Biosaintifika 18 (1) (2026): 1-10 p-ISSN 2085-191X | e-ISSN 2338-7610
Journal of Biology & Biology Education https://journal.unnes.ac.id/nju/index.php/biosaintifika

High-Performance Thermotolerant Local Strains for Sustainable Tropical Bioethanol Production via Adaptive Laboratory Evolution

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Submitted: 2025-11-23. Revised: 2026-01-28. Accepted: 2026-03-31.

Abstract. High fermentation temperatures will remain a major limitation to competitive bioethanol production in tropical countries and regions. The objective of the present study was to enrich indigenous microbial strains, improve their performance through Adaptive Laboratory Evolution (ALE), and identify a promising candidate for sustainable bioethanol production. A design an exploratory-comparative laboratory study design was used. Four isolates, *Candida tropicalis* (CT-BKL01), *Kluyveromyces marxianus* (KM-BKL02), *Bacillus coagulans* (BC-BKL03), and *Zymobacter palmae* (ZP-BKL04), were isolated, characterised, and evolved at elevated temperatures (up to 60 generations). Statistical methods included t-tests (paired and unpaired), fold-change values from proteomics, and nonlinear regression using the Monod-Haldane model. ALE results showed ALE significantly increased the specific growth rate, the activity of the enzyme ($p < 0.05$), and promoted overexpression of HSP70, GroEL, and ADH-II, whereas its improvement in ethanol titer was observed at 45 °C, with BC-BKL03 as the best strain exhibiting μ_{max} of 0.63 h^{-1} that had strong model-appropriate fits ($R^2 \geq 0.98$). From a scientific point of view, this study offers an excellent non-recombinant approach for strain improvement without genetic modification. Operationally, this work contributes to SDGs 7 (Affordable and Clean Energy) and SDGs 13 (Climate Action) by enabling efficient production without active cooling. It enables regional energy sovereignty and reduces carbon emissions, offering a scalable solution for the renewable energy industry in tropical countries.

Keyword: Adaptive Laboratory Evolution; Bioethanol; Fermentation kinetics; Lignocellulosic biomass; Thermotolerance

How to Cite: Heryensi, E., Yanti, F. A., Wardana, R. W., Firdaus, M. L., Khamis, N., & Rahim, S. S. B. A. (2026). High-Performance Thermotolerant Local Strains for Sustainable Tropical Bioethanol Production via Adaptive Laboratory Evolution. *Biosaintifika: Journal of Biology & Biology Education, 18(1)*, 1-10.

DOI: http://dx.doi.org/10.15294/biosaintifika.v18i1.38316

INTRODUCTION

Global demand for low-carbon energy is increasing in line with rising CO_2 emissions due to the use of fossil fuels and the impact of climate change (Nong et al., 2025). Bioethanol is considered one of the prospective renewable energy sources owing to its environmentally friendly nature and potential in minimizing dependency on fossil fuels (Al-Hammadi et al., 2025; El-Araby, 2024). Yet, so far, the majority of the world's bioethanol production relies on food-based feedstock, including corn and sugar beet (Tsafara et al., 2022). This presents new challenges, as it may threaten food security and lead to competition for land use. Thus, non-food lignocellulose biomass is getting attention recently as an abundant and renewable feedstock for raw materials (Devi et al., 2022; Zhang et al., 2024).

Lignocellulose biomass resources are also abundant in tropical countries, such as Indonesia (coconut coir *Cocos nucifera*, banana fronds *Musa paradisiaca*, and nipah waste *Nypa fruticans*, which can be used to produce fuels through thermochemical processes. (Helmi & Karsiningsih, 2024). The biomass is composed of cellulose, hemicellulose, and lignin, which can be hydrolysed to reducing sugars as a fermentation substrate. Nonetheless, this potential is only partially exploited, particularly in connection with red biotechnology, which employs indigenous microorganisms adapted to the hot-humid conditions of the rainforest area (Ndapamuri et al., 2021). On the other hand, high potential and many challenges should be faced when converting

lignocellulose biomass to bioethanol application, particularly in the process of fermentation, because the lab-scale efficiency of pretreated enzymatic hydrolysis sugar utilization is influenced by the ability of the used microorganisms (Li et al., 2022; Tsegaye et al., 2024; Yao et al., 2023).

These are most efficient at a temperature of 30-35 °C, and their activity drops significantly above 38 °C (Frohwitter et al., 2024). This is a concern in tropical countries such as Indonesia, where ambient temperatures and industrial fermentation temperatures can range from 35 to 45 °C. As a result, the use of non-tropical microbes requires additional cooling systems that actually increase energy consumption by 25-30% (Gozal et al., 2024; Miah et al., 2022; Pattanakittivorakul et al., 2024; Sengupta et al., 2022).

This method has been helping to increase the thermal tolerance of microorganisms through genetic engineering; however, there are significant limitations in some environments, such as the financial and advanced equipment required to develop the microorganism itself, which may not always ensure a stable strain suitable for industrial-scale processes in tropical countries (Lechtenberg et al., 2024). Instead, ALE is a non-recombinant approach that allows microbes to obtain temperature tolerance through gradual selection under conditions of repeated stress, which is still rarely applied in Indonesia (Huang et al., 2018; Sánchez-Adriá et al., 2025).

Although ALE has been implemented in several studies, most have focused on a single species, have not compared across strains, and have remained limited to ethanol yield evaluations (Hua et al., 2024; Xu et al., 2022). Indeed, co-fermentation of bacteria and yeast or cross-feeding between these microbes can lead to synergistic effects on fermentation, resulting in enhanced efficiency in sugar-to-ethanol conversion. (Echa et al., 2024; Sun et al., 2021). In addition, no studies have integrated enzymatic, proteomic, and fermentation-kinetics approaches to understand the mechanisms of thermal adaptation in tropical microorganisms simultaneously (Zentou et al., 2021). Until now, no studies have explored local microbial strains derived from Bengkulu tropical biomass as candidates for high-temperature fermentation.

Hence, the objective of this study is: (1) to screen and characterize four candidate local microbial strains namely CT-BKL01, KM-BKL02, BC-BKL03, and ZP-BKL04; (2) To enhance strengthening temperature tolerance and overcome more fermentative ability by using ALE method; (3) to examine changes in enzyme activities, protein expression profiling as well as fermentation kinetic parameters kinetics based on Monod-Haldane model; (4) Identify the best performance strain which will be utilized as a forefront candidate for sustainable high temperature bioethanol production for tropics (Sonego et al., 2025).

METHOD

This hypothesis-testing comparative study adopted a quantitative, empirical approach in the isolation, development, and assessment of strains under high-temperature fermentation. This design facilitates comparison among strains before (pre-ALE) and after (post-ALE) the experimental evolution, considering that physiological, biochemical, proteomics, and fermentation performance data are in agreement with the microbial evolution research methodologies currently recommended in the literature (Lee & Kim, 2020; Hirasawa & Maeda, 2023; Wang et al., 2023).

Sample Collection

Tropical lignocellulosic biomass samples were collected from three distinct ecological zones in Bengkulu, Indonesia. Coconut fiber from Pasar Seluma Village, banana stems from Pino Raya District, and nipah pulp from the Bengkulu River Estuary (Figure 1). Each 500 g sample was collected using sterile techniques and stored at 4°C to maintain physicochemical stability before microbial isolation.

Microbial Isolation and Identification

Isolates were obtained through serial dilution and plating on three specific growth media: YPD for yeast, Nutrient Agar for bacteria, and a lignocellulosic synthetic liquid medium to select for fiber-degrading functional strains. Four potential fermentative strains were isolated and designated as CT-BKL01, KM-BKL02, BC-BKL03, and ZP-BKL04. Isolation was performed based on morphological appearance, Gram staining, and catalase activity, followed by molecular identification using either 16S rRNA gene sequence analysis for bacterial strains or the ITS region sequencing for yeast strains.

(A) (B) (C)

Figure 1. Coconut fiber (A), Banana stems (B), and Nipah pulp (C)

Adaptive Laboratory Evolution (ALE)

In order to improve thermotolerance and fermentation efficiency, four isolates were subjected to a systematic ALE. Inocula growth was carried out in YPD-NB medium at 35°C until the mid-exponential phase ($OD_{600} \geq 0.6$). Thermal shock was imposed by raising the temperature by 2 °C every 48 h from 35°C to 50°C during each cycle, and 10% seeding was resumed in a new medium. The adaptive selection lasted 60 generations. Evolved strains that grew consistently at 48-50°C were stored in a 20% glycerol solution at −80 °C.

Enzymatic, Proteomic, and Fermentation Analysis

It was confirmed that enzyme activity for cellulase, pNPG assay for β-glucosidase and the absorbance at 340 nm (in monitoring NADH) for ADH. Proteomic analysis with 2D-PAGE and MALDI-TOF-MS was performed to examine the expression of stress-related proteins, heat shock protein HSP70, GroEL, and ADH-II. Fermentation was carried out at 35°C and 45°C in anaerobic (pH 5.5, 50 g/L glucose) conditions for 48 h. The ethanol concentration was assayed by Gas Chromatography (GC-FID) using an HP-INNOWax column, an injector temperature of 200°C, and a detector temperature of 250°C.

Data Analysis and Kinetic Modeling

Data for (n = 3) biological replicates are shown as mean ± standard error. SD, technical duplicates where appropriate, as determined by laboratory reporting standards for microbial evolution studies (Hirasawa & Maeda, 2023). Statistical significance was set at 5% (α = 0,05) in SPSS 26 and GraphPad Prism 9. Statistical comparisons of microbial performance before and after ALE were performed using paired t-tests, with an independent t-test being used to compare strains at 45 °C. The efficiency of ethanol production is expressed by the Ethanol Yield ($Y_{p/s}$) formula:

$$Y_{p/s} = \frac{P}{S_0 - S_r}$$

Explain: P = final ethanol concentration (g/L); S_0 = initial substrate (g/L); S_r = residual substrate (g/L). Growth kinetics were analyzed using the Monod-Haldane model via non-linear regression in GraphPad Prism 9:

$$\mu = \mu_{max} \frac{S}{K_s + S + \frac{S^2}{K_i}}$$

Explain: μ = specific growth rate (h^{-1}); μ_{max} = maximum specific growth rate; K_s = half-saturation constant, K_i = inhibition constant, S = substrate concentration (g/L). The coefficient of determination ($R^2 \geq 0.98$) was used to assess model fit.

RESULTS AND DISCUSSION

Isolation and Molecular Identification of Potential Local Microbial Strains

Four native isolates were obtained from different tropical bioresources collected in Bengkulu, Indonesia: CT-BKL01, KM-BKL02, BC-BKL03, and ZP-BK04. Sampling sites (coconut fiber, banana stems, and nipah pulp) had been carefully chosen to richly represent local biodiversity. Morphological and molecular testing results showed that the four strains had distinct characteristics, differed both microscopically and genetically (Table 1).

Table 1. Morphological and molecular characteristics of the four local tropical strains of Bengkulu

Isolate code	Colony Shape	Gram	Biomass Sources	Optimum Temperature (°C)	Homology Genetics (%)	Target Gene
CT-BKL01	Beige, convex	-	banana stems	35	99.6	ITS
KM-BKL02	Milky white, smooth	-	coconut fiber	37	99.3	ITS
BC-BKL03	Round, pale yellow	+	nipah pulp	40	99.1	16S Rrna
ZP-BKL04	Clear yellow, soft	-	banana stems	36	98.9	16S Rrna

According to Table 1, the assumed CT-BKL01, KM-BKL02, BC-BKL03, and ZP-BKL04 were successfully isolated from various tropical biomass in Bengkulu. All four strains exhibit the typical morphology of saccharification microbes and can grow at temperatures above 37 °C. Using the 16S rRNA gene (bacteria) and the ITS gene (yeast) for molecular analysis, we confirmed that the authenticity and integrity of our isolated strain are supported by ≥ 98% sequence similarity to NCBI databases. This is in agreement with the report of Oluwole et al (2023), where it was reported that tropical environments harbour a large population of fermentative organisms adapted to hyper-tropical conditions, thereby suggesting a promising resource for biotechnology without recourse to gene manipulation. Furthermore, these findings are consistent with Huang et al. (2018), who found that KM-BKL02 and CT-BKL01 were the two tropical yeasts most tolerant of fermentation temperature oscillations.

Enhancement of Thermal Tolerance and Fermentation Efficiency via ALE

ALE spanning 60 generations (±25 subculture cycles) successfully shifted the thermal thresholds and efficiency of the isolates. Accelerated growth kinetics of ALE-derived strains at 45 °C are depicted in Figure 2.

Quantitative increases in maximum temperature tolerance, specific growth rates (μ), and fermentation ability are indicative of a definite, statistically significant ($p < 0.05$) enhancement throughout all ALE-derived strains (Table 2).

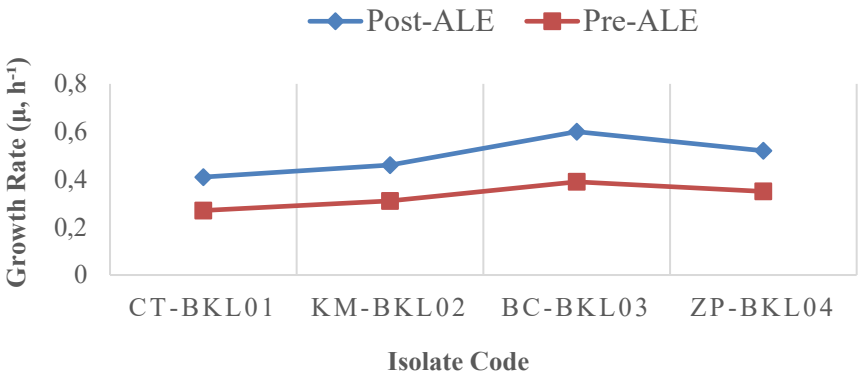

Figure 2. Growth kinetics at 45°C; ALE-derived strains exhibit superior density and shorter lag phase relative to ancestral strains

Table 2. Integrated performance: Growth kinetics and fermentation improvement (35°C to 45°C) after ALE

Isolate code	Temperature (°C)	Early μ (h^{-1})	μ After ALE (h^{-1})	μ Increase (%)	Ethanol (g/L)	Rendemen (Yp/s)	Efficiency (%)
CT-BKL01	35 – 45	0.27	0.41	51.8	17.8 - 23.4	0.44 - 0.56	78.4 - 92.1
KM-BKL02	35 – 45	0.31	0.46	48.4	18.5 - 25.0	0.45 - 0.61	80.2 - 95.6
BC-BKL03	35 – 45	0.39	0.60	53.8	19.2 - 27.8	0.46 - 0.68	81.4 - 98.8
ZP-BKL04	35 – 45	0.35	0.52	48.6	17.2 - 22.0	0.42 - 0.54	76.5 - 89.4

Note: Numerical values demonstrate a statistically significant ($p < 0.05$) increase across all ALE-derived strains.

There is a remarkable "metabolic coupling" in this sense between growth kinetics and bioprocess efficiency. As indicated in Figure 2, the parental strains had significantly extended lag phases and extremely reduced peak biomass at 45°C in comparison with 37°C, which implied that their enzymatic machinery was seriously suppressed by heat. In comparison with this, the ALE strains showed an immediate exponential increase in specific growth rate (μ) as recorded in Table 2. For example, when the specific growth rate (μ) of BC-BKL03 is stepped up from 0.39 to 0.60 h^{-1} (and still being able to grow at 50°C), this already demonstrates systemic physiological and genetic response to thermal stress, which is believed to be due to cumulative mutations in heat-shock regulons. This is in accordance with Dewi et al (2024) and Logan et al (2024), who state that gradual thermal exposure allows microbes to maintain membrane integrity and enzymatic protein stability. In line with the research of Sánchez-Adriá et al (2025), who stated that adaptive evolution can equal or even surpass genetic engineering in optimizing the performance of industrial microbes (Gozal et al., 2024; Tekarslan-Sahin, 2022).

The increase in kinetics observed in the fermentation results shown in Figure 2 is the key to the high ethanol levels in Table 2. Because the cells grow healthier and more stable, they are much more efficient at converting sugar into ethanol, with BC-BKL03 producing the highest ethanol yield (27.8 g/L), a yield (Yp/s) of 0.68 g/g, and an efficiency of 98.8%. The present productivity increase is consistent with the Koukoumaki et al (2024), findings that thermotolerant strains originating in hot climates use an efficient redox system and heat-resistant membranes to stabilize ethanol. Moreover, Miah et al (2022) showed an increase of 15-20% in *C. tropicalis* and *Bacillus sp*., indicating that metabolic load sharing and co-fermentation kinetics could further enhance productivity. In this context, for strategic reasons, ALE presents a most favorable avenue as it generates non-recombinant strains with greater genetic stability and fewer bioethical risks than GE (Gibson et al., 2020; Lee & Kim, 2020). Adaptive evolution, combined with local strain selection employed in the present study, provides tangible evidence of its feasibility for sustainable bio-ethanol production even in tropical areas that lack conditioned environments (Pattanakittivorakul et al., 2024).

Biochemical, Proteomic, and Kinetic Adaptation Analysis

The molecular adaptation underlying thermotolerance was validated by increased tolerance, enhanced enzymatic activity, and proteomic shifts. Table 3 presents the primary enzyme activities at 45°C.

The activities of the main enzymes, namely cellulase, β-glucosidase, and alcohol dehydrogenase (ADH), were significantly increased ($p < 0.05$) in the ALE resulting strains compared to the initial strain (Table 3). The highest activity was detected in BC-BKL03, with 2.52-4.12 U/mg of ADH activity. An acquired increase in enzyme activity corresponds to metabolic adaptation, resulting in a higher sugar-to-ethanol conversion rate at high temperatures. This high ADH activity is necessary because it catalyzes the direct conversion of acetaldehyde to ethanol in anaerobic glycolysis. This observation is consistent with Khamwachirapithak et al (2023), who demonstrated that high ethanol production in thermotolerant yeast was due to elevated ADH expression levels.

Moreover, the activities of cellulase and β-glucosidase, which hydrolyze lignocellulose to glucose, were enhanced. Consistent findings were obtained by Cagnin et al. (2021) and Sengupta et al. (2022), who reported that ALE promotes the secretion of hydrolyzing enzymes by reinforcing the protein secretion system and strengthening cell walls. The fact that activity increased in the absence of a genetic manipulation continues to support the uniqueness of this study, the only known local tropical microbes so far able to evolve naturally via directed evolution.

The proteomic basis of this adaptation is reflected in the expression of specific proteins, as shown in Table 4.

Table 3. Primary enzyme activity (U/mg protein) in the initial strain and ALE yield at 45°C

Isolate code	Cellulase	β-glukosidase	ADH	Information
CT-BKL01	1.21 - 1.95	0.98 - 1.60	2.10 - 3.25	increased significantly ($p < 0.05$)
KM-BKL02	1.35 - 2.28	1.12 - 1.82	2.25 - 3.41	increased significantly ($p < 0.05$)
BC-BKL03	1.42 - 2.76	1.25 - 2.10	2.52 - 4.12	increased very significantly ($p < 0.01$)
ZP-BKL04	1.18 - 1.92	0.89 - 1.45	2.05 - 3.02	increased significantly ($p < 0.05$)

Table 4. Dominant proteins expressed after ALE at 45°C

Types of Protein	Main Role	Average Expression Increase (Fold Change)	Isolate code
HSP70	Protein fold protector from heat denaturation	2.8×	BC-BKL03
GroEL	Chaperonin for enzyme stability	2.5×	KM-BKL02
ADH-II	Catalyst for the conversion of acetaldehyde to ethanol	2.1×	CT-BKL01
GAPDH	Enzyme glycolysis metabolism	1.9×	ZP-BKL04

2D-PAGE and MALDI-TOF-MS analysis. For all 19 ALE-producing strains, the synthesized heat-protective proteins HSP70 (44 kDa), GroEL (57 kDa), and ADH-II were detected by 2D-PAGE and mass spectrometry (Table 4 BC-BKL03 showed a 2.8 increase in HSP70 content, showing that it may have an important function in maintaining enzyme structure to avoid protein denaturation under high temperature. According to Dupuy et al. (2023), HSP70 and GroEL are molecular chaperones that help repair misfolded proteins and protect enzymatic activities from heat stress. The positive association between high ADH activity and HSP70 expression indicates that thermal adjustment was evident at both the metabolic and proteinomic levels (Asefi et al., 2024; Yoshida et al., 2021). These results also support the theory of Sun et al. (2021) that HSPs stabilize complexes involving glycolysis enzymes, leading to higher rates and thereby increasing glucose-to-ethanol conversion at temperatures close to physiological limits for microorganisms.

Among the classical heat-shock marker proteins HSP70, GroEL, and ADH-II (9-11), post-ALE expression of GAPDH suggests a parallel metabolic adjustment rather than a mere stress response. GAPDH overexpression has been described as a typical response for microorganisms to adapt to higher temperature, which contributes to enhanced glycolytic flux and NAD+/NADH ratio in demand for efficient ethanol synthesis (Huang et al., 2018; Xu et al., 2022). Thus, this reply shows that ALE rescues not only thermotolerance but also rewires metabolic networks to maintain ethanol production at 45°C. Systemic validation was further confirmed by the Monod-Haldane kinetic model (Table 5).

To demonstrate the effect of substrate concentration on growth rate, the Monod-Haldane model is applied. The ALE strains were also observed to be substrate-tolerant and consequently good in metabolic performance, which was demonstrated by their μ_{max}, Ks, and Ki values (Table 5), calculated from the regression analysis. The model was particularly appropriate for BC-BKL03, as it had the highest μ_{max} (0.63 h-1) and Ki (125 g²/L²) values, with a coefficient of determination (R^2) of at least 0.98. The capacity of microorganisms to continue growing in the presence of high substrate concentrations is indicated by an increase in Ki values (Lechtenberg et al., 2024). In contrast, a decrease in Ks indicates a higher substrate affinity of the enzyme. These findings are in agreement with the study conducted by Tsegaye et al. (2024), who observed that thermotolerant strains are stable, and confer stability on essential glycolytic enzymes, mitigating substrate inhibition. Tropical bioethanol production has been directly boosted by the ALE method, which has been shown to increase temperature tolerance, growth kinetics, and fermentation efficiency.

Determination of Optimal Strain for Sustainable Tropical Bioethanol

Based on growth kinetics, enzymatic robustness, and proteomic stability data, BC-BKL03 is the best potential candidate for high-temperature bioprocesses. The superior performance of BC-BKL03 is attributed to the ability to maintain a high specific growth rate (μ = 0.60 h^{-1}) and high fermentation efficiency up to 98.8% at 45°C (Figure 3), where this isolate surpasses all other isolates, showing their metabolic adaptation is better in converting substrates into ethanol under extreme thermal stress conditions.

Table 5. Growth Kinetics of Tropical Local Isolates by the Monod-Haldane Model after ALE

Isolate code	μ_{max} (h^{-1})	K_s (g/L)	K_i (g^2/L^2)	R^2
CT-BKL01	0.46	2.2	85	0.97
KM-BKL02	0.50	2.0	110	0.96
BC-BKL03	0.63	1.8	125	0.98
ZP-BKL04	0.47	2.4	90	0.95

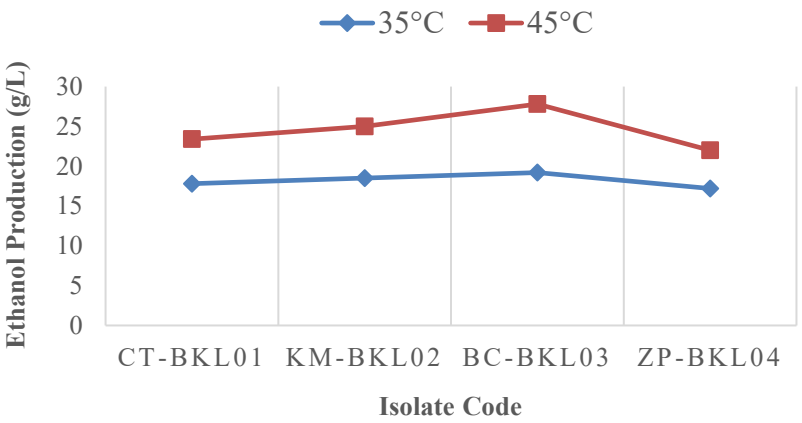

Figure 3. Ethanol Yield Conversion at 35°C and 45°C

The vitality of BC-BKL03 is obviously derived from a stable redox system and the generation of protective proteins, in particular HSPs such as HSP70 and GroEL, which function efficiently. This process is designed to keep the "engine" of cell metabolism running smoothly despite the vagaries of the outside environment. These findings support Koukoumaki et al (2024) A theory proposal on energy flux regulation in thermotolerant bacteria. In addition, it is in line with Miah et al (2022) and Pattanakittivorakul et al. (2024), who state that the optimized use of local bioresources through ALE is the best strategy for biotechnological independence in the tropics.

Thus, the novelty of this study lies in the successful development of non-recombinant tropical local strains that provide three layers of defense simultaneously, physical, molecular, and systemic. Unlike earlier research that relied heavily on costly genetic engineering, which tended to leave microbes "spoiled" and hard to stabilize. It is demonstrated here that ALE can lead to more robust and "natural" industrial biocatalysts. This is a breakthrough in the process of developing industrial microbes planting by utilizing the resources of Indonesia.

From a scientific perspective, the research provides a new piece in our understanding of how microbes adjust their energy to stay alive in high temperatures. In practical terms, the industry gets a true alternative to avoid the higher cost of cooling systems required in tropical bioethanol plants. Using microbes that thrive at 45-50°C greatly lowers the cost of producing renewable energy, so even a poor community can afford it.

Moreover, this study contributes to SDGs 7 (Affordable and Clean Energy) and SDG 13 (Climate Action). Contribution to SDGs 7 is in the form of low-cost, high-efficiency bioethanol production lines on local feedstocks and hastening the on-schedule transition to sustainable energy in the tropics. It also feeds to SDGs 13, Goal 13, in that it saves energy and carbon intensity due to the deployment of industrial cooling installations (Sonego et al., 2025). Therefore, using thermo-resistant microorganisms, this study offers a durable biological alternative to industrial productivity in the face of global warming.

CONCLUSION

This work establishes that 60 generations of ALE are sufficient to effectively convert native tropical isolates into industrially robust biocatalysts. Acceleration in growth kinetics at 45°C, as evidenced by higher specific growth rates (μ) and drastically shorter lag phases, is directly associated with improved fermentation performance and near-theoretical ethanol yield. At the molecular level, this resistance is associated with constant expression of the protective proteins HSP70 and GroEL, as well as continuously active ADH-II at high temperatures. Based on these parameters, BC-BKL03 was selected as the optimal candidate, achieving a maximum fermentation rate of 98.8%. The implications of these findings, including the potential for a cost-effective way to eliminate the cooling requirement in tropical biorefineries, directly contribute to SDGs 7 and SDGs 13. Prospective studies would focus on genomic mapping of precise adaptive mutations and on pilot-scale validation using absolute lignocellulosic waste for accelerating the sustainable commercial implementation.

ACKNOWLEDGEMENT

This work was supported by Hibah Penelitian

Disertasi Doktor from the Ministry of Higher Education, Science and Technology, Republic of Indonesia under contract No. 2870/UN30. 15/PT/2025. The authors are also grateful for the support of the Science Education Study Program, Faculty of Teacher Training and Education, University of Bengkulu.

AUTHOR CONTRIBUTION STATEMENT

All authors contributed significantly to the work reported in this manuscript. EH conceived and designed the study, developed the methodology, provided overall supervision, and drafted the original manuscript. FAY was responsible for data curation, performed the formal analysis, and contributed to the manuscript writing, review, and editing. RWW managed the resources and supported the methodology, formal analysis, and data visualization. MLF, NK, and SSBAR conducted the study's validation and investigation and provided critical feedback during the writing, review, and editing process. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this paper.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that no artificial intelligence (AI) tools were used in the generation, analysis, or writing of this manuscript. All aspects of the research, including data collection, interpretation, and manuscript preparation, were carried out entirely by the authors without the assistance of AI-based technologies.

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