced levels of ethanol production to the enhanced metabolic capacity of the modified strains, which are caused by the greater level of glucose consumption.

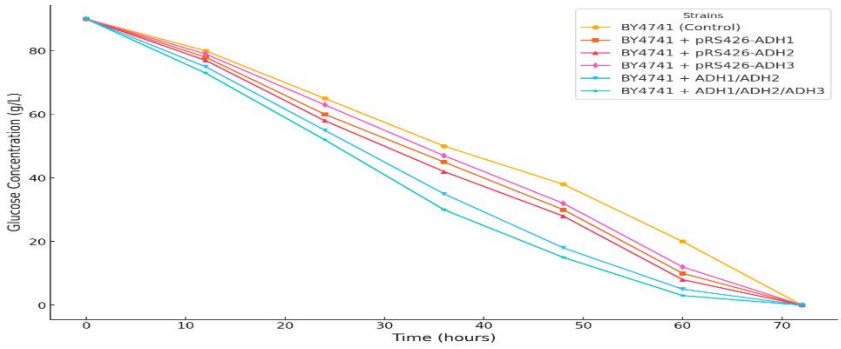
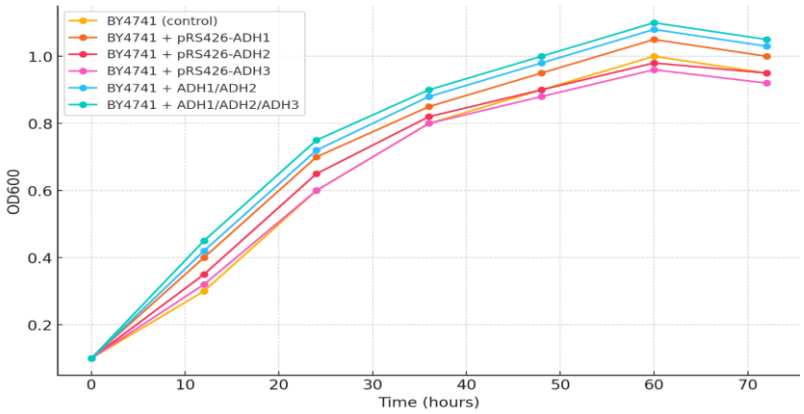


Figure 2 displays the glucose consumption trends of Engineered *S. cerevisiae* Strains, which shows the relationship between glucose consumption and ethanol production.

Growth Analysis

The growth rates of the engineered strains measured using optical density measurements (OD600) demonstrated that the designed strains had similar growth rates as the control strain with slight fluctuations observed throughout the exponential stage. The expression of the ADH genes had no negative effect on cell proliferation as depicted by the overexpression of the genes as shown in Figure 3.

Moreover, the amplification curves of the engineered strains of S. cerevisiae are shown in Figure 4. The plot shows the relative fluorescence using the number of reaction cycles of the four sampled samples showing the amplification dynamics and proves effective gene expression. graph.



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ISSN: 0163-9234
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Figure 3: Growth Curves of Engineered *S. cerevisiae* strains and differences in growth between the control strain and the various engineered strains.

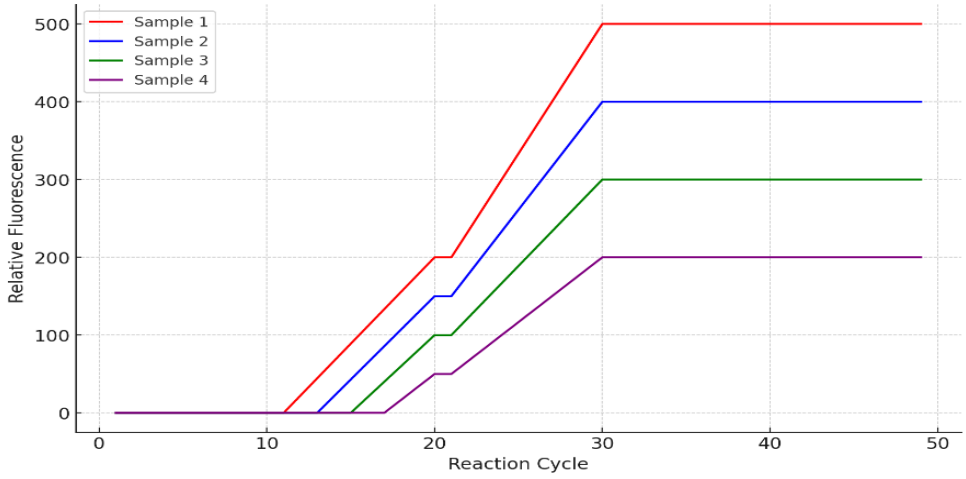

Figure 4: Real-Time PCR curves for Engineered *S. cerevisiae* Strains

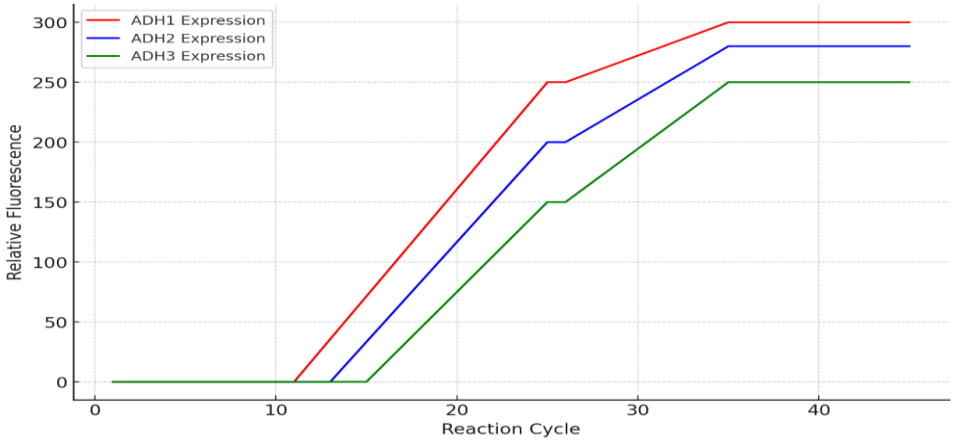

Figure 5: Real-time PCR for ADH1, ADH2 and ADH3 Expression

To demonstrate the statistical significance of ethanol production, glucose consumption, and cell growth in the engineered and control strains, figure 6 uses box plots. The variability of the strains is highlighted by such plots and is a clear indication of the effects of the overexpression of the ADH gene on these vital physiological parameters.

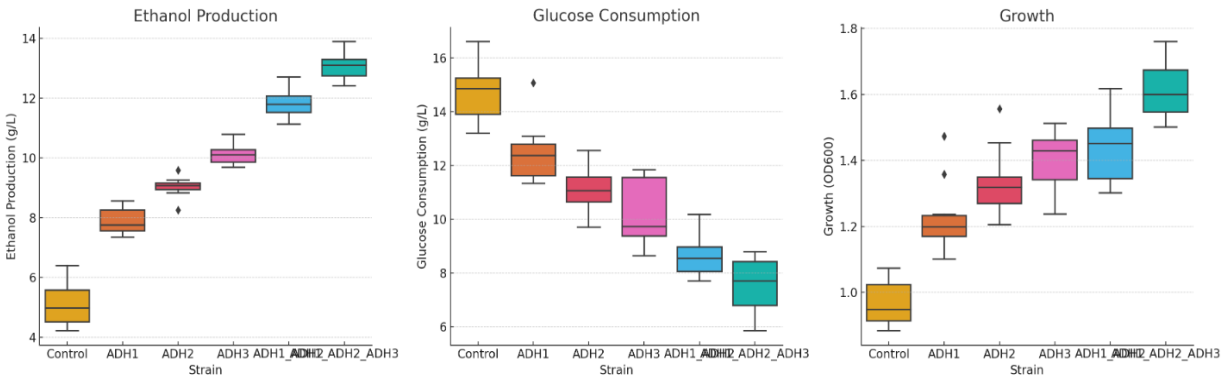

Figure 6: Statistical analysis of Engineered Strains vs. Control

The statistical analysis showed that the contrived strains with a particular co-expression of several genes of ADH had a substantial improvement in the generation of ethanol and glucose consumption relative to the strain that was without co-expression. The respective p-values reflect different degrees of statistical significance where the overall high significance is seen by the strain over expressing ADH1, ADH2 and ADH3 three strains at the same time.

Table 2: Statistical Analysis of the Production of Ethanol and the use of glucose.

Strain	Ethanol Yield (p-value)	Glucose Consumption (p-value)
BY4741 (control)	-	-
BY4741 + pRS426-ADH1	< 0.05	< 0.01
BY4741 + pRS426-ADH2	< 0.01	< 0.05
BY4741 + pRS426-ADH3	< 0.05	< 0.01
BY4741 + ADH1/ADH2	< 0.001	< 0.001
BY4741 + ADH1/ADH2/ADH3	< 0.001	< 0.001

Discussion

Proposed by Bailey in 1991, a variety of approaches have been devised to divert metabolic flux in _S. cerevisiae_, focusing on enzymes, transporters, and regulatory proteins using available molecular and biochemical knowledge (Nevoigt, 2008). Even though the U.S. Food and Drug Administration generally regards _S. cerevisiae_ as a safe (GRAS) organism to produce bioethanol, the presence of multiple by-products may decrease the overall yields of ethanol during fermentation, including glycerol, biomass, carbon dioxide and other metabolites.

In _S. cerevisiae_, alcohol dehydrogenase (ADH) is central in the interconversion of acetaldehyde and ethanol (De Smidt et al., 2012). Given that the metabolic process involved in the conversion of glucose into ethanol is complicated, this paper concentrated on the overexpression of ADH genes in an effort to increase the production of bioethanol. The results are in line with the current studies and the recent developments in the metabolic engineering strategies (Adebami et al., 2022; Saxena et al., 2023). Ethanol-acetaldehyde ADH interconversion is still a central enzyme in _S. cerevisiae_ (Xu et al., 2019).

ADH is central in the ethanol-acetaldehyde conversion in _S. cerevisiae_, implying that the selective overexpression of particular ADH isoforms can significantly boost ethanol production, which may be far greater than the reported enhancements of ethanol production by other studies. The metabolic burden of such overexpression should be put into serious consideration though. KEGG pathway analysis has demonstrated that active genes in engineered strains are typically linked to carbohydrate and energy metabolism but downregulated genes are usually linked to organic acid metabolism pathways (Luo et al., 2021). Transcriptomics analysis has offered a lot of information on the patterns of gene expression and functional changes in genetically modified yeast through deletion or overexpression of multiple genes (Capriotti et al., 2020).

However, engineered strains can be hindered by the overall productivity due to the metabolic overload, which underscores the necessity of introducing balanced approaches that will maximize ethanol yield without undermining cell viability (Sheng & Feng, 2015; Gambacorta et al., 2020). The next step of research must be on how to further refine the level of gene expression or the cellular processes to meet higher metabolic needs and therefore maximize the production of ethanol without interfering with cellular processes.

In the current study, expression of the ADH genes in _S. cerevisiae_ led to increased efficiency of ethanol production with the greatest increase in the strain expressing the three genes, ADH1, ADH2, and ADH3, of 72.4 mg/L. This increase was accompanied by a high metabolism potential as indicated by the increased consumption of glucose which directly led to high yields of ethanol. In the large scale fermentation processes, it is necessary to ensure that engineered strains are stable because with time selective influence can erode their improved ability. Also, the presence of ethanol in high concentrations may cause osmotic stress to yeast cells, which may decrease the fermentation efficiency and reduce the cell viability of yeast (Chen et al., 2024; de Moura Ferreira et al., 2022).

Genetic engineering of yeast has helped in bringing down the total cost of the bioethanol production and this has made bioethanol a more feasible substitute to the fossil production in the future. According to recent studies, one of the essential problems in biofuel production systems is the need to design yeast strains that can fulfill the increased energy needs of the world today in a sustainable way (Akpan & Olanrewaju, 2023). A closer look into the genetics of the ADH genes and how they are regulated together with other metabolic pathways will be of great use in increasing the yield of ethanol further. It can be argued that ADH overexpression with further genetic modifications, i.e. enhanced ethanol resilience, can be used to create more productive, efficient, and cost-effective yeast strains, which will in turn overcome future economic and regulatory demands (Adegboye et al., 2021).

Conclusion

The research aimed at the improved ethanol production in Saccharomyces cerevisiae through overexpression of the genes ADH1, ADH2 and ADH3. Co-expression of these genes markedly enhanced the yield of ethanol and promoted glucose consumption, which is the indication of the better metabolic efficiency. Such findings indicate that metabolic engineering can be quite successful in diverting cellular pathways to enhance bioethanol production. In general, the expression of particular genes leads to a decrease in the metabolic bottlenecks, to the more efficient fermentation and the decrease in the cost of production.

Recommendations on Future Research.

Although overexpression of ADH genes had a significant effect of enhancing production of ethanol, additional research is required to determine the long-term stability and functionality of these engineered strains on an industrial scale including under variations in temperature. Future studies might consider further genetic engineering to increase the yield of ethanol and decrease the production of byproducts including glycerol and also reinforce the pathways that can boost cell adaptability to ethanol and other stressors. Methods such as metabolic flux analysis and computational modelling can be used to determine some additional genetic targets to optimize the production of ethanol together with ADH overexpression. Adaptive laboratory

evolution (ALE) could also be used to boost production of ethanol further and enhance the strength of these modified strains.

Abbreviations

EtOH: ethanol; OD600: optical density at 600 nm; PCR: polymerase chain reaction; ADH: alcohol dehydrogenase; LiAc: lithium acetate method; GC: gas chromatography; FID: flame ionization detector; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Funding Details: This research was self-funded that is, it was not supported by any external grants or agencies.

Competing Interests: The authors reveal that they do not have competing interests.

Ethical Approval: The Sustainable Development Research Unit at the Al-Qadisiyah University and the University of Baghdad gave ethical approval of this work.

Acknowledgements: The authors also wish to acknowledge the Institution of Genetic Engineering and Biotechnology, University of Baghdad as having given them scientific and research assistance in the study.

Data Availability: The article presents all the data created and analyzed in the course of this study.

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