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Ethanol Production Potential of *Saccharomyces cerevisiae* A10, an Indigenous Yeast Strain from Assam

Research Article

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Abstract

This study investigates the ethanol production potential of the *Saccharomyces cerevisiae* A10 isolated from the traditional fermented alcoholic beverage from apple juice by native people of Assam, India. The strain A10 was obtained through an enrichment culture in TGE (tryptone, glucose, and yeast extract) medium, followed by serial dilution, plating, and repeated streaking to establish a pure culture. Identification of the strain was accomplished using morphological assessments and sequence analysis of the D1/D2 regions of the 26 rRNA gene. Sequence analysis and phylogenetic tree construction confirmed A10 as *Saccharomyces cerevisiae*, which shared a high level of similarity, with 99.42% identity and 100% coverage with *S. cerevisiae* NRRL Y-12632. The ethanol production efficiency of this strain was evaluated in batch fermentation using TGE medium. Optimal fermentation conditions, including temperature, pH, and sugar concentrations, were determined to maximize ethanol yield. Results showed that *S. cerevisiae* A10 achieved an impressive ethanol yield of 34.5 g/L after 24 hours of fermentation at 32°C with 8% glucose. This promising ethanol yield underscores the potential of this indigenous yeast strain for large-scale industrial bioethanol production at a low cost and in a shorter time frame and its significance in promoting traditional fermentation practices in Assam. These results provide a foundation for further research into sustainable strategies for bioethanol production.

Introduction

The rising global demand for renewable energy has intensified interest in bioethanol as a sustainable alternative to fossil fuels. Yeasts particularly, *Saccharomyces cerevisiae* play a key role in bioethanol production due to their potential to efficiently ferment sugars and starchy crops into ethanol. To harness this potential, it is crucial to isolate yeast strains from a variety of unexplored sources and conduct extensive screenings. Various food substrates, including fermented milk [1], sugarcane molasses [2], traditional fermented foods and beverages [3-10], homemade fermented cow milk [11], kefir [12], and fruit wastes [13, 14] have proven to be rich source for isolating yeast strains to evaluate their ethanol production potential and stress tolerance, particularly ethanol toxicity. However, the maximum ethanol yield from yeasts is limited, which requires a large quantity of substrates and a prolonged time for fermentation [15,16]. Therefore,

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to advance sustainable bioethanol production, it is essential to explore new yeast strains from unexplored geographical regions.

This present study focuses on an indigenous yeast strain, *Saccharomyces cerevisiae* A10, which was isolated from traditional apple juice fermentation in Assam, India. This beverage is prepared from fermenting apple juice with Bakhar, a starter cake made from rice dust and various plant root extracts [17], traditionally by local tribal communities. It contains a consortium of microorganisms [18] and is widely consumed by the tribes of Assam. *S. cerevisiae* A10 was confirmed through both morphological examination and molecular techniques, specifically analysing the D1/D2 region of the large ribosomal subunit. The potential of this indigenous yeast strain A10, in terms of its low-cost ethanol production in a shorter fermentation time along with its ability to produce bioethanol from various sugars, is remarkable compared to other yeasts, as reported in this study. Further optimization and physicochemical characterization using glucose-rich waste substrates could lead to the development of a viable strategy for large-scale ethanol production for industrial use.

Materials and methods

Isolation and morphological features of *Saccharomyces cerevisiae* A10

The fermented apple juice sample was serially diluted from 10^{-1} to 10^{-8} in water and a 50 µL sample from 10^{-7} and 10^{-8} dilution was spread onto TGE agar (tryptone, glucose and yeast extract (HI-Media), at pH 6.5 [19] The plates were incubated at 32 °C for 48 h. A few single colonies with distinct morphology were repeatedly streaked on TGE-agar to obtain the pure culture.

Scanning electron microscopy

For Field Emission-Scanning Electron Microscopy (FE-SEM) the strain A10 was prepared without chemical fixatives. The strain grown overnight in a Tryptone-Glucose-Yeast extract medium was inoculated into a fresh 1% medium and incubated for 30 minutes. After centrifugation at 4000 rpm for 7 minutes, the supernatant was discarded, and the pellet was washed with autoclaved distilled water, repeating this process three times. The cell pellet was resuspended in 10µl of water. A 95 µL aliquot of autoclaved distilled water was mixed with 5µL of the cell suspension to create a 100µL cell suspension. For slide preparation, 2-3µL of the suspension was placed on clean coverslips and dried at 32°C for 30 minutes to avoid desiccation. After drying, gold plating was applied for 5 to 7 minutes before imaging with a ZEISS GEMINI SEM 450.

Fermentation conditions and ethanol production

S. cerevisiae A10 was cultured in TGE medium at a temperature of 32 °C. 4 % of an overnight-grown culture was inoculated into fresh TGE medium to enhance growth and ethanol production. Two separate 100 ml Erlenmeyer flasks were used for fermentation, with 20 ml and 100 ml of TGE medium added to allow for varying levels of air exposure. To determine the colony-forming units (CFU), the culture was diluted up to 10^{-8}, and aliquots from each dilution were plated onto TGE-agar plates. Following incubation at 32 °C, the colonies were counted. Ethanol production during fermentation was quantified using the Megazyme enzymatic kit (K-ETOH, Megazyme Inc., Ireland). The flasks were sealed with parafilm, and each experiment was performed in triplicate.

Isolation of Genomic DNA

Genomic DNA of strain A1 was extracted from a pure culture using a previously established protocol [20]. Briefly, cells from an overnight culture grown at 32°C in 5 mL of TGE [19] were centrifuged at 5000 × g for 10 minutes. The cell pellet was then lysed in a mixture of 400 µl lysis buffer (1% SDS and 88 mM sodium acetate), followed by the addition of 400 µl of TE-saturated phenol (pH 8). The samples were incubated at 65°C for 10 minutes before being centrifuged at 5000 × g for 7 minutes at 4°C. The aqueous supernatant was treated with 20 µl of RNase (10 mg/mL) (Genei, India) for 30 minutes at 37°C, and subsequently incubated with 10 µl of Proteinase K (Genei, India) at 50°C for 1 hour. Following this, an equal volume of a phenol/chloroform mixture (1:1, 500 µl) was added. After another centrifugation at 5000 × g for 7 minutes at 4°C, DNA was precipitated using sodium acetate (3M) and isopropanol, and was then resuspended in sterile double-distilled water. The DNA concentration was quantified spectrophotometrically.

PCR amplification of D1/D2 region of 26S rRNA gene of *S. cerevisiae* A10

The D1 and D2 region of the 26S rRNA gene of A10 was amplified using PCR with the conserved fungal primer pair NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) [21]. These primers were commercially synthesized (Bio-Kart India). The PCR was conducted under conditions similar to those described previously [20], utilizing *Pfu* DNA polymerase (Fermentas, Hanover, MD, USA) in an Applied Biosystems 2720 thermal cycler. The thermal cycling program included an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 52°C for 1 minute, and extension at 72°C for 2 minutes, concluding with a final extension at 72°C for 10 minutes. The PCR products were analyzed by electrophoresis on a 1% (w/v) agarose gel, with a 100-bp DNA ladder (New England BioLabs Inc.) serving as a molecular marker. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced commercially (Bio-Kart, India), using both primers. Sequence comparisons for homology assessment were conducted using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). The sequence obtained was submitted to GenBank with the accession SRA: SRR32731888, Bio sample: SAMN47283387 and Bioproject:PRJNA1215549.

Phylogenetic tree

The sequences of the 26S rRNA D1/D2 regions were analyzed using BLAST at the NCBI (National Center for Biotechnology Information) to align them with known 26S rDNA in the GenBank database, generating percent identity scores to identify the yeast strain. Phylogenetic trees were then constructed with MEGA version 11.0 using a neighbor-joining algorithm [22] and the Kimura two-parameter (K2P) distance measure, with *Pachysolen tannophilus* NRRLY-2460 selected as the outgroup species. Bootstrap support for the neighbor-joining tree was evaluated through 1000 replicates, with bootstrap values indicated at the branch nodes. The bar represents 2 base substitutions per 100 nucleotides.

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Results

S. cerevisiae A10 was observed under scanning electron microscope and found to be ovoid with about 3.05 µm in diameter (Figure 1).

The sequence analysis of the D1/D2 region of the 26S rRNA gene for the strain A10 allowed for species-level identification. A BLAST analysis revealed that the strain exhibited a 99.13% identity with *S. cerevisiae* NRRL Y-12632. Consequently, isolate A10 was identified as *S. cerevisiae* and designated *S. cerevisiae* A10. In the phylogenetic tree, this organism clustered with *S. cerevisiae* NRRL Y-12632 (Figure 2) further confirming its identity as established by the BLAST analysis.

To evaluate the ethanol production potential of *S. cerevisiae* A10, seven different sugars-glucose, galactose, sucrose, maltose, starch, fructose, and lactose (all at 2%)-were assessed. Among these, glucose emerged as the most effective sugar for stimulating ethanol production when the yeast was cultured in 20 ml of medium within a 100 ml flask for 24 hours, the ethanol yield was 3.2 g/L, while the ethanol yields from the other sugars varied. However, when A10 was cultured in 100 ml of medium with the same concentration of glucose in a 100 ml flask, the ethanol production was found to be increased to 8.5 g/L, but still lower than the yields obtained from the other sugars (Figure 3A).

To evaluate the impact of varying glucose concentrations on

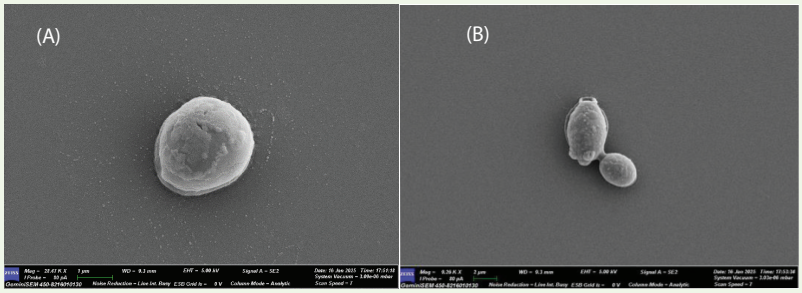

Figure 1: Representation of Field Emission- Scanning Electron Microscopy images captured by ZEISS GEMINI SEM 450, the photograph shows *S. cerevisiae* A10 single cell morphology (A) with a scale bar of 1µm and budding stage of *S. cerevisiae* A10 with a scale bar of 2µm.

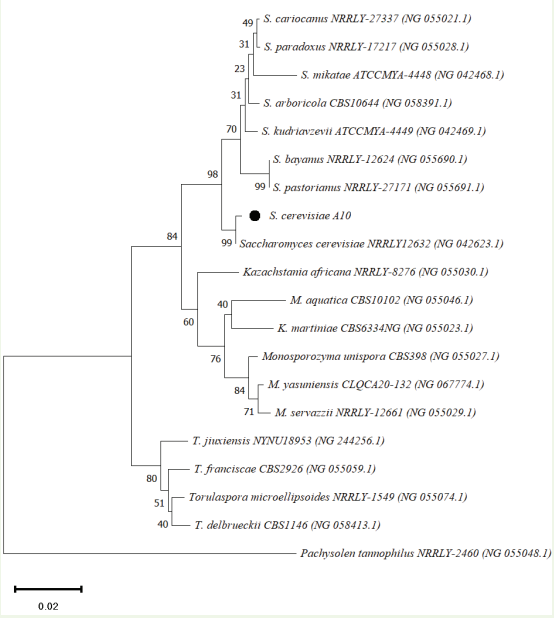

Figure 2: Phylogenetic tree of *S. cerevisiae* A10 (accession SAMN47283387). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). This analysis involved 20 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 542 positions in the final dataset. Evolutionary analyses were conducted in MEGA11

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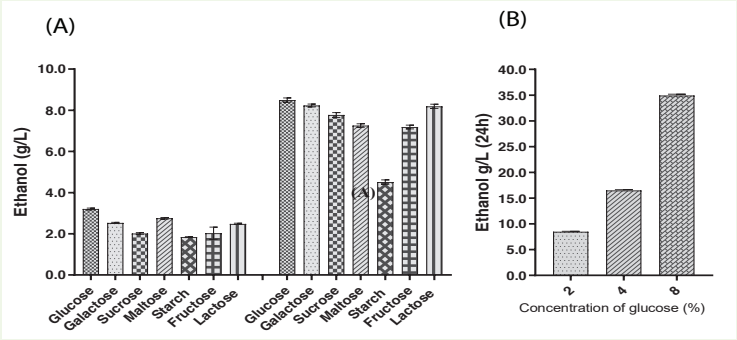

Figure 3: Representation of ethanol produced (g/L). (A) The graph shows the ethanol yielded in 24 hours' both in aerobic (left) and anaerobic like mimic conditions (right) in seven different types of sugar with a fixed concentration of 2% each. (B) The graph shows the ethanol yielded during fermentation by three different concentrations of glucose (2%,4%,8%) in a fixed time point of 24 hours..

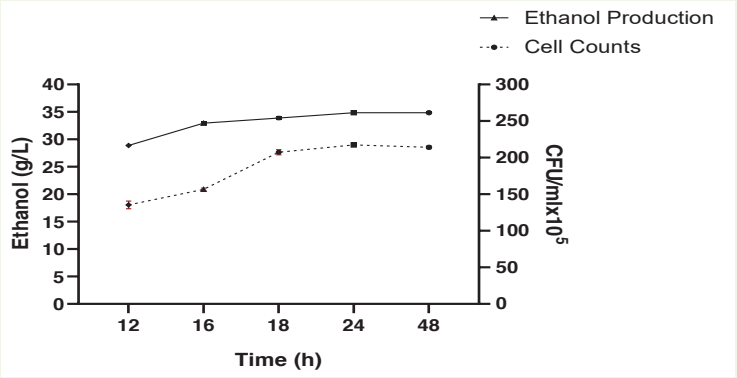

Figure 4: Representation of ethanol production and cell counts (CFU/ml). The graph represents the amount of ethanol (g/L) produced at a concentration of 8% glucose during fermentation at different time intervals (represented in left axis) with the count of cell growth (represented in the right axis) in each time interval.

ethanol production, a range of 2-8% glucose was used in a total volume of 100 ml medium, set for fermentation in 100 ml Erlenmeyer flasks with minimal aeration, the fermentation continued for 48 hours. The highest ethanol production occurred at 24 hours with the 8% glucose concentration (Figure 3B). Analysing ethanol production in relation to growth revealed that *S. cerevisiae* A10 reached its peak ethanol level at 24 hours (Figure 4) coinciding with maximum growth, after which both production and growth remains constant.

Discussion

Traditional fermented beverages have long been valuable sources for isolating valuable yeast strains, significantly contributing to our understanding of the microbial world [23]. This study investigates the unexploited potential of the indigenous yeast strain *S. cerevisiae* A10 in fermenting sugar. The key to proper ethanol yield always lies on its better optimization [24]. In this study under optimized parameters i.e. temperature 32^{0}C in anaerobic like mimic condition and a 4% inoculum size, *S. cerevisiae* A10 showed noteworthy results.

In a 100 ml flask containing 20 ml of medium, A10 produced an ethanol yield of 3.2 g/L. However, in an anaerobic-like environment with nearly no airspace,it achieved an impressive ethanol production of 8.5 g/L in 100 ml of medium. The flask to volume ratio played an efficient role in yielding more ethanol in a lesser amount of time.

It is reported that once excessive aeration was avoided the production was higher [25]. In contrast to other reported yeast strains, which only produce a maximum of 12% to 15% ethanol [26], the ethanol yield was impressive in A10 (34.5 g/L). No doubt A10 yielded higher amount of ethanol in 4% and 8% concentrations of glucose but the major highlight about A10 was its effective amount of ethanol production even from a low concentration of glucose i.e. 2%. Although the lower concentration of glucose took longer span of time to yield a high amount of ethanol, but the important part was its cost- effective value and a higher yield. A10 demonstrates superior efficacy, highlighting the potential of traditional fermentation methods for alcohol production. This research not only illustrates the feasibility of using indigenous yeasts for bioethanol production but also emphasizes their contribution to sustainable fermentation practices in their native region.

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While ethanol production by yeasts typically peaks at 15%, many researchers have engineered yeast strains to enhance this yield. However, the wild-type *S. cerevisiae* A10 does not require genetic modification, although further optimization could be beneficial for improving ethanol production outcomes. For industrial applications, establishing low-cost, large-scale production methods using sugary waste materials under optimized conditions is essential [27].

Conclusion

In summary, the strain A10 exhibits a substantially improved ability to produce bioethanol compared to previously studied strains. This research underscores its promise as a viable renewable energy source and establishes a foundation for future studies aimed at sustainable bioethanol production methods. The results indicate that additional exploration and optimization of this strain could enhance bioethanol production efficiency, advancing the development of more environmentally friendly energy solutions.

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