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Homofermentative Lactic Acid- and Cellulase-Secreting *Oryctes rhinoceros* Larval Gut Bacterial Strains for Lactic Acid Production

(Penghasilan Homofermentatif Asid Laktik dan Selulase Strain Bakteria Usus Larva *Oryctes rhinoceros* untuk Pengeluaran Asid Laktik)

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ABSTRACT

Global demand for lactic acid (LA) has increased significantly in recent years, thus, prompting increased interest in utilising lignocellulosic waste biomass for its production. This study investigated the relationship between cellulase activity and LA production by lactic acid bacteria (LAB) isolated from the gut of rhinoceros beetle (*Oryctes rhinoceros*) larvae. Out of 11 LAB strains tested, three isolates namely L5-5, L2a-1 and L3-2 were observed to exhibit good growth using glucose as substrate and were able to obtain final LA concentrations of 9.04 g/L, 9.26 g/L and 9.34 g/L, respectively. These strains were further screened for cellulolytic activity at various temperatures and pH levels using a carboxymethyl cellulose (CMC) assay and were identified as *Enterococcus thailandicus* through 16S rDNA sequencing. Optimal enzyme activity at 45 °C for strain L5-5 was 0.42 U/mL, L2a-1 was 0.61 U/mL, and L3-2 was 0.62 U/mL. Notably, the strong cellulolytic activity was positively correlated with the elevated LA production. These LAB strains could tolerate a broad temperature range of 30 to 50 °C, salt concentrations of 2.5 to 10 % (w/v), furfural concentration of up to 1% (v/v), and pH levels between 4.5 and 9.0. The findings highlighted the potential of LAB isolates from *O. rhinoceros* larval gut as viable candidates for LA production in industrial applications.

Keywords: Cellulase; *Enterococcus thailandicus*; lactic acid bacteria; lactic acid fermentation; *Oryctes rhinoceros* larva

ABSTRAK

Permintaan global untuk asid laktik (LA) telah meningkat dengan ketara dalam beberapa tahun kebelakangan ini, lalu mendorong peningkatan minat terhadap penggunaan biojisim sisa lignoselulosa untuk pengeluarannya. Penyelidikan ini mengkaji hubungan antara aktiviti selulase dengan pengeluaran LA oleh bakteria asid laktik (LAB) yang dipencilkan daripada usus larva kumbang badak (*Oryctes rhinoceros*). Daripada 11 strain LAB yang telah diuji, tiga pencilan iaitu L5-5, L2a-1 dan L3-2 telah menunjukkan tumbesaran yang baik menggunakan glukosa sebagai substrat dan mampu menghasilkan kepekatan akhir LA masing-masing sebanyak 9.04 g/L, 9.26 g/L dan 9.34 g/L. Kesemua strain tersebut selanjutnya disaring untuk aktiviti selulolitik merentasi pelbagai suhu dan tahap pH menggunakan asai karboksimetil selulosa (CMC) dan dikenal pasti sebagai *Enterococcus thailandicus* melalui penjujukan 16S rDNA. Aktiviti enzim optimum pada 45 °C bagi strain L5-5 adalah 0.42 U/mL, L2a-1 adalah 0.61 U/mL dan L3-2 adalah 0.62 U/mL. Dengan jelasnya, aktiviti selulolitik yang kuat dikorelasikan secara positif dengan pengeluaran LA yang tinggi. Kesemua strain LAB ini mampu bertoleransi dengan julat suhu yang luas iaitu 30 hingga 50 °C, kepekatan garam pada 2.5 hingga 10 % (b/v), kepekatan furfural sehingga 1% (v/v) dan tahap pH antara 4.5 kepada 9.0. Penemuan ini menyerlahkan potensi pencilan LAB daripada usus larva *O. rhinoceros* sebagai calon yang berpotensi untuk pengeluaran LA dalam aplikasi industri.

Kata kunci: Bakteria asid laktik; *Enterococcus thailandicus*; fermentasi asid laktik; larva *Oryctes rhinoceros*; selulase

INTRODUCTION

Lactic acid (LA) is an industrially crucial organic acid with extensive applications in the food, pharmaceutical, medical, cosmetics, and chemical industries (Jurášková, Ribeiro & Silva 2022). Over half of global LA production supports the food industry, functioning as an emulsifier,

acidulant, flavouring agent, preservative, and pH regulator (Kaur, Panesar & Ahluwalia 2022). The global demand for LA is growing fast at 18.7% annually, with an annual market of 1.1 billion US dollars (Ojo & de Smidt 2023). LA exists as L-(+) and D-(-) isomers, with the pure forms being more valuable than the racemic mixture. Microbial fermentation is preferred for producing optically pure L- or D-lactic acid due to its use of renewable resources, mild conditions, eco-friendly methods, cost-effectiveness, and energy efficiency (de Oliveira et al. 2018). Lactic acid bacteria (LAB) are preferable for fermentation due to their safety, pH, and temperature tolerance, and high yield (Yang et al. 2022). LAB are either homofermenters that use the Embden-Meyerhof-Parnas pathway to metabolise glucose into LA as the main end product or heterofermenters that use the phosphogluconate or the phosphoketolase pathway, which produce other by-products such as acetic acid, ethanol and CO_2 along with the LA (Zhang et al. 2023). Homofermentative LA production is more desirable industrially as it reduces the cost of downstream purification and also yields a crystalline polymeric product in suitable applications (Rawoof et al. 2021).

Lignocellulosic biomass, an abundant yet underutilised natural resource, has gained significant attention due to its availability, affordability, and renewability (Baruah et al. 2018). Furthermore, it offers a promising alternative to conventional feedstocks in biochemical production since it comprises three major structural components namely cellulose, hemicellulose, and lignin. Lignocellulose can be hydrolyzed to yield fermentable carbohydrates, which can then be processed into valuable end products through biorefinery techniques (Huang et al. 2023; Kim et al. 2022; Sun et al. 2021; Zhang et al. 2022). Integrating lignocellulosic biomass into circular economy models promotes a more sustainable approach in utilising renewable resources, reducing dependency on finite resources while simultaneously mitigating waste through efficient biorefinery processes that produce energy and value-added chemicals, such as lactic acid. Incorporating biological treatments with physico-chemical methods would enhance bio-based product recovery and improve saccharification and fermentation yields (Chandel et al. 2018; Ubando, Felix & Chen 2020). This conversion process involves physicochemical pretreatments to deconstruct the polymeric matrix, followed by enzymatic hydrolysis of carbohydrates into fermentable sugars, which are subsequently converted into lactic acid by suitable microorganisms. The isolation of cellulase-producing lactic acid bacteria (LAB) plays a vital role in integrating lignocellulosic biomass into the circular economy, as this integration would enhance the valorisation process of lignocellulosic materials by converting them into valuable products, such as fermentable sugars and lactic acid, via a synergistic process that involves both cellulases and LAB (Du et al. 2023). Cellulases, including endoglucanases, exoglucanases, and β-glucosidases, play a central role in enzymatic hydrolysis by synergistically breaking down cellulose, with other accessory enzymes contributing to the overall process (Harindintwali, Zhou & Yu 2020).

Enzymatic hydrolysis poses a significant challenge for cost-effective lactic acid (LA) production due to the high enzyme requirement and related expenses. Efficient enzyme formulations and process optimization are necessary for cost mitigations. The simultaneous saccharification and fermentation (SSF) process integrates hydrolysis with fermentation in a single vessel, offering benefits like reduced inhibition, vessel usage, and processing time, with increased yields (Chacón, Ibenegbu & Leak 2021). However, incompatibilities between optimal conditions for hydrolysis and fermentation could often limit yields. Alternatively, employing microorganisms with inherent hydrolytic and LA-producing capabilities can eliminate or reduce the need for enzymes. While promising, this approach may yield by-products like acetic acid, necessitating purification, which could be addressed by utilising cellulolytic strains producing LA exclusively (Pleissner et al. 2017).

Numerous lactic acid bacteria (LAB) strains have been sourced from various environments, yet the demand for efficient strains necessitates the exploration of unique sources. *Oryctes rhinoceros*, commonly known as the coconut rhinoceros beetle, is a pest prevalent in palm crop regions of Asia and the Pacific Islands and larvae of this beetle feed on the soft core of palm biomass, such as palm shoots. The larval gut harbours symbiotic microbes to aid in digestion of the biomass, deriving energy from cellulose, hemicellulose, and lignin components of biomass, rendering it an ideal reservoir for cellulolytic LAB (Shelomi & Chen 2020). Despite numerous microorganisms being isolated from the larval gut, cellulolytic LAB strains from this organism remain unreported (Marheni, Martono & Sijabat 2021). In this study, it was hypothesized that *O. rhinocerus* larval gut possess potential cellulolytic lactic acid bacteria. This study aimed to isolate and characterize cellulolytic LAB strains from *O. rhinoceros* larvae gut, exploring their potential for direct biomass-to-lactic acid conversion.

MATERIALS AND METHODS

COLLECTION AND PREPARATION OF LARVAL GUT

The *O. rhinoceros* larvae were collected from a compost pile at Biotechnology Research Centre Glami Lemi (PPGBL) Jelebu, Negeri Sembilan, Malaysia (2° 56′ O″ N, 102° 5′ O″ E). These were stored in plastic holders containing the compost and transferred to the laboratory at Universiti Malaya. The larvae were washed several times with tap water and then frozen for 20 min. The larvae were dipped in 70% ethanol, which was allowed to evaporate from the surface before further processing of the samples. The insect larvae were fixed on a foam table and dissected with sterile needles and sterile fine-tip forceps. The gut of the larvae was assayed for lactic acid bacteria (Calumby et al. 2022).

ISOLATION OF MICROBIAL CULTURES

The extracted guts of the larvae were inoculated into 10 mL of sterile normal saline and allowed to stand for 30 min after which they were inoculated into 100 mL of MRS broth (pH 7.0) in 250 mL Erlenmeyer flasks. The flasks were incubated at 37 °C with shaking at 150 rpm for 3 days. Next, 0.1 mL of the enriched culture was plated on MRS agar using the spread plate method. The stock culture was serially diluted, plated on MRS agar plates, and incubated at 37 °C under aerobic conditions for 48 h. The morphological characteristics such as colony size, shape, colour, elevation pattern, edges, and transparency were observed visually (Elzeini et al. 2021; Wang et al. 2022). Small colonies which were opaque with smooth surfaces were chosen and were sub-cultured to obtain pure cultures of the isolates. Pure cultures were kept in 30% glycerol at -20 °C until further use.

CHARACTERISATION OF LACTIC ACID ISOLATES

The isolates were characterised based on different parameters such as appearance on agar plate, gram reaction, catalase test, shape, gel plug test (homo or heterofermentation), and tolerance to environmental conditions (salt, temperature, pH, furfural). A 24-h broth culture of each isolate was centrifuged at 1500 × g for 10 min. The supernatant was decanted, and the cells were washed twice using 0.85% sterile saline. The washed cells were then resuspended in 5 mL of saline. A 50-µL aliquot of the cell suspension was inoculated into bijou bottles containing 5 mL of the basal medium (de Man, Rogosa & Sharpe 1960) with modification. The basal medium contained glucose (20 g/L), K_2HPO_4 (2.0 g/L), $MgSO_4.7H_2O$ (0.20 g/L), $MnSO_4.4H_2O$ (0.03 g/L), peptone (10. g/L), sodium acetate (5.0 g/L), yeast extract (4.0 g/L), and Tween 80 (1.0 mL) with Bromocresol purple (0.17 g/L) added as a colour indicator. The inoculated medium was incubated in a shaker water bath for 7 days at varied conditions depending on the tested parameter. For temperature tolerance, the bottles were incubated at 15 °C, 30 °C, 45 °C, and 50 °C at pH 7.0. Incubation was also done at different NaCl (1.5%, 2.5%, 5%, 7.5%, and 10% w/v) and furfural (1%, 2.5%, and 5% v/v) concentrations at 37 °C and initial medium pH of 7.0. Colour changes were noted. The basal medium was used and adjusted to different pH values (4.5, 7 and 9) using 1 M H_2SO_4 or 1 M NaOH for the pH tolerance test. It was incubated at 37 °C for 48 h and growth was monitored by turbidity changes measured at 600 nm compared with control. Each treatment was tested in triplicate.

GEL PLUG TEST

This procedure was used to test for homofermentative and heterofermentative characteristics of the isolates using a nutrient gelatin medium (Gibson & Abdel-Malek 1945), which contained gelatin (200 g/L), peptone (5.0 g/L), yeast extract (3.0 g/L), and glucose (5.0 g/L). Glucose was sterilised separately and added aseptically to the nutrient gelatin after autoclaving at 121 °C for 15 min. Bromocresol purple (0.17 g/L) was used as an indicator. For the inoculum, 24 h old culture broth of the isolates was centrifuged at 3000 × g for 5 min. The cell pellets were washed twice and resuspended in 5 mL of 0.85% (w/v) saline. A volume of 10 mL of nutrient gelatin with 0.5% (w/v) of glucose was added into a separate test tube, and 1 mL of the suspended cells was inoculated into each tube. After inoculation, sterile molten agar was poured into the test tubes to create airtight tubes for the gel plug culture. The test tubes were incubated for 7 days in the water bath at 30 °C. Production of gas and colour changes in the test tubes were noted and recorded. The change of the medium colour from purple to yellow indicated glucose utilisation. In contrast, splitting the gel plug with the culture medium indicated CO_2 production, which implied that heterofermentation had occurred (Adnan & Tan 2007).

IDENTIFICATION OF THE ISOLATES

Selected isolates were identified by 16S rDNA gene sequencing method using a commercial service (First Base, Singapore). The processing of the isolates was done as follows. The amplification of the bacterial full length 16S rDNA (1.5 kb) was carried out using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5'-TACGGTTACCTTGTTACGACTT-3). The total reaction volume of 25 µL contained gDNA purified using a standard extraction method, 0.3 pmol of each primer, 400 µM of each deoxynucleotide triphosphate (dNTP), 0.5 U DNA polymerase, supplied PCR buffer and water. PCR was carried out as follows: 1 cycle (98 °C for 2 min) for initial denaturation, 25 cycles (Annealing at 98 °C for 10 s, 53 °C for 30 s, 68 °C extension for 1 min) for annealing and extension of the amplified DNA. The PCR products were purified using the standard method and were directly sequenced with primers 785F and 907R using a BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems). Search for sequence similarities was conducted using NCBI BLAST, and the nearest relatives of the 16S rDNA were identified using the GenBank database. The phylogenetic tree of selected LAB strains was constructed using MEGA X software. The maximum likelihood method and bootstrap program with 1000 replicates were employed to determine the relatedness and reliability in the identification of the organisms. Percentage similarities were employed in the identification of the isolates.

SCREENING OF ISOLATES FOR CELLULOLYTIC ABILITY

For inoculum preparation, the isolates were grown to their late log phase, and 50 mL of each broth culture was centrifuged at 3000 × g for 10 min at 4 ºC. The cell pellet was resuspended in 0.1% sterile peptone. The cell suspension was adjusted to OD = 1.0 at 600 nm and it was used as inoculum in the screening experiment.

TABLE 1. Biochemical characterisation of homofermentative LAB isolates from *O. rhinocerus*

Environmental Conditions	Isolates										
	L1-1B	L1-2	L1-2s	L1-3	L2a-1	L2a-3s	L3-2	L3-3	L5-1	L5-3	L5-5
Temperature °C											
30	++ (4.28 ± 0.02)	++ (4.21 ± 0.02)	++ (4.37 ± 0.07)	++ (4.96 ± 0.01)	++ (3.88 ± 0.05)	++ (4.30 ± 0.08)	++ (3.98 ± 0.05)	++ (4.25 ± 0.03)	++ (4.27 ± 0.04)	++ (4.29 ± 0.02)	++ (3.85 ± 0.08)
37	++ (4.33 ± 0.01)	++ (4.33 ± 0.01)	++ (4.34 ± 0.07)	++ (5.03 ± 0.02)	++ (4.10 ± 0.27)	++ (4.51 ± 0.08)	++ (3.98 ± 0.27)	++ (4.37 ± 0.01)	++ (4.35 ± 0.01)	++ (4.36 ± 0.02)	++ (4.16 ± 0.09)
45	++ (5.25 ± 0.04)	++ (5.23 ± 0.04)	+ (5.49 ± 0.06)	+ (5.47 ± 0.01)	++ (4.86 ± 0.02)	+ (5.97 ± 0.01)	++ (5.06 ± 0.02)	+ (5.92 ± 0.05)	+ (5.39 ± 0.03)	+ (5.44 ± 0.02)	++ (5.13 ± 0.30)
50	+ (5.65 ± 0.02)	+ (5.55 ± 0.02)	- (6.98 ± 0.02)	- (6.99 ± 0.02)	+ (5.46 ± 0.05)	- (6.99 ± 0.02)	+ (5.65 ± 0.02)	+ (5.78 ± 0.04)	- (6.98 ± 0.01)	+ (5.54 ± 0.01)	+ (5.76 ± 0.01)
NaCl Concentration (% w/v)											
1.5	++ (4.26 ± 0.01)	++ (4.27 ± 0.07)	++ (4.26 ± 0.03)	++ (4.16 ± 0.06)	++ (4.12 ± 0.02)	++ (4.16 ± 0.03)	++ (4.14 ± 0.04)	++ (4.23 ± 0.03)	++ (4.22 ± 0.04)	++ (4.22 ± 0.03)	++ (4.18 ± 0.02)
2.5	++ (4.34 ± 0.04)	++ (4.36 ± 0.02)	++ (4.33 ± 0.04)	++ (4.37 ± 0.07)	++ (4.2 1± 0.01)	++ (4.32 ± 0.07)	++ (4.12 ± 0.03)	++ (4.30 ± 00.7)	++ (4.38 ± 0.03)	++ (4.35 ± 0.05)	++ (4.19 ± 0.01)
5.0	++ (4.43 ± 0.07)	++ (4.47 ± 0.04)	++ (4.38 ± 0.02)	++ (4.42 ± 0.01)	++ (4.34 ± 0.04)	++ (4.37 ± 0.04)	++ (4.17 ± 0.02)	++ (4.44 ± 0.05)	++ (4.46 ± 0.05)	++ (4.42 ± 0.02)	++ (4.23 ± 0.03)
7.5	++ (4.69 ± 0.05)	++ (4.66 ± 0.01)	++ (4.96 ± 0.03)	++ (4.71 ± 0.02)	++ (4.57 ± 0.03)	++ (4.79 ± 0.06)	++ (4.52 ± 0.04)	++ (4.87 ± 0.06)	++ (4.76 ± 0.02)	++ (4.64 ± 0.08)	++ (4.67 ± 0.05)

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10.0	++ (4.98 ± 0.03)	++ (5.15 ± 0.05)	++ (5.12 ± 0.04)	++ (4.94 ± 0.06)	++ (4.95 ± 0.07)	++ (5.11 ± 0.05)	++ (4.87 ± 0.04)	++ (5.18 ± 0.01)	++ (5.19 ± 0.01)	+ (5.34 ± 0.02)	++ (4.92 ± 0.03)
Furfural Concentration (%v/v)											
1.5	+ (5.67 ± 0.03)	+ (5.38 ± 0.02)	++ (5.20 ± 0.02)	+ (5.24 ± 0.06)	++ (4.61 ± 0.03)	+ (5.44 ± 0.04)	+ (5.33 ± 0.01)	+ (5.36 ± 0.02)	+ (5.29 ± 0.06)	+ (5.31±0.02)	++ (5.2 0± 0.03)
2.5	+ (5.93 ± 0.02)	+ (5.73 ± 0.04)	+ (5.70 ± 0.05)	+ (5.68 ± 0.03)	++ (4.85 ± 0.04)	+ (5.74 ± 0.04)	+ (5.66 ± 0.04)	+ (5.64 ± 0.04)	+ (5.80 ± 0.04)	+ (5.87±0.03)	+ (5.71 ± 0.02)
5.0	+ (6.09 ± 0.03)	+ (6.06 ± 0.02)	+ (5.99 ± 0.01)	+ (6.04 ± 0.01)	+ (5.99 ± 0.01)	+ (6.07 ± 0.01)	+ (6.00 ± 0.01)	+ (6.07 ± 0.01)	+ (6.03 ± 0.01)	+ (6.07±0.01)	+ (6.01 ± 0.01)
pH											
4.5	+ (4.23 ± 0.01)	+ (4.13 ± 0.01)	+ (4.34 ± 0.03)	+ (4.22 ± 0.01)	+ (4.13 ± 0.03)	- (4.53 ± 0.02)	+ (4.19 ± 0.02)	+ (4.16 ± 00.2)	+ (4.23 ± 0.03)	+ (4.36 ± 0.04)	+ (4.16 ± 0.01)
7.0	++ (4.24 ± 0.01)	++ (4.24 ± 0.01)	++ (4.28 ± 0.02)	++ (4.31 ± 0.01)	++ (4.19 ± 0.01)	++ (4.35 ± 0.05)	++ (4.32 ± 0.01)	++ (4.38 ± 0.01)	++ (4.34 ± 0.02)	++ (4.29 ± 0.02)	++ (4.32 ± 0.01)
9.0	++ (4.34 ± 0.01)	++ (4.39 ± 0.01)	++ (4.43 ± 0.02)	++ (4.35 ± 0.01)	++ (4.14 ± 0.02)	++ (4.43 ± 0.03)	++ (4.27 ± 0.02)	++ (4.42 ± 0.01)	++ (4.37 ± 0.02)	++ (4.35 ± 0.01)	++ (4.24 ± 0.03)

For Temperature, NaCl, and Furfural Concentration: (++) indicates a complete colour change from purple to yellow which means high growth; (+) indicates a partial colour change from purple to green, which means low growth; (-) indicates no colour change which means no growth. For pH, (++) indicates an increase in turbidity of more than 50%, (+) indicates an increase of more than 10 %, and (-) indicates no increase or less than 10%

PLATE SCREENING USING CARBOXYMETHYL CELLULOSE AGAR (CMCA)

The standardised inoculum (5 µL) of each isolate was spot-plated on to a fresh CMCA plate in triplicate (Kasana et al. 2008). The plates were incubated for 48 h at 30 ºC, flooded with 0.1% Congo red dye for 30 min, then flooded with 1 M NaCl for another 30 min (Teather & Wood 1982). The ratio of the halo diameter to the colony diameter was recorded by hydrolytic capacity produced by the colony (Hankin & Anagnostakis 1977).

CELLULASE PRODUCTION IN LIQUID MEDIUM

Cellulase production was aerobically carried out in a CMC medium with the following composition in g/L: CMC (10.0), K_2HPO_4 (1.0), KH_2PO_4 (1.0), $MgSO_4$.$7H_2O$ (0.2), NH_4NO_3 (1.0), $FeCl_3$.$6H_2O$ (0.05), $CaCl_2$ (0.02) and yeast extract (5.0). Inoculum was prepared by transferring 2 to 3 colonies of each strain from Tryptic Soy Agar (TSA) plates into 50 mL of Tryptic Soy Broth (TSB) in 250 mL conical flasks. Tryptic soya media was used in this procedure as it can be used to cultivate fastidious and non-fastidious bacteria. The mixtures were incubated for 24 h at 37 °C with shaking at 150 rpm. Five mL of the culture was inoculated into 45 mL of enzyme production medium, which was then incubated at different temperatures (27, 37, 45, 50, and 60 °C) for 96 h with shaking at 150 rpm. The effect of pH on cellulase production was determined by adjusting the initial pH of the production medium to different values (4.0, 4.5, 6.0, 7.0, and 9.0) and conducting fermentation under those conditions. Then, 5 mL of the samples were collected aseptically at 24-h intervals for 96 h. Each sample was centrifuged at 3000 × g for 10 min at 4 °C. The supernatant was used as the crude enzyme for cellulase assay. This experiment was done in triplicate.

CELLULASE ASSAY

The cellulase activity was determined by measuring the reducing sugar liberated after the reaction of 200 µL of the enzyme preparation with 500 µL of 1% CMC and 300 µL of 0.05 M phosphate buffer (pH 7.0) (Zhang, Hong & Ye 2009). The mixture was incubated for 30 min at 50 °C, and the reaction was stopped by adding of 3 mL a DNS reagent; then, the tubes were immersed in boiling water for exactly 5 min. Released sugars were measured as glucose equivalent using DNS reagent (Miller 1959). One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar per mL per minute for the substrate used. A glucose standard curve was utilised to determine the equivalent reducing sugars released.

LACTIC ACID PRODUCTION BY SELECTED ISOLATES

Shake flask studies of selected isolates were carried out using 300 mL basal MRS broth in a 500 mL Erlenmeyer flask to test for LA production. The carbon source used was 10% glucose. The basal MRS broth and glucose solution were autoclaved separately at 121 °C for 15 min. After cooling, the broth and the glucose solution were mixed.

PREPARATION OF INOCULUM AND FERMENTATION

Each isolate was inoculated into 50 mL MRS broth, and the mixture was incubated on a shaker incubator (Protech model-SI-50D AX9) at 37 °C, 100 rpm for 18 h. The broth was centrifuged at 2000 × g for 10 min at 4 °C, and the cell pellets were washed twice and resuspended in 5 mL of 0.85% normal saline. Ten mL of the resuspended cells were inoculated into conical flasks, each containing 300 mL of MRS medium (pH 7), and the flasks were incubated at 37 °C under 100 rpm agitation for 24 h. Samples were collected at intervals throughout the fermentation. The experiment was done in triplicate.

ANALYTICAL METHODS

QUANTIFICATION OF LACTIC ACID IN THE BROTH

Aliquots (1 mL) of the samples were centrifuged at 2000 × g at 4 °C for 10 min. The supernatant was diluted tenfold with ultra-pure water. The diluted samples were filtered into autosampler vials using a 0.2 µm size membrane filter (Thermo Scientific® Nylon) to prevent contamination, after which the vials were arranged into the sample column. The quantification of lactic acid in the culture was determined using high-performance liquid chromatography (HPLC) (Agilent 1260) with a reflective index detector and a Biorad Aminex HPX-87H. The mobile phase 0.005 M sulfuric acid was used after it was filtered using 0.2 µm and degassed. A sample volume of 20 µL was taken at 0.6 mL/min flow rate and run time of 35 min. The column temperature was 50 °C. A standard curve was used to obtain several concentrations of L-lactic acid (1.0 g/L, 2.5 g/L, 5.0 g/L, and 10 g/L). The concentration of lactic acid in the samples was measured by comparing peak areas against the standard curve. The experiment was done in triplicate. The amount of glucose consumed within 24 h was also quantified.

RESULTS AND DISCUSSION

ISOLATION, SCREENING, AND IDENTIFICATION OF ISOLATES

The physico-chemical tolerance tests were conducted to select potential isolates with industrial applications. The screening for isolates that could tolerate high temperatures was done because it would be able to inhibit the growth of potentially undesirable microbes thus reducing the risk of contamination (Pitiwittayakul, Bureenok & Schonewille 2021). Furthermore, isolates functioning at higher temperatures could enhance the overall fermentation rate, especially for simultaneous saccharification and fermentation (Chacón, Ibenegbu & Leak 2021). During the acid fermentation process, to maintain pH, the acid produced by the microbial culture

would be neutralised by alkali-producing salts, thus, changing the osmotic conditions towards hypertonic conditions in the fermentation media, affecting the process. A non-salt-tolerant microorganism could reduce the overall fermentation rate. Utilising salt-tolerant isolates would prevent this event from happening as they could tolerate the change in the osmotic conditions (Yang, He & Wu 2021). The presence of furfural in cultivation media would inhibit microbial activities. Utilising furfural-tolerant isolates in fermenting lignocellulosic hydrolysates would be beneficial as they could tolerate furfural presence. It was found that some furfural-tolerant LAB strains have demonstrated improved performance in fermenting lignocellulosic hydrolysates containing furfural (Chen et al. 2023). pH-tolerant isolates are valuable in fermentation as they can thrive at a lower starting pH during cultivation and thus out-compete spoilage microorganisms (da Cunha et al. 2024).

A total of 56 isolates were recovered from *O. rhinoceros* larval gut. Lactic acid bacteria from this group were identified through gram staining and catalase tests, as most of the LAB have been identified as Gram-positive and catalase-negative (Sharpe 1976). Eleven of twelve of the isolates were identified as homofermentative via the gel plug test. Identifying the isolates as homofermentative is crucial if they are considered potential industrial strains, as they produced solely lactic acid via fermentation (Stephen & Saleh 2023). The eleven isolates were assessed for potential industrial application via physico-chemical properties such as pH tolerance, temperature tolerance, salt tolerance, and furfural tolerance. The results obtained are summarized in Table 1. All the isolates were able to tolerate temperatures of 30 °C and 37 °C. At 45 °C, very few of the isolates were able to grow and tolerate at that temperature and subsequent temperature of 50 °C, all the isolates displayed partial colour change except for L1-2s, L2a-3S, L1-3, and L5-1 which did not display any positive colour change. Using thermotolerant strains in lactic acid fermentation would reduce the energy needed for the cooling process of production.

Additionally, employing these strains can decrease the risk of microbial contamination during fermentation (Chatgasem et al. 2023). Abdel-Rahman et al. (2015) reported that LAB are Gram-positive, non-sporing, catalase-negative and mesophilic microorganisms (10-50 °C) which are in accordance with the present study. For growth in different NaCl concentrations, the isolates could grow at varying concentrations of salt, ranging from 1.5% to 10%; only isolate L5-3 showed a weak reaction at 10% salt concentration. Salt tolerance tests have been used to classify many bacteria based on their propensity to grow in varying amounts of sodium chloride (NaCl). Some species of the genus *Enterococcus* have been reported to tolerate high salt concentrations, including *E. faecalis, E. zymogenes, E. liquifaciens,* and *E. durans* (Dong et al. 2017). Using salt-tolerant strains would increase the concentration of lactic acid produced as the strains would be able to withstand the increase in the osmotic pressure in the media as fermentation progresses (Yang, He & Wu 2021). For furfural tolerance, Table 1 also shows that only L1-2s, L2a-1, and L5-5 could fully tolerate 1% furfural (v/v) compared to other isolates that could only partially tolerated that concentration. At higher furfural concentrations (2.5% and 5%), a partial change in the growth medium from purple to green was observed for all isolates except for L2a-1, which displayed complete tolerance at 2.5%, indicating that most isolates showed the ability to partially tolerate these furfural concentrations. During the pretreatment of lignocellulosic biomass, furfural compound is one of the inhibitory by-products that are produced, and its presence inhibits the fermentative microorganisms that utilise the lignocellulosic hydrolysates (Grewal et al. 2020). The ability to tolerate furfural would be an advantage for microbes in LA production utilising carbon sources from lignocellulosic hydrolysates. The ability to tolerate inhibitory substances is desirable in industrial strains (Peng et al. 2013). Thus, the isolates from this study are promising since they were able to tolerate the presence of furfural. For growth at different pH, the isolates could partially tolerate pH 4.5 except L2a-3s while for pH 7.0 and 9.0, all isolates displayed high growth, showing an increase in turbidity of more than 50%. The tolerance of these isolates to different pH levels also showed their versatility in performing fermentation at various acidity levels. This ability is a favourable trait in the isolates, which is valuable and important in the fermentation process (Suwannaphan 2021).

IDENTIFICATION OF ISOLATES

The three (3) selected isolates from the larval gut of *O. rhinoceros* were chosen based on their exceptional physico-chemical properties and were identified using the 16S rDNA, namely *E. thailandicus* L2a-1, L5-5 and L3-2. As shown in Figures 1-3, the phylogenetic trees illustrated similarities of the identified isolates to various microorganisms. All three identified isolates had 100% similarity with *E. thailandicus. E. thailandicus* strains L2a-1 had similarity percentages of 84%, 64%, and 79%, L5-5 had 88%, 65%, 80% while L3-2 had 86%, 63%, and 79% similarity with *E. hirae*, *E. faecium*, and *E. durans*, respectively, while Figure 4 showed the gel electrophoresis of the three selected isolates. Figure 4(A) displayed the PCR amplification of the 16S rDNA gene in the agarose gel, while (B) showed the DNA ladder or marker with an amplification band of 2000 bp for the isolates. Shao et al. (2014) carried out a metagenomic study of the intestine of the cotton leaf worm where it was discovered that the genus *Enterococcus* was predominant, and their metabolic activeness throughout the whole of the larval life cycle showed the importance of this genus as a gut colonizer of larva from the members of *Lepidoptera*. Previous studies have found *Enterococci* to be beneficial organisms of the

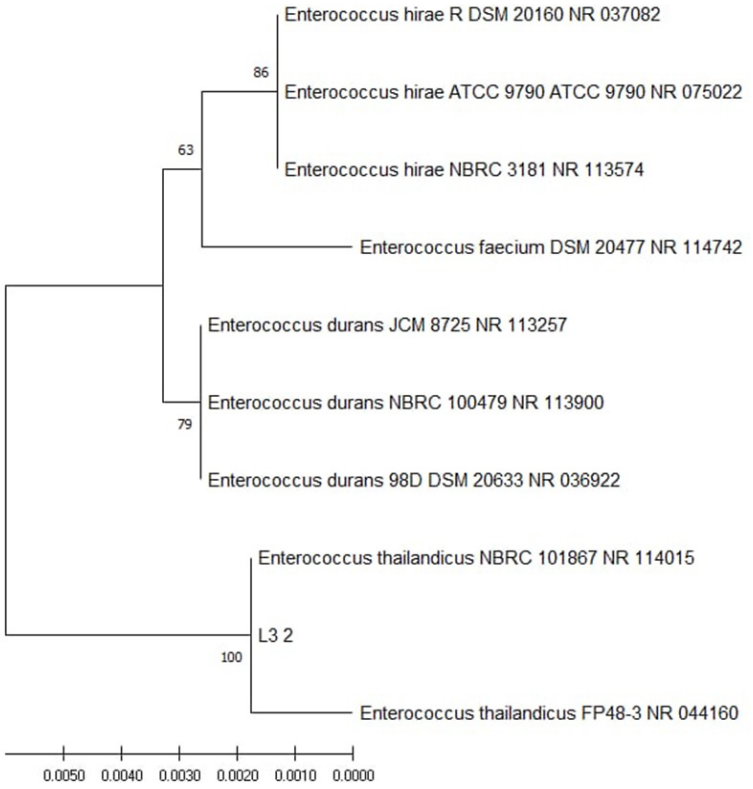


FIGURE 1. The maximum likelihood phylogenetic tree for isolate L3-2

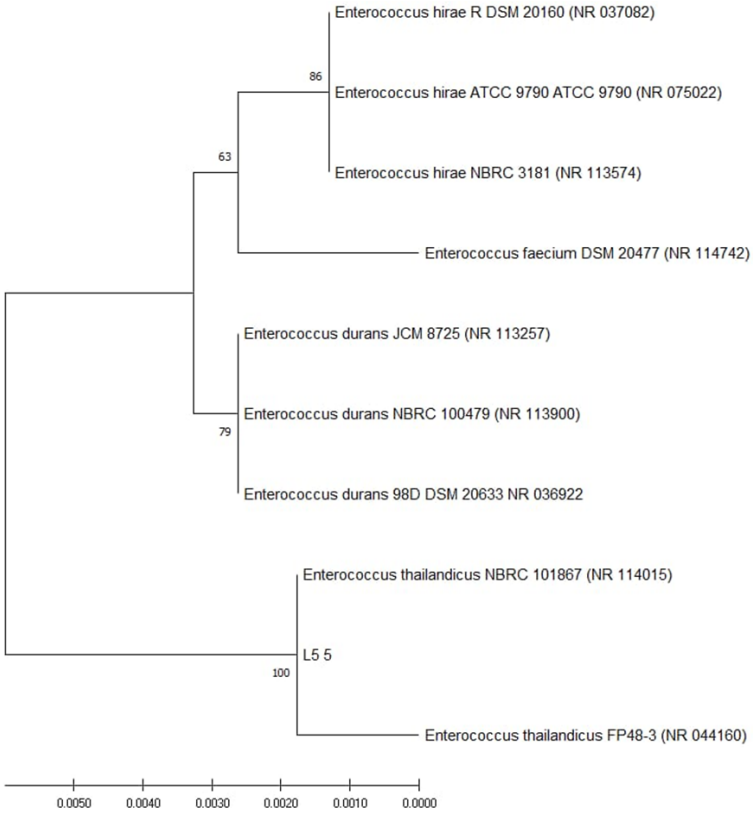


FIGURE 2. The maximum likelihood phylogenetic tree for isolate L5-5

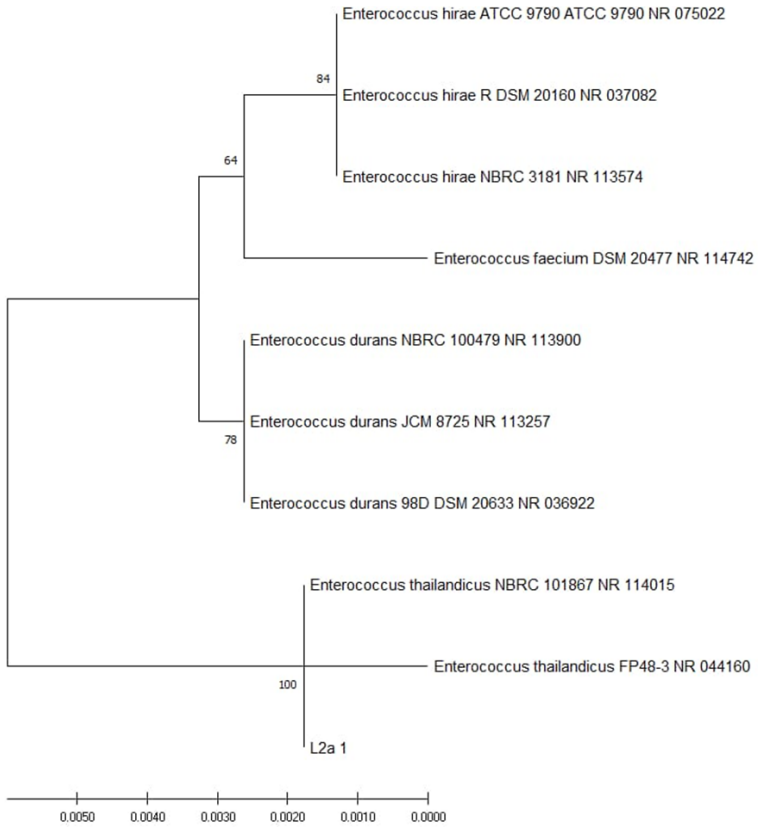


FIGURE 3. The maximum likelihood phylogenetic tree for isolate L2a-1

1. Gel Photo

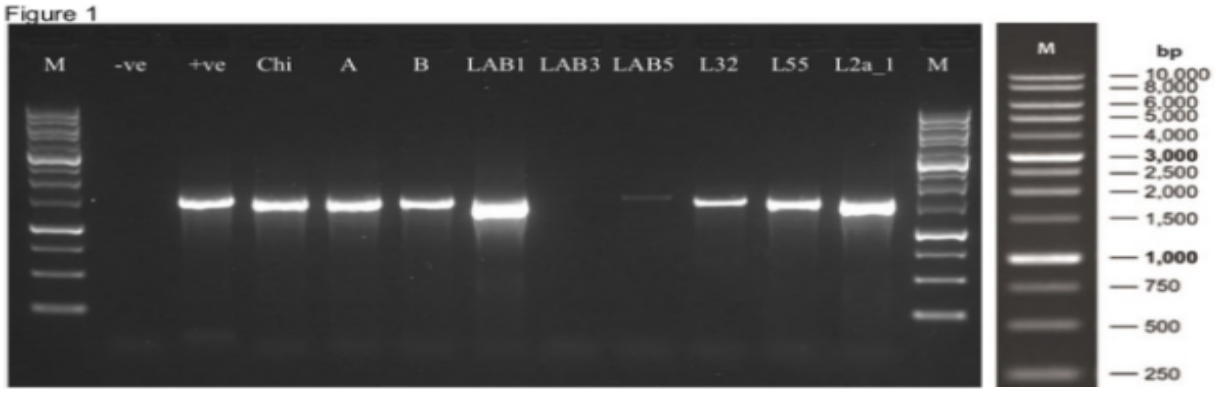


FIGURE 4. A) Gel electrophoresis of L3-2, L5-5, and L2a-1 isolates shows the PCR amplification of the 16SrDNA gene on an agarose gel; B) DNA ladder or marker, which serves as an indicator for the length of the isolates (L3-2, L5-5, and L2a-1)

gut microbiota of animals and insects as their presence would enhance the immune defense against pathogens (Motta & Moran 2024; Upfold et al. 2023; Zhang, Zhang & Lu 2022). Marheni, Martono and Sijabat (2021) reported the isolation of LAB from the larval gut of *O. rhinoceros* from oil palm empty fruit bunches. *E. termitis* and *Lactococcus taiwanensis* were isolated from the hind and mid-gut, respectively. However, the species in this study has not been isolated from the larval gut.

CELLULASE ACTIVITY OF THE IDENTIFIED ISOLATES

The cellulolytic ability of three isolated *Enterococcus* strains was determined. On the CMCA plate, it was

observed that there were clear zones of inhibition, with the broadest diameter (cm) being L5-5 (1.06 ± 0.05), followed by L2a-1 (1.03 ± 0.05) and L3-2 had the least diameter zone of 0.86 ± 0.05.

EFFECT OF TEMPERATURE ON CELLULASE PRODUCTION

The optimum temperature at which the three strains produced cellulase enzyme was determined at 45 °C as shown in Figure 5. At 45 °C, the enzyme titers were observed to increased compared to the 24- to 96-h incubation period. *E. thailandicus* strain L5-5 had the highest cellulase activity (0.42 ± 0.02 U/mL) at 45 °C, and the least (0.16 ± 0.04 U/mL) was at 37 °C. At 27 °C, 50 °C and 60 °C, the titers were 0.24 ± 0.03 U/mL, 0.18 ± 0.01 U/mL, and 0.32 ± 0.01 U/mL, respectively. *E. thailandicus* strain L2a-1 also had its highest cellulase activity (0.61 ± 0.04 U/mL) at 45 °C, while the least cellulase activity (0.21 ± 0.03 U/mL) was at 50 °C. At 27 °C, 0.54 ± 0.03 U/mL titer of enzyme was produced, which was the second highest activity, while 0.38 ± 0.03 U/mL and 0.33 ± 0.02 were the titers of enzyme produced at 60 °C and 37 °C by this strain, respectively. *E. thailandicus* strain L3-2 had its highest cellulase activity (0.62 ± 0.03 U/mL) at 45 °C and the least (0.22 ± 0.03 U/mL) at 50 °C. At 27 °C, 37 °C, and 60 °C, the titers were 0.53 ± 0.00 U/mL, 0.36 ± 0.01 U/mL and 0.35 ± 0.01 U/mL, respectively. Dantur et al. (2015) isolated cellulolytic *E. casseliflavus* from *Diatraea saccharalis* larva but found that it produced very low cellulolytic activity. Ngouénam et al. (2021) demonstrated that the cellulase activity of LAB changed not only among isolates from various biotopes but also among isolates from the same biotope. Similar findings were made in this study, where strain L3-2 cellulase displayed the highest activity level, producing 0.62 U/mL. LAB isolates' types and origins, cellulase volume produced, and the physico-chemical conditions may all contribute to the variations in cellulase activity between the isolates examined. All three isolates had their optimum cellulase production at 45 °C.

EFFECT OF pH ON CELLULASE PRODUCTION

The optimum pH at which the enzyme activity was highest was 4.5. Surprisingly, *E. thailandicus* strain L5-5 that had a slightly larger zone of inhibition (1.06 ± 0.05) in the plate screening test had the least activity for all the pHs (4.0, 4.5, 6.0, 7.0, 9.0) and temperatures (27, 37, 45, 50, 60) as shown in Figure 5. It was observed that for all the strains, the enzyme titer increased as the time of cultivation increased (i.e., 24 to 96 h). *E. thailandicus* strain L5-5 had the highest activity (0.68 ± 0.02 U/mL) at pH 4.5. At pH 7.0, strain L5-5 had the lowest activity (0.16 ± 0.04) while at pH 4.0, 6.0, and 9.0, the titer was 0.33 ± 0.02 U/mL, 0.40 ± 0.01 U/mL, and 0.25 ± 0.03 U/mL, respectively. *E. thailandicus* strain L2a-1 had its highest activity (0.62 ± 0.04 U/mL) at pH 6.0 and the least activity (0.32 ± 0.03 U/mL) was at pH 7.0. At pH 4.0, 4.5 and 9.0, the titer was 0.35 ± 0.01 U/mL, 0.50 ± 0.00 U/mL and 0.45 ± 0.03 U/mL, respectively. At pH 4.5, *E. thailandicus* strain L3-2 had its highest activity (0.73 ± 0.01 U/mL) and the least (0.33 ± 0.01 U/mL) at pH 4.0. At pH 6.0, the titer was 0.60 ± 0.02 U/mL while at pH 7.0 and 9.0, the titers were 0.36 ± 0.01 U/mL and 0.43 ± 0.01 U/mL. The three (3) *E. thailandicus* strains isolated and identified in this study had higher enzyme activity (L5 5:0.42 U/mL, L3 2:0.62 U/mL and L2a 1:0.61 U/mL, respectively) compared to a LAB (*Pediococcus acidilactici* MK 20) isolated from Mentok (*Anas maschanta*) with 0.0153 U/mL as reported by Herdian et al. (2018). Cellulase activity in LAB could be valuable and helpful for the simultaneous saccharification and fermentation of lignocellulosic biomass into lactic acid (Malacara-Becerra et al. 2022). Table 2 compares enzyme activity in some LAB, other bacteria and the LAB isolated in this study. This trait also shows that these isolates are potential candidates for cost-effective LA production as they have the potential to reduce the need for enzyme supplements in the saccharification and fermentation process by having the ability to produce more cellulase enzyme at higher temperature and lower pH, respectively, compared to other cellulase enzyme producers at 30 °C and pH 7. Using isolates that could produce cellulases that could function effectively at elevated temperatures and acidic pH levels in saccharification and fermentation allows for more efficient saccharification of lignocellulosic biomass into fermentable sugars to be utilised for lactic acid fermentation (Xavier et al. 2024; Yankov 2022).

PRODUCTION OF LACTIC ACID AND CONSUMPTION OF GLUCOSE BY THE IDENTIFIED ISOLATES

Production of lactic acid and utilization of glucose by the three *E. thailandicus* strains were determined. There was an increase in lactic acid production by the three strains from the 6th h to the end of incubation time (24 h), as shown in Figure 6. The range of LA produced was 6.18 ± 0.08 g/L to 9.34 ± 0.14 g/L. At the 6th h, *E. thailandicus* strain L5-5 produced the highest concentration of 6.34 ± 0.07 g/L, 7.99 ±0.01 at the 12th h, 8.72 ±0.09 at the 18th h and 9.04 ±0.11 at the 24th h. *E. thailandicus* strain L2a-1 produced 6.28 ± 0.04 g/L of LA at the 6th h of incubation. At the 12th h, an 8.08 ± 0.04 g/L concentration of LA was produced, an increment from the concentration of LA produced at the 6th h. Subsequently, there was an increase in the concentration of LA produced at the 18th h (8.08 ± 0.04 g/L) to the 24th h (9.26 ± 0.13 g/L). *E. thailandicus* strain L3-2 produced 6.18 ± 0.08 g/L of LA at the 6th h of incubation which was the least LA concentration produced by the three strains at this hour. At the 12th h, 8.02 ± 0.07 g/L of LA was produced as the second highest producer. From the 18th h of incubation, *E. thailandicus* strain L3-2 produced the highest LA concentration (8.89 ± 0.07 g/L) up to the 24th h (9.34 ± 0.14 g/L), and this strain produced the overall highest concentration of LA. At the end of the experiment, *E. thailandicus* strain L3-2 produced the highest LA with

TABLE 2. Cellulolytic activities of some microorganisms

Bacteria	Enzyme activity (U/mL)	Isolate source	References
Bacillus (BE 8)	2.16	Rice bran	Jannah et al. (2018)
Bacillus (BE 14)	1.31	Rice bran	Jannah et al. (2018)
B. cereus BR0302	0.121 ± 0.006	Thai coastal wetland soil	Chantarasiri (2015)
B. pumilus kd101 TUC-EEAOC	0.32 ± 0.002	Intestine of *Diatraea saccharalis* larvae	Dantur et al. (2015)
E. thailandicus L2a-1	0.61 ± 0.04	Gut of *O. rhinoceros* larvae	This study
E. thailandicus L3-2	0.62 ± 0.03	Gut of *O. rhinoceros* larvae	This study
E. thailandicus L5-5	0.42 ± 0.02	Gut of *O. rhinoceros* larvae	This study
Pediococcus acidilactci MK 20	0.0153	Mentok (*Anas moschata*) gastrointestinal tract	Herdian et al. (2018)

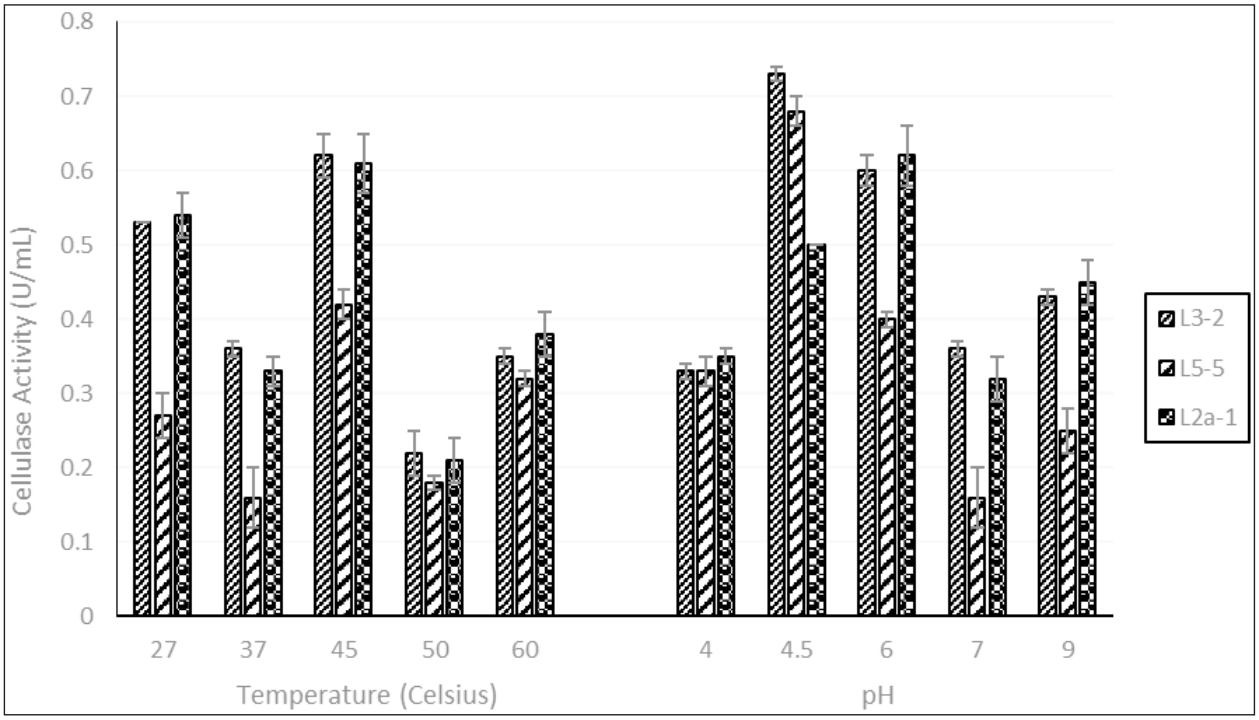
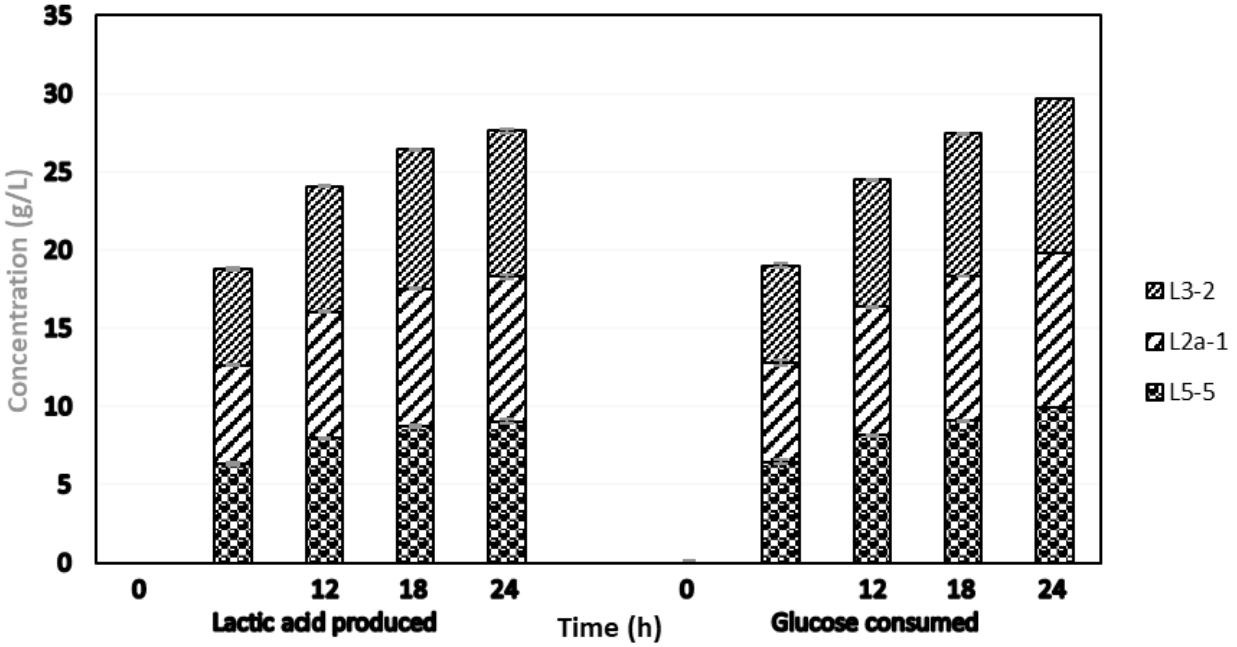


FIGURE 5. Effect of Temperature and pH on cellulase production for strains L3-2, L5-5 and L2a-1

9.34 ± 0.14 g/L, followed by *E. thailandicus* strain L2a-1 with 9.26 ± 0.13 g/L and *E. thailandicus* strain L5-5 with 9.04 ± 0.11 g/L.

The quantity of glucose consumed by the three *E. thailandicus* strains within 24 h of incubation was also investigated. Figure 6 shows the trend of consumption of glucose. Consumption of glucose by the three *E. thailandicus* strains increased from the 6th h to the end of the incubation period. All three strains could almost consume all the glucose in the medium. At the 6th h of incubation, strain L5-5 consumed 6.47 ± 0.19 g/L of glucose, which was 64.7% of the total amount of glucose initially added to the culture broth. There was an increase in the concentration of glucose consumed from the 6th h up to the 24th h of incubation. At the 12th and 18th h of incubation, strain L5-5 consumed 8.16 ± 0.18 g/L and 9.08 ± 0.02 g/L glucose, respectively, as the second and third positions to consume such amount. At 24th h, strain L5-5 consumed 9.95 ± 0.02 g/L, the highest glucose concentration consumed by any of the strains. *E. thailandicus* strain L2a-1 consumed 6.31 ± 0.16 g/L (63.1%) at 6th h and consumed 8.20 ± 0.17 g/L and 9.28 ± 0.04 g/L at 12th and 18th h, respectively. Strains L2a-1 consumed the second highest glucose concentration at



Values represent the mean of 3 replicates ± SD

FIGURE 6. Lactic acid produced and glucose consumed by the identified isolates within 24 h

24th h of incubation. *E. thailandicus* strain L3-2 consumed 6.23 ± 0.18 g/L (62.3%) of glucose at 6th h while 8.13 ±0.16 g/L and 9.12 ± 0.06 g/L of glucose were consumed at 12th and 18th h of incubation, respectively. Strain L3-2 consumed the least concentration of glucose after 24 h. As shown in Figure 6, at the end of incubation, strains L5-5, L2a-1 and L3-2 consumed 9.95 ± 0.02 g/L, 9.90 ± 0.00 g/L, 9.87 ± 0.02 g/L which were equivalent to 99.5%, 99%, and 98.7%, respectively. The amount of glucose consumed to the amount of lactic acid produced was approximately 1:1. Invariably, 1 mol of glucose produced 2 mol of lactic acid. It was observed that *E. thailandicus* strain L5-5 consumed the highest concentration of glucose but produced the least concentration of LA while strain L3-2 consumed the least concentration of glucose but produced the highest concentration of LA after 24 h. Using ANOVA ($p < 0.05$), there was a significant difference among the isolates regarding glucose consumption. Trinh et al. (2018) reported the isolation of some thermophilic LAB strains which were able to produce LA ranging from 6.0 g/L to 14.1 g/L at 37 °C and the highest percentage yield was 72.97% whereas in this study, the highest percentage yield was 95%.

Numerous studies have demonstrated the potential of LA production using lignocellulosic wastes as substrates (Grewal et al. 2020). In addition, several species of LAB have been used to produce LA from these feedstocks (Rawoof et al. 2021). Although some researchers have demonstrated lignocellulolytic activity in some LAB strains (Ngouénam et al. 2021), they however paid little attention to the possibility of producing LA directly from biomass in a one-pot process using the innate cellulolytic enzymes of LAB strains. Mohamad Zabidi et al. (2020) evaluated cellulolytic and hemicellulolytic enzyme production by *Lactobacillus plantarum* RI 11 on rice straw, palm kernel cake and molasses as carbon sources. Similarly, *Pediococcus acidilactici* MK 20 isolated from the gut of muscovy duck (*Anas moschata*) displayed CMCase activity on 1% CMC as a carbon source (Herdian et al. 2018). However, these studies did not evaluate homofermentative LA production alongside cellulase production. As mentioned earlier, this ability is a potential way to reduce the purification costs of LA when such a route is followed. As seen in this report, it is possible to use naturally cellulolytic LAB strains to produce pure LA from lignocellulosic hydrolysates in a way that saves enzyme costs and the cost of downstream purification.

CONCLUSION

In this study, we successfully isolated three *E. thailandicus* strains with the dual property of homofermentative LA and cellulase production. To the best of our knowledge, this is the first report of the isolation of homofermentative cellulolytic *E. thailandicus* from *O. rhinoceros.* The strains were robust and tolerated varying conditions of pH, temperature, salt and furfural. They produced between 9.04 and 9.34 g/L of LA and between 0.42 to 0.62 U/mL cellulase utilising glucose as the main carbon source. These findings showed that these strains are promising for the cost-effective production of LA from various substrates. The potential for scaling up the industrial application of

thermophilic and acidophilic cellulase-producing lactic acid bacteria (LAB) is quite significant, especially in the context of bioprocessing of lignocellulosic biomass into valuable products. These microorganisms offer unique advantages, but several challenges must be addressed to facilitate their effective translation from laboratory research to industrial settings such as setting large-scale physico-chemical treatment plants for lignocellulosic waste that would require large monetary investments and could be prohibitive to the production of lactic acid from lignocellulosic waste.

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