Improvement of ethanol stress tolerance in *Saccharomyces cerevisiae* through modulation of Ehrlich pathway via overexpression of *BAT2* gene

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Abstract

Ethanol functions both as a primary product and a stressor in yeast fermentation, particularly affecting Saccharomyces cerevisiae at high concentrations. Elevated ethanol levels disrupt membrane integrity, enzyme activity and redox balance, reducing yeast viability and fermentation efficiency. Enhancing ethanol tolerance is vital for optimizing industrial fermentation processes. This study investigates the role of the BAT2 gene, which encodes a cytosolic branched-chain amino acid aminotransferase involved in the Ehrlich pathway- a metabolic route converting branched-chain amino acids into fusel alcohols.

Overexpressing BAT2 significantly increases higher alcohol production and is correlated with improved ethanol stress tolerance. These findings show that engineering the Ehrlich pathway via BAT2 overexpression can strengthen yeast tolerance. This approach offers a promising metabolic engineering strategy to develop yeast strains better suited for industrial fermentation under ethanol stress conditions.

Keywords: Ethanol stress, *Saccharomyces cerevisiae*, Branched-chain Amino Acid Transaminase 2 (*BAT2)* gene, Ehrlich pathway, Branched-chain amino acids, Higher alcohol production, Yeast fermentation, Metabolic engineering, Stress tolerance.

Introduction

Ethanol is not only a primary product of yeast fermentation, but also a key environmental stress factor that can limit yeast viability and performance, especially at high concentrations[10,26]. In industrial fermentations including those for alcoholic beverages, yeast cells are frequently exposed to ethanol levels that can impair membrane integrity, inhibit enzyme activity and disrupt redox homeostasis. Therefore, enhancing ethanol tolerance in yeast is an important aspect of strain improvement for robust and efficient fermentation. Enhancing yeast tolerance to ethanol is therefore critical for efficient large-scale fermentation[4,11].

According to Koonthongkaew et al[7] the *BAT2* gene, encoding a cytosolic branched-chain amino acid aminotransferase, plays important role in the Ehrlich pathway, a secondary metabolic route that converts branched-chain amino acids into fusel alcohols. Overexpression of *BAT2* enhances the catabolism of valine, leucine and isoleucine, leading to increased production of higher alcohols like isobutanol and isoamyl alcohol[9]. This not only aids in maintaining redox balance under anaerobic fermentative conditions but also improves cellular tolerance to ethanol stress, making *BAT2* a key candidate gene for metabolic pathway engineering in ethanol-producing yeast strains[25]. Historically, ethanol and fusel alcohols such as isobutanol and isoamyl alcohol were produced through chemical synthesis methods like petrochemical cracking or catalytic hydration of ethylene. While these approaches offered high yields and rapid production, they were heavily reliant on non-renewable resources, energy-intensive and generated significant environmental pollutants[13].

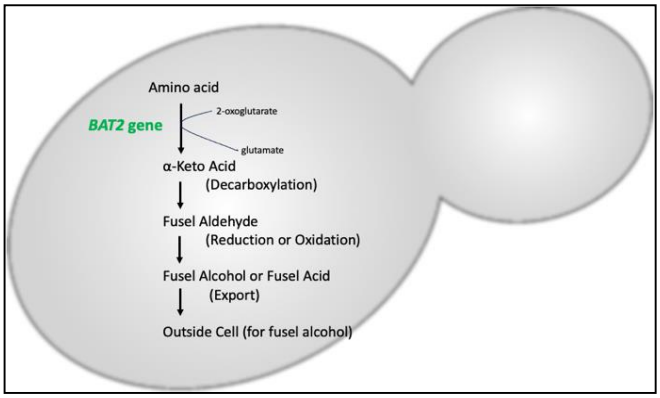

Figure 1: Schematic representation of the Ehrlich pathway showing the role of *BAT2* gene in the conversion of branched-chain amino acids into fusel alcohols

Additionally, chemical synthesis lacked specificity, often resulting in mixed or impure alcohol products requiring extensive downstream purification[15]. In contrast, microbial fermentation especially using *Saccharomyces cerevisiae* provides a renewable, eco-friendly and highly selective platform for ethanol and fusel alcohol production. Through metabolic pathways like glycolysis and the Ehrlich pathway, yeast can efficiently convert sugars and amino acids into ethanol and higher alcohols under mild, anaerobic conditions[29] (Figure 1). Advances in synthetic biology and metabolic engineering, such as the overexpression of gene like *BAT2*, have further enhanced the ability of microbes to tolerate ethanol stress and increase the biosynthesis of valuable fusel alcohols. This makes microbial production a sustainable and scalable alternative to conventional chemical methods, particularly in the biofuel and beverage industries[14].

According to Ma et al[12], the higher alcohols are essential secondary metabolites that yeast produces during the fermentation of alcohol. Excessive buildup might produce unwanted sensory qualities and possible health risks, even while modest amounts enhance the flavour and aroma of fermented beverages[13]. *BAT1* and *BAT2* in *Saccharomyces cerevisiae* encode branched-chain amino acid transaminases, which are important enzymes in higher alcohol production.

Previous studies have mostly focused on lowering increased alcohol production by either overexpressing *BAT1* or deleting *BAT2*. For instance, in Chinese rice wine fermentation, *BAT2* deletion in an industrial strain resulted in comparable decreases in higher alcohol production without affecting fermentation performance, whereas in wine yeast YZ22, replacing *BAT2* with a *BAT1* expression cassette significantly decreased isobutanol and 3-methyl-1-butanol levels[31].

Yet, despite its potential outcomes, the function of *BAT2* overexpression remains underexplored. Most current research emphasizes gene deletion or suppression strategies, while overlooking how enhanced *BAT2* expression might affect higher alcohol formation, yeast stress tolerance and overall fermentation kinetics. Thus, this study represents a key research gap in our understanding of the regulatory mechanisms underlying higher alcohol biosynthesis. Our study aims to address this gap by focusing on the functional characterization of *BAT2* under conditions like overexpression, thereby providing a more balanced and comprehensive view of *BAT2*'s role in yeast metabolism.

In this study, the *BAT2* gene was amplified using gene-specific primers, sequenced and uploaded to the NCBI GenBank database. The *BAT2* open reading frame (ORF) was later cloned into a yeast expression vector and transformed into *S. cerevisiae* (BY4741) by the lithium acetate (LiAc) method. Transformants were selected using SD-uracil dropout plates. The *BAT2* enzyme's functional activity was evaluated using a Branched-Chain Aminotransferase (BCAA) activity assay. Furthermore, the ethanol tolerance of the recombinant yeast strains was tested using a yeast spot assay at different ethanol concentrations of up to 10% (v/v). These methodologies offer biochemical and physiological insights into the effects of *BAT2* overexpression, laying the groundwork for future applications in yeast performance and flavour modulation in fermented drinks.

Material and Methods

Strains, Media and Reagents: *S. cerevisiae* BY4741 was employed as the host strain. *E. coli* DH5α was used for construction of plasmid. A YPD medium composed of 10 grams/Liter (g/L) yeast extract, 20 g/L peptone and 20 g/L dextrose, was used for cultivating yeast strains. *E. coli* strains were grown in a Luria-Bertani medium 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl at 37 °C. SD-URA (synthetic medium with uracil omitted) were used for selection and cultivation of yeast transformants. Restriction endonucleases and T_4 DNA ligase were sourced from Thermo Scientific (Thermo Scientific, USA). The DNA polymerase (Hifi-Taq) was used for amplification of all DNA fragments. Antibiotics like ampicillin were purchased from Thermo-Fisher Scientific.

Plasmid construction: Initially, the *BAT2* gene was amplified from the genomic DNA of *Saccharomyces cerevisiae* strain BY4741 using gene specific forward (5'-CGGGATCCATGACCTTGGCACCCCTAGAC-3') and reverse (5'-CGAAGCTTTCAGTTCAAATCAGTAACAA CCCTTGA-3') primers containing BamHI and HindIII restriction sites respectively. The PCR amplification was carried out for 35 cycles, with an initial denaturation at 95 °C for 5 minutes, subsequently by denaturation at 95 °C for 30 seconds, annealing at 50.5 °C for 45 seconds and extension at 72 °C for 1.3 minutes, with a final extension step at 72 °C for 10 minutes (Fig. 2). Both the amplified *BAT2* fragment and the empty p406TDH3 plasmid backbone were subjected to double digestion with BamHI and HindIII enzymes.

The digested DNA fragments were then ligated using T4 DNA ligase for 16 hours at 16 °C following protocol from Qi's[13] literature. The recombinant plasmid, designated p406TDH3+*BAT2*, was constructed using the p406TDH3 yeast expression vector (obtained from Addgene Plasmid #15977) as a backbone, which carries a TDH3 promoter, a multiple cloning site (polylinker), a CYC1 terminator and a URA3 gene as a selectable marker. The constructed plasmid was propagated and maintained in *Escherichia coli* DH5α cells and plasmid DNA was extracted using the Qiagen Miniprep kit as per the manufacturer's protocol[6].

Construction of yeast strain: The recombinant plasmid p406TDH3+*BAT2* was transformed into *Saccharomyces cerevisiae* BY4741 cells to achieve overexpression of *BAT2* gene using the standard lithium acetate (LiAc) transformation method[5].

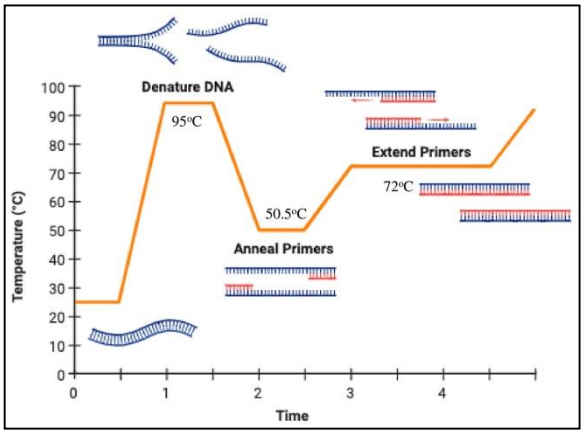

Figure 2: PCR thermal cycling profile showing steps of initial denaturation, annealing, extension and final extension used for *BAT2* gene amplification.

Following transformation, positive transformants were selected by plating the cells on SD-URA (Synthetic Dropout medium lacking uracil) plates and incubating them at 30°C for 2-3 days. Colonies that grew under these selective conditions were verified and designated as BY4741-*BAT2*OE (where "OE" stands for "overexpression").

BCAA Assay for yeast transformed with *BAT2* gene: Quantification of branched-chain amino acids (BCAA's) in *Saccharomyces cerevisiae* strain was conducted using the BioVision Branched Chain Amino Acid (BCAA) assay kit following the manufacturer's instructions with slight modifications. Yeast strains (BY4741 and BY4741 + *BAT2*OE) were grown overnight in SD-URA medium at 30°C and sub-cultured to an initial OD_{600} of 0.2-0.3. Cultures were incubated to reach mid-log phase (OD600 of 0.8–1.0) before harvesting by centrifugation at 8500 × g for 5 minutes at 4 °C. Cell pellets were washed twice with ice-cold PBS and re-suspended in BCAA assay buffer. Cells were lysed by glass bead method, followed by centrifugation at 12,000 × g's for 10 minutes at 4 °C to obtain clear supernatants.

Lysates were deproteinized by adding 10% perchloric acid, incubated on ice for 5 minutes and neutralized with potassium carbonate. The deproteinized extracts were used for the assay. Samples and BCAA standards were loaded onto a 96-well plate along with the reaction mixture provided in the kit. After incubation at room temperature for 60 minutes protected from light, absorbance was measured at 450 nm using a microplate reader. BCAA concentrations were determined by comparison with the standard curve and normalized to total protein content[32].

Yeast Spot Assay for Ethanol Tolerance: To evaluate ethanol tolerance, a yeast spot assay was executed by comparing the control strain BY4741 (transformed with empty p406TDH3) and the recombinant strain BY4741+*BAT2*OE (transformed with p406TDH3+*BAT2*) by following protocol from the study of Sahu et al[19]. Overnight grown cultures were plated on SD-URA media at 30 °C, normalized to an OD600 of 1.0 and serially diluted in 10-fold steps. 3 µL of each dilution was spotted on SD-URA agar plates supplemented with different concentrations of ethanol (0%, 3.5%, 7% and 10% v/v). Plates were incubated at 30 °C for 2-3 days and growth was assessed visually. No notable difference in growth was observed between the two strains on plates containing 0%, 3.5% and 7% ethanol. However, at 10% ethanol, the BY4741-*BAT2*OE strain exhibited noticeably higher ethanol tolerance compared to the control strain.

Results and Discussion

In this study, a systematic approach was undertaken to enhance ethanol tolerance in *Saccharomyces cerevisiae* BY4741 through the overexpression of the *BAT2* gene. The overall workflow is summarized in fig. 3. Initially, BY4741 yeast cells were cultured on YPD plates and propagated in liquid medium, followed by transformation with the recombinant p406TDH3+*BAT2* plasmid using the lithium acetate (LiAc) method. Successful transformants were selected on SD-URA dropout plates and further confirmed by colony PCR and restriction digestion analysis.

To assess the impact of *BAT2* overexpression, yeast spot assays were conducted under ethanol stress conditions (10% v/v), revealing improved growth performance of the engineered BY4741+*BAT2*OE strain in comparison to the control strain. Additionally, BCAA (branched-chain amino acid) metabolic activity was evaluated using microplate reader assays, linking *BAT2* overexpression to higher ethanol tolerance, thus improving fermentative conditions under ethanol stress.

Amplification and Sequencing of the *BAT2* Gene: The BAT2 gene was efficiently amplified using gene specific primers, yielding a distinct band of approximately 1131 bp as observed on ethidium bromide (*EtBr*) pre-stained 1% agarose gel (Fig. 4, Lanes 1-4), with lane 1 representing the DNA ladder used as a standard DNA marker. Lane 2 is non-template control. Lanes 3 and 4 are *BAT2* PCR amplified product. The amplified PCR product was sequenced from Barcode Biosciences (Bangalore, India). The obtained sequence data were managed to sort clean reads, which were

subsequently analysed[2,28]. No point mutations were detected when the obtained sequence was compared to the reference sequence, confirming the accuracy of the amplification. The final validated sequence of the *BAT2* gene was submitted to the NCBI GenBank database with the accession number PV399958.

Transformation and Confirmation of *BAT2* Gene Cloned into p406TDH3 Yeast Expression Vector: The *BAT2* gene was cloned into p406TDH3 yeast expression vector following successful ligation and transformation procedures. For plasmid propagation and maintenance, *Escherichia coli* DH5α was employed. The *BAT2* insert and the p406TDH3 vector were restriction digested with *BamHI* and *HindIII* restriction enzymes and the digested products were ligated at 16°C for 16 hours using T_4 DNA ligase. The ligation mixture was transformed into *Escherichia coli* DH5α cells using the heat shock method. Transformants were selected and colony PCR targeting the *BAT2* gene was performed, successfully amplifying a 1131 bp product, confirming the presence of the insert (Fig. 5).

Recombinant plasmids (p406TDH3+*BAT2*) were extracted from positive colonies and further verified by double digestion with *BamHI* and *HindIII*, showing two clear bands corresponding to the *BAT2* gene at approximately 1131 bp and the empty vector backbone at approximately 5244 bp (Fig. 6).

Following confirmation, the recombinant p406TDH3+*BAT2* plasmid (Fig. 7) was transformed into the *S. cerevisiae* BY4741 strain using a standard lithium acetate transformation method. Positive yeast transformants were confirmed on SD-URA (-uracil) dropout plates, as shown in fig. 8, confirming successful uptake of the recombinant plasmid.

BCAA activity assay: The BCAA enzyme activity assay demonstrated a substantial increase in enzymatic activity upon overexpression of the *BAT2* gene in *Saccharomyces cerevisiae* strain BY4741. As shown in the standard curve (Fig. 9), BCAA concentration correlated linearly with absorbance at 450 nm, validating the assay's accuracy.

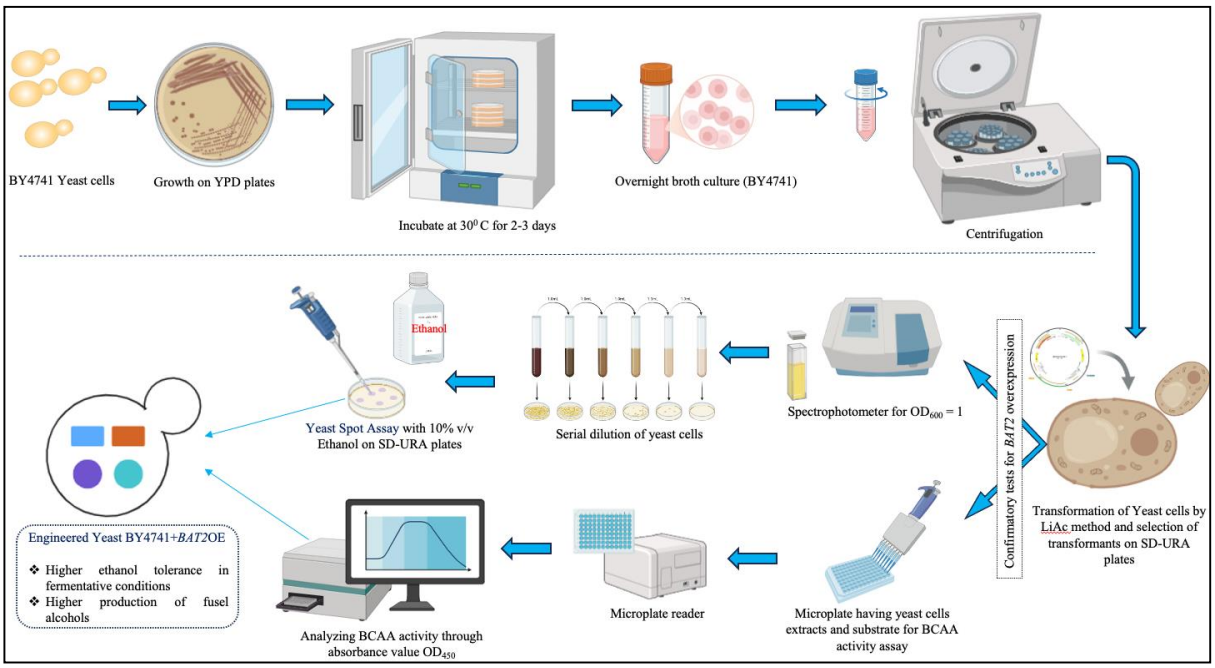

Figure 3: Schematic workflow illustrating the experimental design: starting from BY4741 yeast culture, transformation with p406TDH3+*BAT2* plasmid via LiAc method, selection on SD-URA plates, confirmation of transformants and evaluation through yeast spot assays under ethanol stress and BCAA activity assays using a microplate reader.

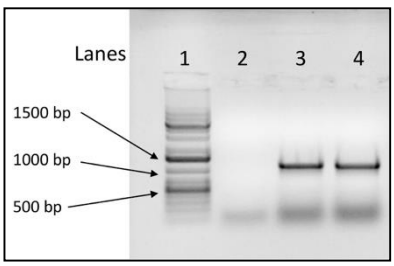

Figure 4: EtBr stained 1% agarose gel image showing amplification of the *BAT2* gene (approximately 1131 bp) using gene-specific primers. (*1 kb+ Thermoscientific ladder)
Lane 1: 1 kb+ ladder, Lane 2: Non template control, Lane 3-4: *BAT2* PCR product

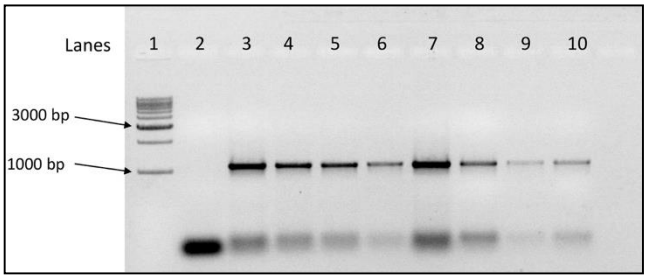

Figure 5: EtBr stained 1% agarose gel image showing colony PCR of positive transformants harbouring p406TDH3+*BAT2*. Lane 1:1 Kb+ Thermoscientific ladder), Lane 2: Non-template control, Lane 3-10: Positive transformants

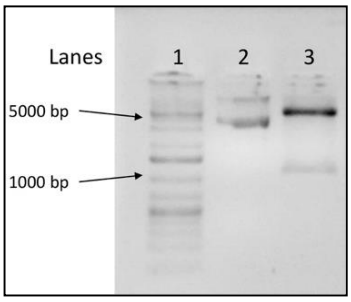

Figure 6: Restriction digestion of p406TDH3+*BAT2* plasmid with *BamH1* and *HindIII*, showing BAT2 gene released in lane 3 at 1131 bp.
Lane 1: 1kb+ ladder, Lane 2: Empty undigested p406TDH3, Lane 3: Double digested plasmid

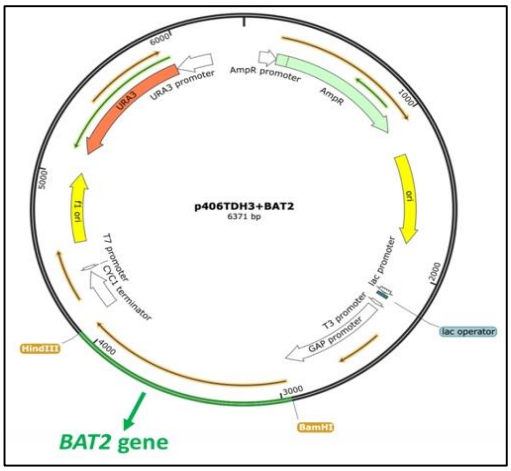

Figure 7: Circular map of the recombinant plasmid p406TDH3+*BAT2* showing the presence of *BAT2* gene between BamHI and HindIII restriction sites.

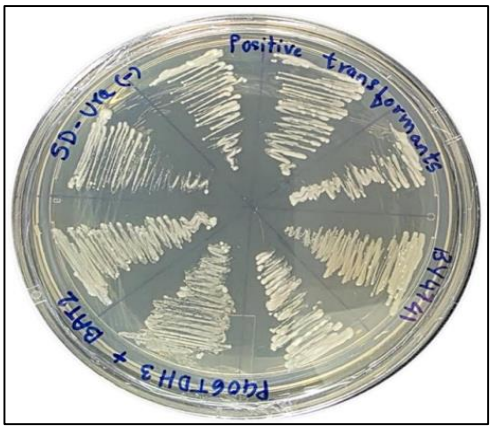

Figure 8: Growth of positive BY4741 transformants on SD-URA plates confirming successful transformation with p406TDH3+*BAT2*.

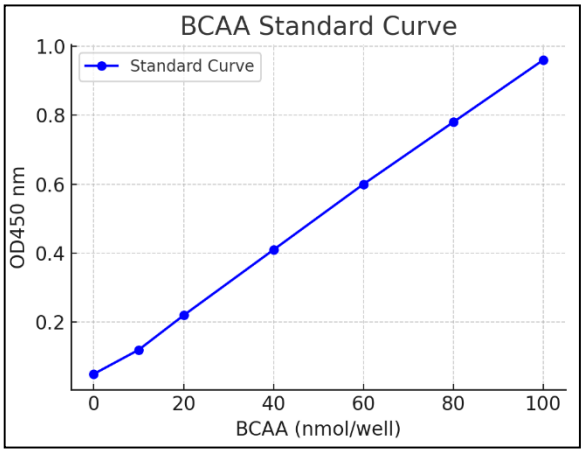

Figure 9: Standard curve for BCAA concentrations generated using the BioVision BCAA Assay Kit; absorbance measured at 450 nm.

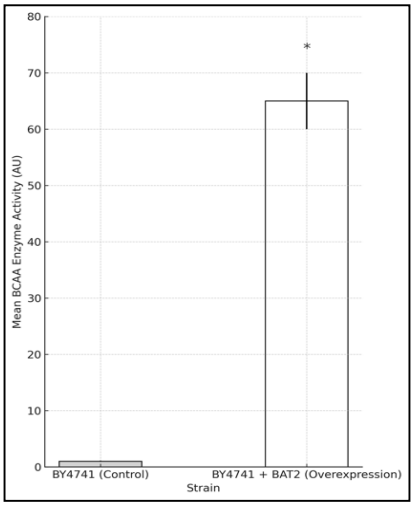

Figure 10: Bar plot showing a Control BY4741 strain and BY4741+*BAT2*OE strain BCAA enzyme activity

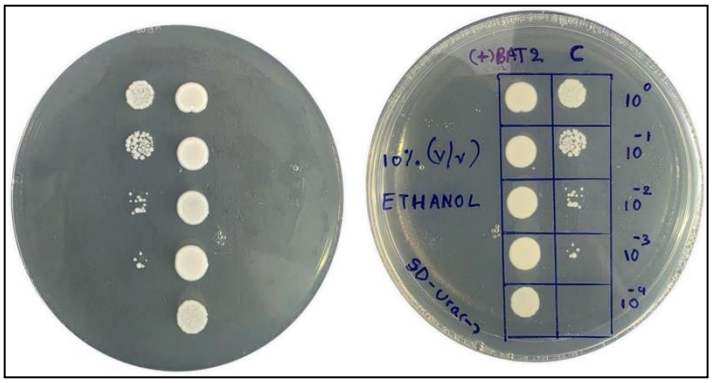

Figure 11: Yeast spot assay under ethanol stress (10%v/v) comparing growth of BY4741 control strain and BY4741+*BAT2*OE strain at different serial dilutions. (Top view and bottom view of SD-URA plates with ethanol)

In the control strain (BY4741 transformed with empty vector p406TDH3), BCAA enzymatic activity remained low, with absorbance values between approximately 0.90 and 1.19 AU. In contrast, the BY4741 strain overexpressing *BAT2* (BY4741+*BAT2*OE) showed a dramatic increase, with activity levels between 58.95 and 70.35 AU, representing an almost 60-fold enhancement relative to the control BY4741 strain (Fig. 10). The consistency across technical and biological replicates confirms the robustness of these findings. These results verify the successful overexpression and functional activity of *BAT2*, highlighting its role in enhancing BCAA transamination in yeast.

Yeast Spot Assay with Ethanol Stress: A yeast spot assay was conducted to assess the ethanol tolerance of *S. cerevisiae* BY4741 strains carrying either the empty p406TDH3 vector (control) or the recombinant p406TDH3+*BAT2* (*BAT2* overexpression). Cells were

grown on SD-URA dropout plates supplemented with 10% (v/v) ethanol. Serial dilutions (10^0 to 10^{-4}) of each culture were spotted on the synthetic uracil-minus plates and maintained at 30°C for 3-4 days. A clear difference in growth was recorded between the two strains. The BY4741 strain overexpressing *BAT2* (BY4741+*BAT2*OE) showed enhanced growth and better tolerance to ethanol stress compared to the control BY4741 strain harbouring the empty vector p406TDH3. These findings indicate that overexpression of *BAT2* improves ethanol stress resistance in yeast (Fig. 11).

In this study, overexpressing the *BAT2* gene in *Saccharomyces cerevisiae* BY4741 resulted in considerable increase in both ethanol tolerance and branched-chain amino acid (BCAA) metabolic activity. The effective amplification and cloning of *BAT2* into the p406TDH3 expression vector were validated by colony PCR, restriction digestion analysis and DNA sequencing. These molecular biology approaches ensured the recombinant plasmid's structural integrity and the precision of the inserted gene sequence, both of which are critical for reliable downstream functional analysis[7].

Positive transformants were chosen on SD-URA dropout plates after the recombinant plasmid was transformed into the BY4741 yeast strain using the lithium acetate (LiAc) method, confirming efficient plasmid uptake and maintenance. In contrast to the control strain carrying the empty vector, the subsequent yeast spot assay, which was carried out under ethanol stress conditions (10% v/v ethanol), demonstrated both quantitatively and visually that *BAT2* overexpression considerably enhances the proliferation and survival of yeast cells. By preserving amino acid balance and lowering the buildup of reactive oxygen species[12,18], *BAT2* may help cellular processes that lessen ethanol-induced damage, as evidenced by the enhanced development of BY4741+*BAT2*OE under ethanol stress.

The functional overexpression of *BAT2* was further confirmed by the BCAA activity assay, which showed a remarkable 60-fold increase in enzymatic activity in comparison to the control strain. Increased BCAA transaminase activity is probably going to promote the breakdown of branched-chain amino acids like leucine, isoleucine and valine, which are building blocks for the Ehrlich pathway that produces higher alcohols like isoamyl alcohol and isobutanol[8]. These higher alcohols are recognised to be crucial for both enhancing yeast stress responses in unfavourable situations, such as high ethanol concentrations and for flavour production during fermentation[1,11].

Previous studies have suggested that modifications in amino acid metabolism can indirectly fortify yeast cells against ethanol stress by balancing intracellular redox status, regulating membrane composition and supporting energy metabolism[23,30]. The enhanced growth recorded in the BY4741+*BAT2*OE strain is on par with this hypothesis and indicates that *BAT2* acts as a key regulator linking amino acid catabolism with stress resistance mechanisms[24].

Moreover, the robustness and reproducibility of the data, as shown by consistent results across biological and technical replicates, underscore the reliability of the experimental system employed. This study aligns with and extends previous findings that engineering metabolic pathways, particularly those involving amino acid metabolism, can be a powerful strategy for improving industrial yeast strains[3,27]. It also provides a foundational framework for future research aimed at coupling *BAT2* overexpression with other stress-tolerance genes, or with modifications that further optimize the Ehrlich pathway for enhanced fusel alcohol production, which could have valuable applications in the brewing, bioethanol and bioproduct industries[17,21].

In summary, the overexpression of *BAT2* successfully enhanced ethanol tolerance and BCAA metabolism in yeast, demonstrating that targeted genetic interventions can substantially improve yeast performance under stress. Further exploration into the regulatory networks associated with *BAT2* and branched-chain amino acid metabolism might yield additional knowledge into stress resilience and metabolic engineering strategies[20].

Conclusion

In this research, we successfully demonstrated that overexpression of the *BAT2* gene in *S. cerevisiae* significantly enhances both ethanol stress tolerance and branched-chain amino acid (BCAA) metabolism. The engineered strain BY4741+*BAT2*OE exhibited markedly improved survival and growth under 10% ethanol conditions compared to the control strain, validating the role of *BAT2* in reinforcing cellular resistance against ethanol-induced stress. Additionally, the substantial increase in BCAA enzymatic activity upon *BAT2* overexpression highlights its critical role in boosting the Ehrlich pathway, which contributes to the production of fusel alcohols beneficial for stress mitigation.

These findings not only expand our understanding of *BAT2*'s functional role beyond flavour compound production but also establish its potential as a metabolic engineering target for developing robust yeast strains for industrial fermentations. Future studies could focus on integrating *BAT2* overexpression with other stress-resistance strategies to further enhance yeast performance in high-ethanol and other challenging industrial environments.

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involving recombinant constructs and assays were conducted in the Anita Peter labouratory within the Department of Plant Biotechnology at GKVK, UAS Bangalore.

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