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Original research paper

ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA FROM ANNA APPLE CULTIVAR *(MALUS DOMESTICA* VAR. ANNA*)* AS POTENTIAL EXOPOLYSACCHARIDE PRODUCER

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Abstract: Exopolysaccharides are a type of polysaccharide produced by various bacteria through cellular excretion. Lactic acid bacteria-produced exopolysaccharide has comparable physicochemical properties to certain plant polysaccharides such as guar gum or pectin. Hence, they provide functional roles in the food industry, such as replacement for gluten in baked goods, alternative thickeners, stabilizers, and even providing health benefits. Currently, exopolysaccharide-producing lactic acid bacteria strains have become highly sought after for research and commercial purposes. However, isolation and characterization from indigenous fruit sources remain insufficiently explored, despite the wide diversity of said fruits and their distinct microbial terroirs. Anna apples are indigenous apple cultivar from Malang, Indonesia, known to have a high sugar content, moderate acidity, as well as containing various vitamins and minerals, suitable for lactic acid bacteria growth. This study aims to isolate and identify endophytic lactic acid bacteria from Anna apple cultivar (*Malus domestica* var. Anna) mesocarp, as a potential exopolysaccharide producer. Isolation and purification produced eight isolates, respectively BAA-1 through BAA-8. The isolates were then characterized via Gram staining, endospore staining, catalase activity assay, carbohydrate fermentation assay, and exopolysaccharide production yield. Results indicate that isolates BAA-5 and BAA-8 produced the highest yields of exopolysaccharide, which were 3350 mg/L and 3050 mg/L respectively. Further molecular identification showed that isolate BAA-5 had a 98.68% gene sequence similarity to *Lacticaseibacillus paracasei*, while isolate BAA-8 had a 99.74% sequence similarity to *Lactiplantibacillus plantarum*. Thus, the two isolates can potentially be developed as functional agents in food industrial applications.

Key words: *lactic acid bacteria, exopolysaccharide, Malus domestica var. Anna, isolation, identification*

INTRODUCTION

Exopolysaccharide (*EPS*) are important metabolites which are composed complex biopolymer mixtures and produced by several microorganisms, including lactic acid bacteria (*LAB*). *EPS* hold strategic value in the food, health, and biotechnology industries. *EPS* produced by *LAB*, exhibit non-toxic and biodegradable characteristics, and they are often used as thickeners, stabilizers, and emulsifiers in food-based applications (Korcz & Varga,

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2021). *EPS* are also known to possess health-promoting bioactivities namely antioxidant, immunomodulatory, antitumor, and antimicrobial activities (Ouarabi et al., 2025). Utilization of natural *EPS* is aligned with Sustainable Development Goals (*SDGs*) principles, particularly *SDG* 3 (Good Health and Well-being) through improving public health, *SDG* 12 (Responsible Consumption and Production) by reducing dependency towards synthetic food additives which adversely impacts the environment and *SDG* 2 (Zero Hunger) by improving functional food quality.

Lactic acid bacteria (*LAB*) refer to a group of Gram-positive bacteria which holds essential roles in food fermentation and bioactive compound formation (most notably lactic acid). *EPS* producing *LAB* can commonly be found in fermented foods, dairy products, vegetables, and fruits (Jurášková, Ribeiro & Silva, 2022). Fresh fruits provide an environment rich in simple sugars, vitamins, organic acids, and high moisture, suitable for growth of endogenic microorganisms including *LAB* (Wicaksono et al., 2023). Among various fruits, the apple is known to harbor diverse microbial communities, ranging from epiphytic to endophytic (Shen et al., 2022; Milute et al., 2016). Anna Apple (*Malus domestica* var. Anna) is a popular cultivar in Indonesia, widely cultivated in highland regions such as Batu-Malang. It is known to have a high sugar content with moderate acidity (Gutiérrez-Villamil et al., 2022), thereby serving as a potential and adaptive *LAB* isolation source unique to Indonesia's tropical agro-climate.

Exploration of indigenous microorganisms from Anna apple cultivar holds great significance. Given Indonesia's tropical climate, microorganisms found are likely to have unique specific characteristics compared to its subtropical counterparts.

This aligns with the concept of microbial terroir and fruit micro-ecology, suggesting that different environmental, regional, and growth conditions may bring about distinct characteristics, such as niche metabolic capabilities, higher *EPS* yields, or different monomeric composition of *EPS* (Vale et al., 2024; Hoefle et al., 2025). These efforts support *SDG* 15 (Life on Land) through the documentation and utilization of local microbial biodiversity.

Apart from scientific values, identification of *EPS* producing *LAB* from local fruits like Anna apple provides additional economic values (Nguyen et al., 2024; Hernández-Figueroa, López-Malo & Mani-López, 2025). High-performing isolates can be further developed as natural biopolymer producing agents, applicable in the food, pharmaceutical, and cosmetic industries. Additionally, *LAB EPS* are known to provide prebiotic properties, aiding in gut health by stimulating growth of beneficial microbes, as well as improving immunity and metabolic health (Yadav et al., 2024). These provide further economic values especially since the global market value of prebiotic has been estimated to experience a 14.4% compound annual growth rate from 2024 to 2030 (Chhabra et al., 2025). The application of natural biopolymers aligns with *SDG* 9 (Industry, Innovation, and Infrastructure) by enhancing biotechnological industry development through a local resource basis.

Previous studies have shown how locally-grown fresh fruits act as promising sources of *EPS*-producing *LAB*. Besrour-Aouam et al. (2019) isolated *Leuconostoc lactis* AV1n from Tunisian avocado, with an *EPS* production yield of 2.25 g/L. Lawalata et al. (2023) have also successfully isolated seventeen *LAB* strains from Pakoba fruit (*Syzygium* sp.), endemic to Minahasa, North Sulawesi. The isolates yielded *EPS* ranging from 102 to 1570 mg/L. These studies highlight fruits as valuable ecological niches for *EPS*-producing *LAB*, providing a strong rationale basis for investigating Anna apple (*Malus domestica* var. Anna) as potential sources of *EPS*-producing strains.

Exploration of *EPS*-producing microorganisms from local Indonesian horticultural commodities such as Anna apple caries significant scientific urgency and strategy. Indonesia's rich microbial biodiversity remains largely unexplored, hence this study will expand local and potential microflora database. Notably, high-performing *EPS* producing *LAB* isolates will also promote national and international biotechnology industries, especially in biopolymer production.

Furthermore, *EPS* can potentially be applied as a natural stabilizer to support food product quality development in local Micro, Small and Medium Enterprises (MSMEs). Overall, this

study contributes to sustainability principles through the development of natural and eco-friendly raw materials, aligned with the need of global industries.

MATERIALS AND METHODS

This study is designed to explore, isolate, identify, and characterize endophytic, *EPS*-producing *LAB* from Anna apple cultivar. Sample collection utilized simple random sampling method on Anna apples in Batu City, Malang Regency, East Java to minimize selection bias. Details of each research processes can be seen below:

Lactic acid bacteria isolation from Anna apple cultivar (*Malus domestica* var. Anna)

Lactic Acid Bacteria isolation was done from ripe and ready to consume Anna apples. Prior to isolation, the fruits were rinsed through running water and decontaminated using 70% ethanol (PT. SMART LAB Indonesia) to reduce epiphytic microbes. Afterwards, the apples were aseptically peeled and 5 g of mesocarp were homogenized, suspended into 45 mL of sterile peptone water (Sigma-Aldrich®), and then serially diluted to 10^{-5}. Suspensions from dilution 10^{-3}-10^{-5} were inoculated on *MRS* (Mann Rogosa Sharpe) Agar (HiMedia®) which is supplemented with 1% $CaCO_3$ (HiMedia®) (w/v) through pour plate method. The inoculated plates were then incubated at 37 °C for 24–72 hours in anaerobic facultative conditions. Clear zone formation around colonies was used as an initial indicator for *LAB* acid production, due to the dilution of $CaCO_3$ (Veselá et al., 2019). Colonies were also observed for *LAB* morphological characteristics such as white, yellowish or creamy colour, round shape, convex or raised elevation, and entire or smooth margin (Stephen & Saleh, 2023; Tian et al., 2024).

Colonies forming clear zone and having *LAB* morphology characteristics were chosen as *LAB* candidate and then purified using four quadrant streak plates on *MRS* agar until a single colony with stable morphology was found.

The pure isolates were then macroscopically and microscopically analyzed to verify *LAB* characteristics. Common *LAB* characteristics include Gram positive, cocci or bacilli shape, non-spore forming, and negative catalase activity (Mokoena, 2017; Dey et al., 2023).

Macroscopic and microscopic identifications

Macroscopic identifications were done by observing colonies that grow on *MRS* agar with $CaCO_3$ supplementation. These include the colony shape, colour, margin, and elevation. *LAB* colonies generally have white, yellowish, or creamy colour, round shape, entire margin, as well as convex or raised elevation (Stephen & Saleh, 2023; Tian et al., 2024).

Colony diameter was also measured. which varies according to incubation time and species, but is typically 0.5-3.0 mm (Sulmiyati, Fahrodi, Malaka & Maruddin, 2018; Nandhini, Prasanth, Selvi & Sundaresan, 2025). Additionally lactic acids produced by *LAB* will dilute $CaCO_3$, creating a clear zone. Colonies with identical morphologies are assumed to be the same strain (Gupta, Mohanty & Majumdar, 2021).

Microscopic identification was done via Gram staining to cultures after 24 hours of incubation. *LAB* are Gram positive bacteria with cocci or bacilli shape, hence the stained cells should have a purple color (Dey et al., 2023).

Afterwards, endospore staining was done using malachite green (HiMedia®) and safranin (HiMedia®). *LAB,* are non-spore forming, indicated by a red color (fully vegetative cell) with no green stains inside of the cells (Zapaśnik, Sokołowska & Bryła, 2022).

Biochemical test of lactic acid bacteria isolate

Isolates that met both microscopic and macroscopic characteristics of *LAB* were further analyzed using a series of biochemical tests. These included the catalase test, fermentation type characterization, and evaluation of *EPS* production yield.

Catalase test

Catalase test was performed according Dey et al. (2023). One loopful of 24 hour aged *LAB* culture was placed on a slide glass and were given 1-2 drops of 30% H2O2 (HiMedia®). Catalase-positive activity is shown by O2 bubble formation in ≤10 seconds. *Staphylococcus aureus* ATCC 25923 was used as a positive control, while *Lactiplantibacillus plantarum* ATCC 8014 was used as a negative control. Isolates that do not produce bubbles were categorized as catalase-negative, which is a known *LAB* physiological characteristic.

Fermentation type characterization

Fermentation type was determined according to CO_2 production in Durham tubes (Pyrex®). As much as 1% (OD600 = 0,5) were inoculated into *MRS* broth (HiMedia®) with 6.5 pH, placed into a test tube with a Durham tube inside. Samples were then incubated for 48 hours at 37 °C and anaerobic conditions. No gas formation indicates homofermentative isolate, while gas formation indicates heterofermentative isolate (Borowska et al., 2023). An uninoculated medium was used as a control.

Exopolysaccharide (EPS) production yield

EPS production yield was measured following a method by Hernández-Figueroa et al. (2025) with several modifications. Isolates were grown in 25 mL of *MRS* broth and were incubated at 30 °C for 24 hours. Cultures were then centrifuged at 5000 rpm for 30 minutes at 4 °C to separate cell biomass (refrigerated centrifuge from Thermo Scientific, model Sorvall ST8R). The resulting supernatant was collected. To obtain *EPS* precipitate, 10 mL of supernatant was combined with 20 mL of technical acetone (PT. SMART LAB Indonesia) (2:1, v/v). The mixture was then kept overnight at 4 °C. Precipitates were re-centrifuged under the same conditions to that of the cultures and then diluted with 10 mL distilled water (PT. SMART LAB Indonesia). To remove protein, diluted precipitates were combined with 250 µL trichloroacetic acid 80% (HiMedia®) and the mixture was centrifuged for 30 minutes (5000 rpm, 4 °C).

EPS precipitate was dried at 100 °C until a constant weight was achieved (confirmed using a desiccator). The *EPS* mass was calculated from the mass difference before and after drying. The *EPS* production yield was calculated using dry mass per media volume (mg/L).

Molecular identification of lactic acid bacteria isolate

The *16s rRNA* based molecular identification was done to further complement phenotypic characterization of *EPS*-producing isolates. This process is essential to verify if isolates belong to the LAB species group. Identification procedures include genomic *DNA* isolation, *16s rRNA* gene amplification using *PCR* (SimpliAmp™ Thermal Cycler *PCR* System), and sequence analyses.

Genomic DNA isolation

DNA isolation was done using NEXprep™ DNA isolation kit (Genes Laboratories Co., Ltd.). As much as 1,5 mL LAB culture was centrifuged at 10 000 rpm for 2 minutes, supernatant was then discarded. Pellet was then combined using 180 µL GT1 buffer (Genes Laboratories Co., Ltd.) and was homogenized using the vortex mixer. The mixture was then combined with 20 µL Proteinase K (Genes Laboratories Co., Ltd.) and 200 µL GT2 buffer (Genes Laboratories Co., Ltd.). The final mixture was incubated at 56 °C for 10 minutes and test tube was inverted every 5 minutes. Afterwards, 200 µL absolute ethanol (HiMedia®) was added and the mixture was vortexed.

The resulting suspension was transferred to a spin column and centrifuged at 13 000 rpm for 1 minute. The spin column (Genes Laboratories Co., Ltd.) was then washed sequentially using 500 µL W1 buffer (Genes Laboratories Co., Ltd.) and 700 µL W2 buffer (Genes Laboratories Co., Ltd.), each followed by centrifugation for 1 minute. The column was re-centrifuged for 2 minutes to dry thoroughly. *DNA* was then eluted using 50–100 µL elution buffer (Genes Laboratories Co., Ltd.) and centrifuged at 13 000 rpm for one minute. *DNA* obtained was then stored at -20 °C for short-term storage and -70 °C for long-term storage.

16s rRNA gene amplification using PCR

16s rRNA gene amplification was done with 30 µL total volume, which includes: 3 µL primer (5 pmol), 1,5 µL *DNA* template (100 ng/µL), 11,5 µL nuclease free water, and 15 µL NEXpro *PCR* Master Mix. The universal primer used was 27F (5'-AGAGTTTGATC-(A/C)TGGCTCAG-3') and 1492R (5'-TACGG(C/T)TACCTTGTTACGACTT-3') (1^{st} BASE). Partial amplification *PCR* was done.

PCR reactions were done in these temperature conditions: pre-denaturation at 95 °C for 2 minutes; followed by 20 cycles of denaturation at 95 °C for 2 minutes, annealing at 50 °C for 30 seconds, extension at 72 °C for 2 minutes; and final extension at 72 °C for 7 minutes. *PCR* products were analysed using gel electrophoresis (MUPID-exU electrophoresis system, Advance Co. Ltd.). Agarose gel was made at 40 mL quantity with 2% concentration, using agarose powder (Sigma-Aldrich®) and 1X

TAE buffer (HiMedia®) the mixture was heated until a clear white colour was achieved. Afterwards, 4 µL gel red stain (1 µL/10 mL gel) (Sigma-Aldrich®) was added after the gel mixture has reached room temperature. The gel was then poured into provided mould and let set in room temperature while covered from light. Subsequently, samples were injected into well and electrophoresis was run 80 volts for 45-60 minutes. Samples that showed *DNA* ribbons with ±1500 bp were sent for sequencing at 1st BASE laboratories (Mala-ysia).

16s rRNA gene sequencing analysis

Sequencing chromatogram was analyzed using BioEdit software to obtain nucleotide consensus sequence. Species identification was done by comparing consensus sequence to reference sequence from NCBI GenBank database, using *BLAST* tool from the NCBI database with the following parameters: database set to nr/nt, program selection optimized for highly similar sequences (megablast), E-value threshold of 1e-5, match/mismatch scores of +2/-3, and gap costs (existence 5, extension 2). Phylogenetic analysis was done using MEGA 11.0 with Neighbor-Joining method and 1000 bootstrap replicates. *Escherichia coli* O104 strain 2011c-3493 was used as an outgroup (Johnson et al., 2019; Imade et al., 2024; Li et al., 2024). Isolates with ≥99% nucleotide sequence similarity were considered as belonging to the same species. The resulting data was used to verify the species of *EPS*-producing *LAB*.

Data analyses

Qualitative characterizations (macroscopic observation, microscopic observation, catalase test, fermentation type evaluation, and molecular identifications) were analysed descriptively and summarized in tables and figures (Lawalata et al., 2023). Macroscopic observation, microscopic observation, catalase test, fermentation type evaluation was done in triplicate to ensure data reproducibility. *EPS* yield evaluation was done in triplicate, to ensure data reproducibility and was analysed using one way-analysis of variance (*ANOVA*), followed by Tukey's post hoc test ($P<0.05$).

RESULTS AND DISCUSSION

Isolation and characterization of lactic acid bacteria from Anna apple (*Malus domestica* var. Anna)

Eight isolates were successfully isolated from Anna apples (coded as BAA-1 through BAA-8). All isolates showed relatively uniform macroscopic and microscopic characteristics (Table 1).

Macroscopic and microscopic characteristics also showed similarities to *Lactobacillaceae* and *Leuconostocaceae* colony. The *Leuconostocaceae* family, such as *Weissella* sp. and *Leuconostoc* sp., often have white or cream-coloured colonies, round or irregular shape, entire margin, raised or convex elevation (Bhatia et al., 2022; Teixeira et al., 2021).

Table 1.
Macroscopic and microscopic characteristics of lactic acid bacteria isolates from anna apple cultivar (*Malus domestica* var. Anna)

	Macroscopic					Microscopic		
Code	**Colony shape**	**Colony colour**	**Margin**	**Elevation**	**Colony diameter (mm)**	**Cell shape**	**Gram classification**	**Endospore**
BAA-1	Round	Pale white	Entire	Convex	2.1-2.2	Bacilli	Positive	Negative
BAA-2	Round	Yellowish white	Entire	Convex	1.5-1.6	Bacilli	Positive	Negative
BAA-3	Round	Yellowish white	Entire	Convex	1.4-1.9	Bacilli	Positive	Negative
BAA-4	Round	Milky white	Entire	Convex	2.0-2.2	Cocci	Positive	Negative
BAA-5	Round	Milky white	Entire	Raised	2.0-2.1	Bacilli	Positive	Negative
BAA-6	Round	Milky white	Entire	Convex	2.3-2.5	Bacilli	Positive	Negative
BAA-7	Round	Pale white	Entire	Convex	2.0-2.2	Cocci	Positive	Negative
BAA-8	Round	Milky white	Entire	Raised	2.2-2.3	Bacilli	Positive	

Furthermore, the *Lactobacillaceae* family, such as *Lacticaseibacillus* sp., *Lactiplantibacillus* sp. and *Lactobacillus* sp., often have creamy or white coloured colony, entire margin, convex or raised elevation, and round shape (Bhatia et al., 2022; Rabiei et al., 2019). LAB colony diameter will vary according to incubation time, nutrition, and species metabolism, but will generally range from 0.5 to 3.0 mm (Sulmiyati et al., 2018; Nandhini et al., 2025). However, it is important to note that macroscopic and microscopic characterization act as preliminary data, and hence cannot be used as a sole basis for microbial identifycation.

Colony colour variations, ranging from pale white to milky white, indicated production of exopolysaccharide (*EPS*), where in opaque colony colour are often related to *EPS* accumulation (Yadav et al., 2024).

Colony elevations are often convex, however raised elevations as found in *EPS*-producing isolates, such as BAA-5 and BAA-8 often correlates to relatively higher metabolic activity or *EPS* matrix formation. Colony diameter was found ranging from 1.4-2.5 mm, which is consistent to *LAB* colony characteristic in *MRS* medium and strain variety commonly found in fresh (Linares-Morales et al., 2022).

Uniformity of macroscopic characteristic among isolates showed a similar metabolic pattern in *MRS* medium as well as ability to utilize glucose from glycolysis or phosphoketolase pathway, hence resulted in a relatively stable colony phenotype (Jawaid, Ashfaq, Al-Ghouti & Zouari, 2024). $CaCO_3$ as a fermentation indicator showed clear zones around colonies, suggesting that lactic acid production was enough to solubilize $CaCO_3$ and that high fermentative isolates were successfully isolated. Variation of clear zone clarity among isolates also implies a difference in metabolic efficiency and *EPS* production potential (Juráš-ková et al., 2022).

Colony phenotype homogeneity indicated that Anna apples provide a conducive and suitable habitat for *LAB*. This is due to the high sugar content and low pH level of the apples, which allows acid producing bacteria to dominate the environment. This aligns with Hoefle et al. (2025), who suggests that fruit microecology affects the composition and prevalence of epiphytic and endophytic *LAB* (Hoefle et al., 2025). Microscopically, six isolates were found to be bacilli while two were cocci shaped. All isolates were Gram positive, a general characteristic of *LAB*, due to their peptidoglycan-rich cell wall (Zhang et al., 2025).

Cell morphology showed that isolates BAA-1, BAA-2, BAA-3, BAA-5, BAA-6, dan BAA-8 were bacilli shaped, while isolates BAA-4 dan BAA-7 were cocci shaped. This morphological difference is common finding in *LAB* groups, especially those isolated from fruits. Typically, *Lactiplantibacillus* genus is bacilli shaped, while *Lactococcus* or *Leuconostoc* genera are cocci shaped. Shape variation also indicates a possibility of having different physiological capabilities, since cellular structure may affect adhesion, stress tolerance, and secondary metabolite production capabilities (such as EPS) (Zhang et al., 2025).

Endospore staining indicated that all isolates were non-endospore forming. This was shown by no green structure or coloration after staining by malachite green. The nonexistence of endospore confirmed that isolates did not originate from spore forming species such as *Bacillus* or *Clostridium*.

This finding is important since endospore typically indicate high resistance towards environmental stress, a physiological characteristic not found in *LAB* (Rama, Bucker, Salazar, Ray & Granada, 2024).

The presence of endophytic *LAB* on Anna apple mesocarp holds significant ecological relevance. This also corresponds to the characteristics of apple as a substrate, a sugar rich, low pH, and microaerophilic environment which support endophytic *LAB* growth.

Concurrently, recent findings show that horticultural fruit commodities are potential *LAB* sources, with enzymatic activities and biosynthetic capacity including organic acids, bacteriocins, and exopolysaccharides (Vaishnav, Upadhyay, Tipre & Dave, 2016; Panthavee, Noda, Danshiitsoodol, Kumagai & Sugiyama, 2017; Joshi, Salini, Mohan, Nandagopal & Arakal, 2024). Hence, bacilli and cocci morphological findings in this study are in line with previous findings regarding diversity of *LAB* community from plant-related sources.

Table 2.
Biochemical analyses and exopolysaccharide yield of LAB isolated from Anna apples (*Malus domestica* var. Anna)

Isolate Code	Catalase	Fermentation Type	*EPS* (mg/L)
BAA-1	(-) Negative	Homofermentative	1950±0.21[a]
BAA-2	(-) Negative	Heterofermentative	2950±0.18[bc]
BAA-3	(-) Negative	Heterofermentative	1750±0.30[a]
BAA-4	(-) Negative	Heterofermentative	2100±0.20[a]
BAA-5	(-) Negative	Heterofermentative	3350±0.18[c]
BAA-6	(-) Negative	Heterofermentative	2750±0.23[b]
BAA-7	(-) Negative	Homofermentative	1750±0.22[a]
BAA-8	(-) Negative	Heterofermentative	3050±0.21[bc]

[a,b,c]*Different superscript letters within the column indicate significant differences among treatments ($p < 0.05$)*

Biochemical analyses and exopolysaccharide yield of lactic acid bacteria from Anna apples (*Malus domestica* var. Anna)

Biochemical analyses included catalase test and fermentation type test to ensure physiological characteristics of *LAB* isolates. *LAB* isolates produced exopolysaccharide (*EPS*) with varying concentrations. Biochemical analyses and *EPS* yield of *LAB* isolated from Anna apples can be seen in Table 2. Catalase test showed that all isolates (BAA-1 through BAA-8) had negative reactions, with no oxygen bubble formation after H_2O_2 was added. This result is consistent to the general characteristic of *LAB* as a catalase-negative group of bacteria, unable to degrade hydrogen peroxide into water and oxygen. This characteristic also correlates to *LAB* as facultative-anaerobic bacteria, which produces more peroxidase enzyme compared to catalase (Rama et al., 2024).

Fermentation type characterization showed metabolic variations among isolates. Isolate BAA-1 and BAA-7 was classified as homofermentative as no CO_2 gas was present in the Durham tube. On the contrary six other isolates (BAA-2, BAA-3, BAA-4, BAA-5, BAA-6, and BAA-8) showed gas production and was classified as heterofermentative. Heterofermentative *LAB* can convert hexose into lactic acid, CO_2, and ethanol, while homofermentative *LAB* produce lactic acid as its main product.This composition indicated that both metabolic groups of *LAB* can be found in Anna apples, with heterofermentative having a higher proportion. Recent studies have shown that heterofermentative LAB often has additional metabolic pathways that aid *EPS* biosynthesis by forming sugar precursors. However, this highly depends on species and fermentation conditions (Zhang et al., 2025).

All *LAB* isolates showed *EPS* producing capabilities ranging from 1750-3350 mg/L with the same amount of initial substrate, which indicated a difference in metabolic capacity among isolates. Isolate BAA-5 produced the highest *EPS* yield (3350 ± 0.18 mg/L), followed by BAA-8 (3050 ± 0.21 mg/L) and BAA-2 (2950 ± 0.18 mg/L). Generally, *Lactiplantibacillus plantarum* used in traditional fermentations have been reported to produce *EPS* at yields around 1200–2100 mg/L (Han, Naveen, Zhang, Sathiyaseelan & Kim, 2025), while commercial *Lactobacillus casei* and *Lactobacillus rhamnosu*s produced around 800–1600 mg/L on standard *MRS* medium (Jurášková et al., 2002). Additionally, *Leuconostoc mesenteroides* isolated from plant material produced 1800–2600 mg/L of *EPS* on sucrose medium (Yu et al., 2025). Ageeli and Mohamed (2025), isolated *L. plantarum* from fresh fruits and reported *EPS* production of 3000 mg/L on optimum fermentation conditions. Compared to these studies, isolate obtained from Anna apples can be concluded as having high biotechnological potential for producing *EPS*.

Variation of *EPS* production among isolates was caused by internal and external factors, including media composition carbon and nitrogen source, pH, temperature, oxygen, and metabolism patterns of each strain (Zhang et al., 2024; Zang et al., 2025). Non-optimal temperatures or availability of certain carbons may improve *EPS* synthesis as an adaptive cellular response (Xiong, Liu & Huang, 2023). Molecular identification was performed on the two highest *EPS*-producing isolates to identify *LAB*

species with potential as starter culture candidate in development of functional fermented foods.

Molecular identification of high *EPS*-producing lactic acid bacteria isolated from Anna apples (*Malus domestica* var. Anna)

Quantitative analyses of *DNA* purity showed that the two chosen isolates (BAA-5 and BAA-8) had an A260/A280 ratio of 1.91 dan and 2.00 (respectively), which are ideal ratios for PCR process (in the range of 1.8–2.0). Generally, this value represented that isolated *DNA* was free from protein and phenol contamination, suitable for future genetic analyses and amplifications. Furthermore, *DNA* concentration sufficiently high, measured at 460 ng/µl for BAA-5 and 510 ng/µl for BAA-8, which showed that DNA extraction method was effecttive. High *DNA* concentration was important and essential for *16s rRNA* gene amplification success, correlating to primer binding efficiency and sequence reading accuracy (Dinu, Al-Zaidi, Matache & Matei, 2024). Quantitative characteristics of *DNA* for both isolates have met quality standard requirements for *LAB* taxonomy study, especially for potential metabolite producers such as *EPS*.

Electrophoresis of *DNA* isolate showed clear, single *DNA* bands at around 1000 bp for both isolates, indicating that genome was successfully isolated without degradation (Fig. 1A). *DNA* bands had thick patterns with absence of smearing, indicated that there were no random cuts on *DNA* strands due to nuclease or *RNA* contamination. This condition is important since *DNA* cuts or degradation may interfere with *16s rRNA* gene amplification and produce sequencing artefact (Bartoš, Chmel & Swierczková, 2024).

DNA visualization was successful and stable. This showed that isolation methods used was suitable for *LAB* isolates, correlating to their thick cell walls and peptidoglycan content which requires optimal lysis process (Kusmiyati, Wicaksono & Sukarno, 2022). This is consistent with recent studies which report *DNA* extraction effectivity as a crucial factor in determining molecular identification validity (Rozanov, Shaposhnikov, Bondarenko & Sazonov, 2025).

16s rRNA gene amplification with Polymerase Chain Reaction (*PCR*) (partial amplification) technique showed ±750 bp band on isolates BAA-5 and BAA-8 (Fig. 1B). The obtained fragment size was consistent with general length of *16s rRNA* amplicon, ranging at 750-800 bp. This indicated that primer was able to recognize conservative region of *16s rRNA* gene accurately.

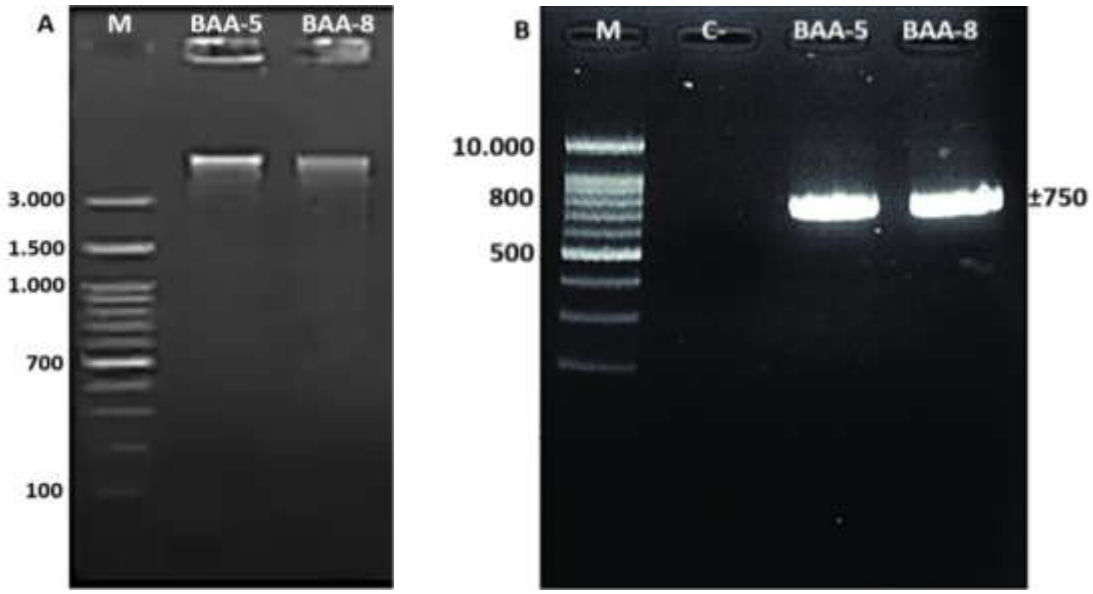

Figure 1. Qualitative Analyses; A = *DNA* isolate electrophoresis, B = *PCR* product amplification using *16s rRNA* gene amplification which produces bands ±750 bp of size, (M: *DNA* marker; C-: negative control, BAA-5 and BAA-8: isolate code of amplified samples)

BAA-5 Isolate

GCATGGTTCTTGGCTGAAAGATGGCGTAAGCTATCGCTTTTGGATGGACCCGCGGCGTATTAGCTA
GTTGGTGAGGTAACGGCTCACCAAGGCGATGATACGTAGCCGAACTGAGAGGTTGATCGGCCACA
TTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACG
CAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTGGA
GAAGAATGGTCGGCAGAGTAACTGTTGTCGGCGTGACGGTATCCAACCAGAAAGCCACGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCG
AGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAAGCGCATCGGAAAC
TGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATA
TGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCATG
GGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAATGCTAGGTGTTGGAG
GGTTTCCGCCCTTCAGTGCCGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGACCGCAAGG
TTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCA
ACGCGAAGAACCTTA

BAA-8 Isolate

CTATACATGCAGTCGAACGAGTTCTGGGGCTAATACATGCAGTCGACGAACTCTGGTATTGATTGG
TGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCC
AGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCC
GAGTTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGG
GTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTG
AGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGA
TGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACA
TATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGG
CGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAAC
TTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAA
CACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAA
ACAGGATTA

Figure 2**.** Sequencing analysis result of lactic acid bacteria isolate from Anna apple cultivar

PCR success also showed that no reactional inhibitors were present on *DNA* extraction phase, such as polysaccharides or polyphenols, commonly found in fruit-derived isolates (Bartoš et al., 2024). This finding strengthened isolate BAA-5 and BAA-8 feasibility for sequencing process, which is essential for definitively determining species and differentiate *EPS* producing strains. Results of sequencing analyses can be seen in Fig. 2.

The *16s rRNA* gene sequencing analysis for isolates BAA-5 and BAA-8 showed that two isolates had conservative motifs which is a common characteristic of *LAB*, especially at the V3-V4 domain which is often used as phylogenetic marker during bacterial identification or phylogeny reconstruction (Olivier et al., 2023).

BAA-5 isolate showed nucleotide patterns that were stable, with GGGAGGCAGCAG motif, a typical characteristic of *Lactobacillaceae* family and is often used as a signature sequence to identify this family (Riesco & Trujillo, 2024). This indicated that isolate BAA-5 might be closely related to *Lactiplantibacillus* genus or other related fermentative bacteria.

Isolate BAA-8 also had the same conservative motif, although showing nucleotide variation on some variable regions, especially V3 dan V4. Variation on said hypervariable domains were important on species or strain separation in *16s rRNA* analysis (Buetas et al., 2024).

This variation pattern showed a phylogenetic difference which allowed isolate BAA-8 to have different species or strain to that of BAA-5, while still being in the *LAB* group. This sequential difference may reflect ecological adaptation or natural variation among strains in *Lactobacillaceae* genus (Olivier et al., 2023).

Table 3.
BLAST Sequence lactic acid bacteria isolate from Anna apple cultivar (*Malus domestic*a var. Anna)

Isolate code	Scientific name	Query cover (%)	E value	Per. ident. (%)	Accession
BAA-5	*Lacticaseibacillus paracasei*	100	0.0	98.68	MZ379454.1
		100	0.0	98.68	MT545052.1
		100	0.0	98.68	OP804282.1
		100	0.0	98.68	MT463826.1
		100	0.0	98.68	MT544967.1
		100	0.0	98.49	PV270180.1
		100	0.0	98.49	PV300337.1
		100	0.0	98.49	MZ379451.1
		100	0.0	98.49	MT464050.1
		100	0.0	98.49	MT505564.1
		100	0.0	98.49	MT515984.1
		100	0.0	98.49	OR083512.1
		100	0.0	98.49	PX311416.1
		100	0.0	98.49	OR362758.1
		100	0.0	98.49	KP165843.1
		100	0.0	98.49	MW392570.1
		100	0.0	98.49	MT613527.1
		100	0.0	98.49	MT463628.1
		100	0.0	98.49	MT544958.1
		100	0.0	98.49	KY287768.1
		100	0.0	98.49	MT473355.1
		100	0.0	98.49	MT510250.1
BAA-8	*Lactiplantibacillus plantarum*	100	0.0	99.61	MT604628.1
		100	0.0	99.74	MT611686.1
		100	0.0	99.61	OM265411.1
		100	0.0	99.74	MT544853.1
		100	0.0	99.49	PQ012471.1
		100	0.0	99.74	MG890194.1
		100	0.0	99.74	MT544889.1
		100	0.0	99.74	MT512153.1
		100	0.0	99.49	MT604683.1
		100	0.0	99.49	OL589473.1
		100	0.0	99.74	MT538472.1
		100	0.0	99.74	MT538527.1
		100	0.0	99.49	OL519107.1
		100	0.0	99.49	MT510468.1
		100	0.0	99.49	MT538464.1
		100	0.0	99.61	MG551252.1
		100	0.0	99.74	MT799877.1
		100	0.0	99.61	KM497500.1
		100	0.0	99.49	MT538790.1
		100	0.0	99.49	OL423285.1
		100	0.0	99.74	KY584253.1
		100	0.0	99.49	ON506122.1

BLAST analysis showed that isolate BAA-5 had a 98.68% similarity towards *Lactiplantibacillus paracasei*, while BAA-8 had a 99.61% similarity towards *Lactiplantibacillus plantarum* (Table 3). E-value score obtained for both isolates was 0.0, which indicated a very high significance and no random match probability.

Lactiplantibacillus paracasei (98.68% similarity towards BAA-5) and *L. plantarum* (99.61% similarity towards BAA-8) have both previously been reported to include strains that possess *EPS*-producing capabilities. Previous studies have reported that *L. plantarum* included productive strain in *EPS* synthesis, especially heteropolysaccharides (*HePS*) (Ouarabi et al., 2025). For example, *L. plantarum* SP8 with *HePS* yield of 280.105 mg/L (Zhang et al., 2020) while *L. plantarum* NTMI05 and *L. plantarum* NTMI20 exhibited *EPS* yield of 0.956 g/L and 0.827 g/L respectively (Imran et al., 2016). Simultaneously, *L. paracasei*, has also been reported to include *EPS* producer strains, such as *L. paracasei* GL1 (460.5 mg/L) (Wang et al., 2022), *L. paracasei* CIDCA 8339 (130–145 mg of *EPS* per liter of fermented kefir milk) (Bengoa, Dueñas, Prieto, Garrote & Abraham, 2023), *L. paracasei* 2333 (2.38 g/L using maltose substrate) (Fuso et al., 2023).

However, it should be noted that *EPS* production is strain dependent and even the same species of *LAB* may have different *EPS*-producing capacity with different strains (Hernández-Figueroa et al., 2025).

Sequence identity percentage is generally required to be above 97% for *LAB* species identification (Riesco & Trujillo, 2024). Therefore, this sequencing result gave a strong basis that both isolates were strong and valid candidates for further study of *EPS* development from Anna apple *LAB*. *BLAST* analysis result showed that isolate BAA-5 and BAA-8 had high sequence similarity with *L. paracasei* dan *L. plantarum* respectively, this was further confirmed from a consistent clustering on the same clade of phylogenetic tree based on *16s rRNA* gene (Fig. 3).

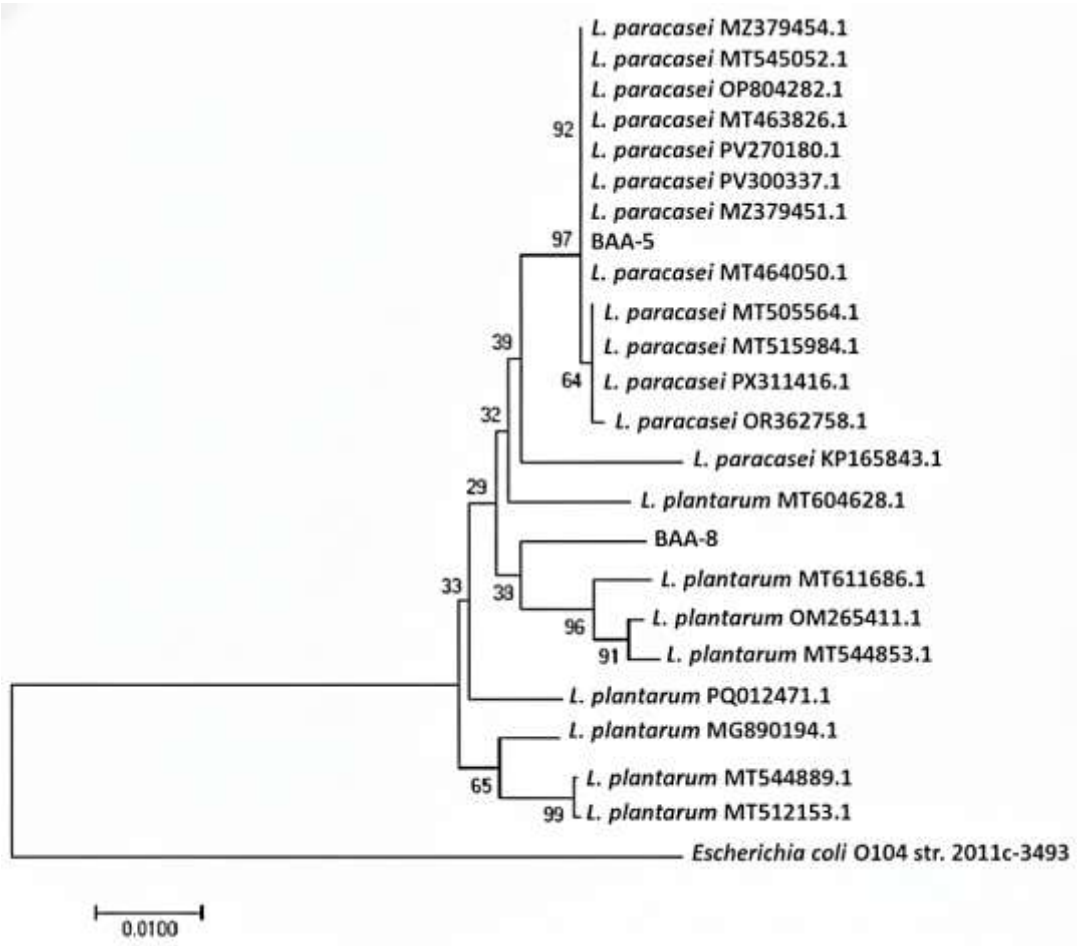

Figure 3. Phylogenetic reconstruction

BAA-5 isolate was consistently grouped *L. paracasei* clade. This isolate is located in the same cluster as some other reference sequences of *L. paracasei* from GenBank, such as MT464050.1, MT505564.1, and MT515984.1 with a relatively high bootstrap support value of 97%. This high bootstrap value shows strong reliability of BAA-5 isolate placement in *L. paracasei* species and reflects high genetic sequence similarity to said species. Moreover, BAA-5 had a 98.7% *16s rRNA* sequence similarity to *L. paracasei*, which again indicated that the isolate can be identified as having a same species (Buetas et al., 2024).

Conversely, isolate BAA-8 was clearly classified in *Lactiplantibacillus plantarum* clade. This species showed a strong phylogenetic relation with reference sequences such as *L. plantarum* MT611686.1, OM265411.1, and MT544853.1, with a 96–99% bootstrap support value. Although this species has been known to have a relatively high genetic diversity, however cluster consistency was still found during *16s rRNA* gene analysis (Cen et al., 2020).

Bootstrap support values greater than 70% are widely considered indicative of reliable phylogenetic support, as adopted from Hillis and Bull (Lemoine & Gascuel, 2024). Furthermore, bootstrap values exceeding 90% are generally accepted as representing strong support (Lozano-Fernandez, 2022). These criteria confirm the identity of isolates BAA-5 and BAA-8 as *Lacticaseibacillus paracasei* and *Lactiplantibacillus plantarum*, respectively.

Branching of *L. paracasei* and *L. plantarum* clades was clearly separated, although some internal nodes had a low to medium bootstrap support value (29–65%). This has been seen as a common phenomenon during *16s rRNA* gene-based phylogenetic analyses in the *Lactiplantibacillus* genus, given a high gene conservation degree which resulted in a limited species resolution between closely related species (Swanson et al., 2020; Li et al., 2024). Thus, it can be concluded that even though low bootsrap support values were found on some internal clusters, the separation of major clades weres till valid and acceptable taxonomically.

Overall, *16s rRNA* gene phylogenetic analyses had successfully confirmed that isolate BAA-5 had high sequential similarities with *L. paracasei*, while isolate BAA-8 had high sequential similarities with *L. plantarum*. High bootstrap support value on main clades strengthened the validity of this molecular identification. Additionally, these findings provided strong scientific basis for future studies, especially in correlation to functional potential, metabolic activity, and application of both isolates in food biotechnology.

CONCLUSION

This study successfully isolated indigenous Lactic Acid Bacteria (*LAB*) from Anna apple cultivar (*Malus domestica* var. Anna) which held a potential exopolysaccharide (*EPS*) producer. From eight isolated microorganism, isolates BAA-5 and BAA-8 showed the highest *EPS* yield, at 3.350 mg/L and 3.050 mg/L respectively. Molecular identification based on *16s rRNA* gene sequencing confirmed that isolate BAA-5 was closely related to *Lacticaseibacillus paracasei*, while isolate BAA-8 was closely related to *Lactiplantibacillus plantarum*. These results showed that both isolates can potentially be developed as functional agents in food industry applications, especially as a source of natural *EPS*. Further studies should focus on *EPS* physicochemical characterization, application on fermented food products, effects on food products, as well as isolate safety and stability evaluation as *LAB* starter culture.

AUTHOR CONTRIBUTIONS

Conceptualization, methodology, N.K. and E.Z.; Investigation, data analysis, N.K.; Validation, E.Z., Methodology, X.Y.Z. and A.B.C.; Data collection, E.R.L. and U.U., Visualization, E.R.L., Resource provision, U.U., Drafting of the original manuscript, N.K. and E.R.L., Writing-review and editing, E.Z.; Supervision, N.K.

DATA AVAILABILITY STATEMENT

Data contained within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE MANUSCRIPT PREPARATION PROCESS

The authors used ChatGPT during the preparation of this work to assist with language refinement, grammar checking, and improving the clarity of the manuscript. The authors carefully reviewed and edited the content as needed and take full responsibility for the accuracy and integrity of the published article.

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IZOLACIJA I IDENTIFIKACIJA BAKTERIJA MLEČNE KISELINE IZ JABUKE SORTE ANNA (MALUS DOMESTICA *VAR. ANNA)* KAO POTENCIJALNIH PROIZVOĐAČA EGZOPOLISAHARIDA

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Sažetak: Egzopolisaharidi predstavljaju vrstu polisaharida koje različite bakterije proizvode putem ćelijskog izlučivanja. Egzopolisaharidi koje sintetizuju bakterije mlečne kiseline poseduju fizičko-hemijska svojstva uporediva sa pojedinim biljnim polisaharidima, kao što su guar guma ili pektin. Zbog toga imaju značajnu funkcionalnu ulogu u prehrambenoj industriji, gde se mogu koristiti kao zamena za gluten u pekarskim proizvodima, alternativni zgušnjivači i stabilizatori, ali i kao komponente sa potencijalnim zdravstvenim benefitima. U poslednje vreme, sojevi bakterija mlečne kiseline sposobni za produkciju egzopolisaharida privlače značajnu pažnju kako u naučnim istraživanjima, tako i u komercijalnoj primeni. Međutim, izolacija i karakterizacija ovih mikroorganizama iz autohtonih voćnih izvora i dalje su nedovoljno istražene, uprkos velikoj raznovrsnosti takvog voća i specifičnim mikrobnim zajednicama koje ih naseljavaju. Sorta jabuke Anna predstavlja autohtonu sortu jabuke iz regiona Malang u Indoneziji, poznatu po visokom sadržaju šećera, umerenoj kiselosti, kao i prisustvu različitih vitamina i minerala pogodnih za rast bakterija mlečne kiseline. Cilj ovog istraživanja bio je izolovati i identifikovati endofitne bakterije mlečne kiseline iz mezokarpa jabuke sorte Anna (*Malus domestica* var. Anna) kao potencijalne proizvođače egzopolisaharida. Izolacijom i purifikacijom dobijeno je ukupno osam izolata, označenih kao BAA-1 do BAA-8. Izolati su dalje karakterisani primenom Gramovog bojenja, bojenja endospora, testa katalazne aktivnosti, testa fermentacije ugljenih hidrata i određivanjem prinosa egzopolisaharida. Rezultati su pokazali da su izolati BAA-5 i BAA-8 ostvarili najveći prinos egzopolisaharida, od 3350 mg/L, odnosno 3050 mg/L. Dalja molekularna identifikacija pokazala je da izolat BAA-5 ima 98,68% sekvencne sličnosti gena sa vrstom *Lacticaseibacillus paracasei*, dok izolat BAA-8 pokazuje 99,74% sličnosti sekvence sa vrstom *Lactiplantibacillus plantarum*. Na osnovu dobijenih rezultata, navedeni izolati imaju potencijal za dalji razvoj kao funkcionalni agensi u primenama prehrambene industrije.

Ključne reči: *egzopolisaharidi, bakterije mlečne kiseline, sorta jabuke Anna, izolacija bakterija, prehrambena industrija*

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