Jurnal Biodjati 10 (2): 361–377, November 2025

e-ISSN : 2541-4208
p-ISSN : 2548-1606

JURNAL BIODJATI

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Bioprospecting of Potential Microorganisms as Agents for Biodiesel Production from Sago Pulp Waste Feedstock

Alice Chaela Lethulur[1], Cecilia Anna Seumahu[*2], Edwin Thomas Apituley[3]

Received: July 09, 2025
Revise from: August 01, 2025
Accepted: November 11, 2025

DOI: 10.15575/biodjati.v10i2.48431

[1,2]Department of Biology, Faculty of Science and Technology, Universitas Pattimura, Jl. Ir. M. Putuhena, Ambon, 97233, Indonesia. [3]Department of Biotechnology, Faculty of Science and Technology, Universitas Pattimura, Jl. Ir. M. Putuhena, Ambon, 97233, Indonesia.

e-mail:
[1]alicelethulur02@gmail.com
*[*2]ceciliaseumahu@gmail.com*
[3]edwinapt2017@gmail.com

**Corresponding author*

Abstract. *The rising global energy demand and environmental issues associated with fossil fuel use have accelerated interest in renewable alternatives, such as biodiesel. This study aimed to provide a preliminary screening of potential indigenous microorganisms among isolates with high ability to saccharify lignocellulosic sago pulp waste, an abundant, non-food biomass in Maluku, and to accumulate lipids. Laboratory experiments were conducted to isolate, characterize (macro and microscopically), saccharify, and accumulate lipids. Bacterial isolates were evaluated for saccharification ability using two parameters: the cellulolytic index (CI), determined by the clear zone on solid CMC media, and reducing sugar production in modified liquid CMC media using pretreated sago waste, measured by Benedict's test. Yeast and mold isolates were assessed for lipid accumulation through Sudan Black B staining and gravimetric lipid extraction under nitrogen-limited conditions. Results identified four bacterial isolates capable of saccharification, with isolate BAS 1B showing the highest cellulolytic index, and all isolates produced detectable reducing sugars by Benedict's test. All yeast and mold isolates accumulated intracellular lipids, with YAS 2 and KAS 2 isolates accumulating the highest lipid compared to the positive control (Saccharomyces cerevisiae). As this study was based on a single-point observation (n=1), the results are exploratory and should be interpreted cautiously. Nevertheless, isolates BAS 1B, YAS 2, and KAS 2 show promising potential for further biodiesel-related research using lignocellulosic waste. Further studies with replication and quantitative validation are required prior to any industrial consideration.*

Keywords: *accumulation, biodiesel, cellulose, lipid, saccharification, sago*

Citation
Lethulur, A. C., Seumahu, C. A., & Apituley, E. T. (2025). Bioprospecting of Potential Microorganisms as Agents for Biodiesel Production from Sago Pulp Waste Feedstock. *Jurnal Biodjati*, 10(2), 361–377.

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INTRODUCTION

Rapid population growth and industrial development have driven rising energy demand, posing a serious challenge for many nations. As the population grows, so does energy consumption. Indonesia, one of the most populous countries in the world with over 281 million people (Badan Pusat Statistik, 2024), consumes significant amounts of energy annually. Like many other countries, Indonesia continues to rely on fossil fuels as the primary energy source. Unrestricted use of fossil fuels can increase atmospheric levels of Greenhouse Gases (GHGs), further contributing to climate change, including extreme weather and rising sea levels (Filonchyk et al., 2024).

Renewable energy development has become a priority in response to this urgent energy and environmental crisis. One promising renewable energy source as an alternative to fossil fuel is biodiesel due to its biodegradability, non-toxicity, low emissions, and compatibility with existing diesel engines. Biodiesel has garnered increasing attention in Indonesia, yet its production remains heavily dependent on Crude Palm Oil (CPO) and its derivatives as the feedstock. Massive oil palm plantations in Indonesia increased CPO production by 30.6 million tons in 2016 (Yunsari et al., 2019). Besides its advantages of high oil content and availability, CPO as a biodiesel feedstock has been linked to several issues, including intensive deforestation, increased emissions, declining soil and water quality, and biodiversity loss (Hidayah, 2025). Over the past two decades, land clearing for oil palm plantations has been Indonesia's primary driver of forest loss (Austin et al., 2019). About 174 million tons of GHGs are emitted when forests are converted to oil palm plantations (Carlson et al., 2012).

Exploring new alternative organic materials as substitutes for biodiesel feedstock is necessary to face this problem and reduce CPO's dependency. One promising substitute is sago pulp, which is rich in organic matter and a byproduct of sago processing, commonly discharged by residents and used as a plant fertilizer (Andayani et al., 2024) or livestock feed (Syadik et al., 2022). This study used sago pulp waste as an organic material for biodiesel feedstock, enabling its transformation into a high-value energy source. A previous study successfully produced bioethanol from sago pulp (Numberi, 2022), but it has limitations, including lower compatibility with diesel engines than biodiesel. This study addresses this research gap by shifting the focus from bioethanol to biodiesel production using sago pulp as a substrate.

Specifically, this study aims to provide a preliminary screening of indigenous microorganisms within a group of isolates with high saccharification and lipid accumulation abilities. Intracellular lipids are formed in response to limited nitrogen in the environment and to a sufficient or redundant carbon source (Garay et al., 2014). Previous studies have successfully identified cellulolytic bacteria (Faizah et al., 2020) and examined fungal activity in sago pulp waste (Asben & Irawadi, 2013), but did not investigate lipid production for biodiesel. Additionally, Hebbale et al. (2019) demonstrated microbial hydrolysis of seaweed for biofuels, yet studies applying these insights to sago pulp remain limited.

MATERIALS AND METHODS

Sago pulp waste (sample) was collected from a sago production site in Hutan

Sago Rutong Village, Ambon, Moluccas, during February 2025. From February to April 2025, indigenous microorganisms were isolated from the sample, saccharified, accumulated, and lipid extracted at the Microbiology Laboratory, Pattimura University. The materials used in this study included a range of laboratory instruments and biological reagents. Equipment comprised Petri dishes, Olympus BX51 microscope, analytical balance, micropipettes, laminar airflow cabinet, Erlenmeyer flasks, test tubes, Boeco OS-10 orbital shaker, Eppendorf 5702 centrifuge, bunsen burner, Memmert oven, hot plate, Boeco MSH 300 magnetic stirrer, inoculating loops, sieves, grinding apparatus, microscope slides and coverslips, separatory funnel, beakers, measuring cylinders, and an autoclave. The study utilized sago pulp waste and pretreated sago waste collected from a sago production facility in Rutong Village, Maluku Province, Indonesia. Reagents and media included phosphate buffer, Nitrogen Limited Media (NLM), Nutrient Agar (NA), Carboxymethyl Cellulose (CMC) media, Potato Dextrose Agar (PDA), and various biological stains such as Sudan Black B, Congo Red, Gram stain (crystal violet and safranin), and Lugol's iodine. Other chemicals used were 95% ethanol, propylene glycol, Benedict's reagent, distilled water (aquadest), chloroform-methanol solvent, and 1 M NaCl. The microbial cultures used consisted of bacterial, yeast, and mold isolates, as well as Saccharomyces cerevisiae as a positive control grown in NLM and a negative control grown in PDA media.

Data Analysis

All quantitative measurements in this study—including the cellulolytic index, wet biomass weight, and lipid yield—were obtained as single-point observations (n=1) without technical or biological replication. This approach reflects the research's preliminary nature, which aimed to screen for potential indigenous microorganisms with high saccharification and lipid-accumulating abilities from sago pulp waste. Because each isolate was measured only once, statistical parameters such as standard deviation (SD) and standard error (SE), as well as inferential statistical tests, were not applicable. Accordingly, all values are presented descriptively as single observations. The graphical visualization of total lipid content and the tables for wet biomass and the cellulolytic index were generated directly from these single-observation data and therefore do not include SD values.

Study Limitations

This study provides preliminary screening data, and all measurements were conducted as single-point observations. Consequently, statistical variation—including standard deviation, standard error, and inferential statistics—could not be assessed. Hence, the study's findings should be interpreted with caution, as the lack of replication limits their reproducibility. Replication experiments—whether technical or biological—are needed in future studies to validate and strengthen the quantitative outcomes reported here.

Medium Preparation

Merck Nutrient Agar (NA) and Oxoid Potato Dextrose Agar (PDA) were used in this study as instant, commercially prepared media. Each medium was prepared by weighing the appropriate amount according to the manufacturer's instructions (20 g/L for NA and 39 g/L for PDA), then dissolving each medium in distilled water to a final volume of 150 mL. Carboxymethyl Cellulose or CMC

(in solid form) medium for saccharification assays was prepared manually by dissolving the following components (in g/L): KH_2PO_4 1.0, NaCl 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, $FeSO_4 \cdot 7H_2O$ 0.01, $(NH_4)_2SO_4$ 0.5, CMC 10, and bacteriological agar 15, in distilled water (Hamka et al., 2016). Modified liquid CMC media used the same components as CMC solid media, but did not add bacteriological agar and replaced CMC with pretreated sago pulp (oven-dried at 100°C, sterilized, ground, and sifted) as the first carbon source. Nitrogen Limited Media (NLM in liquid form) for lipid accumulation studies was prepared by mixing (in g/L): KH_2PO_4 0.75, Na_2HPO_4 1.5, $MgSO_4 \cdot 7H_2O$ 0.4, $CaCl_2 \cdot 2H_2O$ 0.4, $FeSO_4 \cdot 7H_2O$ 0.01, yeast extract 1.5, NH_4NO_3 0.285, and glucose 40 (Thakur et al., 1989). All of the media were sterilized in an autoclave at 121°C for 15 minutes. After cooling to approximately 50°C, the media were aseptically poured into Petri dishes (solid media) or Erlenmeyer flasks (liquid media) under a laminar airflow cabinet.

Microorganism Isolation and Characterization

Microorganisms were isolated from sago pulp waste using the serial dilution method. One gram of sago pulp was added to 9 mL of phosphate buffer solution to obtain a 10^{-1} dilution, followed by serial dilutions up to 10^{-9}. Subsequently, 0.1 mL from each dilution was spread onto Nutrient Agar (NA) plates for bacterial isolation and onto Potato Dextrose Agar (PDA) plates for yeast and mold isolation. Bacterial isolates were incubated for 2 days, while yeast and mold isolates were incubated for 7 days, both incubated at room temperature. All isolates were characterized macroscopically and microscopically. For bacterial isolates, macroscopic characteristics included colony shape, margin, elevation, color, and size, while microscopic characteristics included cell shape and Gram staining. Yeast isolates were evaluated macroscopically for colony texture, color, surface appearance, elevation, and margin, and microscopically for cell shape and reproductive structures. Mold isolates were assessed macroscopically for colony color (top and reverse), shape, texture, margin, zonation, and radial lines, and microscopically for reproductive structures. All conducted experiments in this study are preliminary exploratory screening, and future statistical experiments are required to provide rigorous quantitative validation.

Saccharification Assay

Bacterial isolates obtained from sago pulp waste were used as saccharification agents and incubated in both solid and modified liquid CMC media. Isolate saccharification abilities were evaluated by using two parameters, which are Cellulolytic Index (CI) determined by clear zone formation on solid CMC media, and the reducing sugar production measured by Benedict's reagent in modified liquid CMC media. On solid CMC agar, isolates were incubated for 7 days at room temperature, followed by Congo red staining to detect halo/clear zones. Based on the clear zone, the Cellulolytic Index (CI) can be further measured using the following formula (Sutari, 2020).

$$CI = \frac{\text{clear zone diameter} - \text{colony diameter}}{\text{colony diameter}}$$

Based on the clear zone results, a bacterial starter culture was prepared before incubation in modified liquid CMC media. Three mL of selected potential isolates from starter media were further added to 27 mL modified liquid CMC media containing pretreated sago pulp and incubated for 14 days at room temperature. Saccharification efficiency in the modified liquid media was assessed using

Benedict's reagent to detect reducing sugars, and color changes resulting from the reaction were compared to a standard glucose color scale(Pratiwi and Hufni, 2020).

Lipid Accumulation Assay

Due to limited purification equipment, reducing sugars from the saccharification stage were not used directly; instead, glucose monohydrate was used as a carbon source in the lipid accumulation medium. Mold and yeast isolates, along with *Saccharomyces cerevisiae* as a control, served as lipid-accumulating agents. Each yeast isolate was first cultured in Nitrogen-Limited Medium (NLM) to prepare a 10 mL starter, which was then transferred to 90 mL of fresh NLM. Mold isolates were directly inoculated into 100 mL of NLM without starter preparation. All cultures were incubated for 7 days at room temperature in 250 mL Erlenmeyer flasks on a rotary shaker (150 rpm). After incubation, cells were harvested by centrifugation (4000 rpm, 15 min), then stained with Sudan Black B to observe intracellular lipid accumulation under a microscope (Tampitak et al., 2015). Isolates showing visible lipid accumulation were selected for lipid extraction and quantification.

Extraction and Total Lipid Estimation Gravimetrically

Lipid extraction was performed using a modified version of the method described by Stepanus (2023), with solvent volumes adjusted based on the available biomass. In the original protocol, 6 grams of dry biomass were extracted with 30 mL of a chloroform: methanol mixture (1:2, v/v), corresponding to a solvent-to-biomass ratio of 5 mL/g. In this study, the solvent volume was adjusted in the same proportion. Specifically, 5 mL of chloroform: methanol mixture (2:1, v/v) was used per gram of wet biomass in the first extraction step.

The mixture was stirred for 15 minutes using a magnetic stirrer. Following the initial extraction, two subsequent steps were performed. First, 1.67 mL of chloroform per gram of wet biomass was added, and the mixture was mixed for 15 minutes. Then, an equal volume (1.67 mL/g biomass) of distilled water was added and mixed for 15 minutes. After the final phase separation, the chloroform layer containing the lipids was collected, and the solvent was evaporated. The total lipid accumulation was calculated on a wet basis using the following formula (Stepanus, 2023).

$$\text{yield wet basis (\%)} = \frac{\text{weight of extracted lipid}}{\text{Initial wet weight of biomass}} \times 100$$

RESULTS AND DISCUSSION

Isolation and Characterization of Indigenous Microorganisms

Nine bacterial isolates (BAS) were successfully isolated from sago pulp waste collected in Rutong Village. Colony shape, edge, color, elevation, and size are the scope of macroscopic characteristics. Meanwhile, the observation of microscopic characteristics of BAS isolates includes Gram staining and cell shape under a microscope. Macroscopic observations revealed that all BAS isolates exhibited circular colonies with varying edge types, elevations, sizes, and pigmentation ranging from milky white to yellow, as shown in Table 1.

In addition, Gram staining revealed the presence of both Gram-negative and Gram-positive bacterial isolates in the sample, as shown in Table 2. Most of the isolates are Gram-positive with bacil cell shapes. Four

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BAS isolates (BAS 1B, BAS 2A, 2B, and 3A) showed saccharification ability among all of the isolates. Based on microscopic characteristics, four potential BAS isolates showed a bacilli shape and Gram-positive staining, suggesting they are *Bacillus* sp.

Table 1. Results of the macroscopic characteristics of BAS colony isolates

BAS Isolate	Shape	Edge	Elevation	Pigmentation	Size
BAS 1A	Circular	Entire	Convex	Milky white	Punctiform-moderate
BAS 1B	Circular	Undulate	Convex	Yellow	Punctiform-small
BAS 2A	Circular	Entire	Convex	Yellow	Punctiform-small
BAS 2B	Circular	Entire	Convex	Yellow	Small
BAS 3A	Circular	Entire	Convex	Yellow	Punctiform-small
BAS 3B	Circular	Entire	Pulvinate	Milky white	Large
BAS 4A	Circular	Entire	Raised	Clear white	Punctiform-small
BAS 4B	Circular	Rhizoid	Convex	Milky white	Large
BAS 5	Circular	Rhizoid	Convex	Milky white	Small-large

Table 2. Results of the microscopic characteristics of BAS colony isolates

BAS Isolate	Gram*	Cell shapes
BAS 1A	-	Cocci
BAS 1B	+	Bacilli
BAS 2A	+	Bacilli
BAS 2B	+	Bacilli
BAS 3A	+	Bacilli
BAS 3B	-	Cocci
BAS 4A	+	Bacilli
BAS 4B	-	Cocci
BAS 5	+	Bacilli

*) Gram positive (+), Gram negative (-)

The common characteristics of *Bacillus* sp. are rod-shaped cells, sometimes in chains, and the ability to produce endospores (Breed et al., 1957), with diameters of 0.4 to 1.8 µm and lengths of 0.9 to 10.0 µm (De Vos et al., 2009). In line with Cahya et al. (2022), who stated that *Bacillus* sp is classified as gram-positive with bacil or rod-shaped cells. The genus Bacillus is among those that have been successfully isolated from sago pulp waste. A previous study successfully isolated several cellulolytic bacteria, including *Bacillus licheniformis* and *Bacillus cereus*, from a similar source: sago pulp waste (Faizah et al., 2020).

The indigenous microorganisms successfully isolated were yeast and mold. Isolates of sago pulp yeast (YAS) and sago pulp mold (KAS) in this study act as lipid-accumulating agents. In addition, macroscopic and microscopic characteristics of these lipid-accumulating isolates were observed.

Macroscopic observations of yeast isolates include texture, color, surface, elevation, and margin of each colony, as shown in Table 3. Macroscopic observation revealed that most isolates have a butyrous texture, a milky-white color, a glistening, smooth surface, and umbonate and entire. Meanwhile, microscopic characterization of yeast isolates includes cell shape and asexual reproductive structure, as shown in Figure 1. Yeasts are commonly 2-5 times larger than bacteria, with diameters ranging from 2 to 5 µm, but some yeasts can reach 40 µm in diameter (Walker et al., 2002). One feature of yeast is pseudohyphae, branching filamentous cells that resemble a chain of elongated, narrow cells (Mukaremera et al., 2017).

Pseudohyphae are also elliptical, have a narrowing at the cell junction, and many branches (Kadosh & Mundodi, 2020). This is in line with Figure 1 B. where there are elliptical cells that are long and branching to form pseudohyphae structures resembling hyphae in molds. Isolate YAS 1 also showed sexual reproductive structures, namely shmoo and mating cells, when incubated in nitrogen-poor media, as shown in Figure 1E and F. Shmoo are cells that grow at one end and are essential for cell fusion and zygote formation during mating (Goldenbogen et al., 2016). This is because, when grown on nutrient-deficient media such as NLM, yeasts tend to form sexual reproductive structures.

This aligns with Chavez et al. (2024), who stated that, in addition to controlling cell growth, nutrients determine which developmental program a cell follows. Whereas, isolate YAS 2 exhibited a butilliform to oval cell morphology with a unipolar budding type, as shown in Figure 1C. In contrast, isolate YAS 3 displayed an ogival-to-oval cell shape, characterized by unipolar budding, as shown in Figure 1D. Figure 1A shows the control strain (S*accharomyces cerevisiae*) that has circular cells with a multilateral budding pattern. Budding is an asexual reproductive structure that forms in response to environmental stimuli. As long as nutrients remain available in the environment, yeast cells can continue to produce buds through mitotic division (Chavez et al., 2024). Yeasts are classified into unipolar, bipolar, and multilateral types based on their budding patterns. Unipolar budding is characterized by bud formation at only one pole of the cell; bipolar budding involves budding at both poles; and multilateral budding is independent of cell polarity (Chavez et al., 2024).

Table 3. Macroscopic characterisation results of sago pulp yeast isolates

Yeast Isolates	Texture	Color	Surface	Elevation	Margin
YAS 1	Butyrous	White-beige	Glistening & sectored	Umbonate	Entire
YAS 2	Butyrous	Milky-white	Glistening & smooth	Umbonate	Entire
YAS 3	Mucoid	Milky-white	Glistening & smooth	Umbonate	Entire
Control	Butyrous	Milky-white	Glistening & smooth	Convex	Entire

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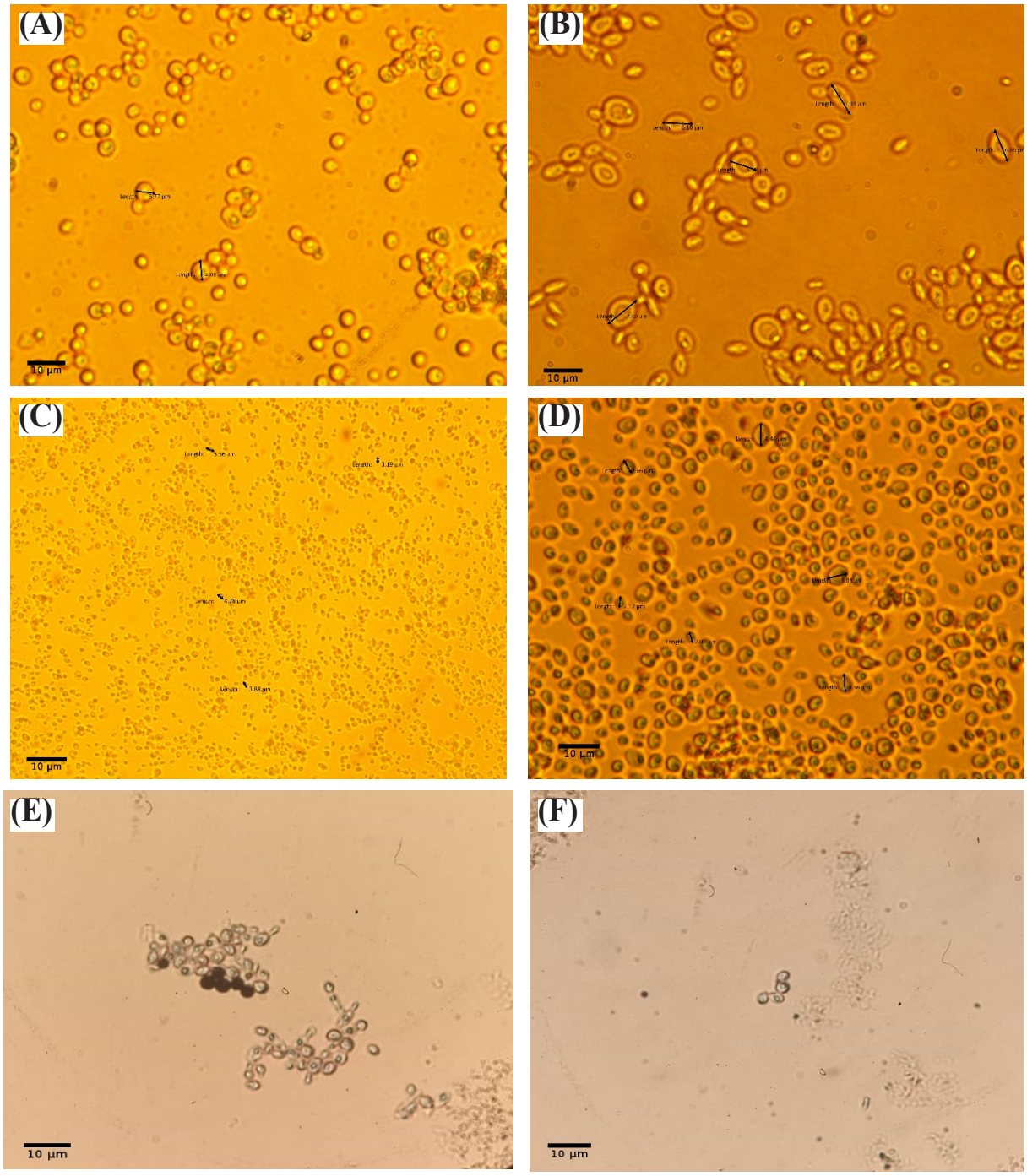

Figure 1. Microscopic characterization results of the YAS isolate and control microorganism (A: Multilateral buds of *S. cerevisiae* with circular cell shape, at 1000x magnification, B: Pseudohyphae and chlamydospores of isolate YAS 1 with cylindrical-oval cell shape, at 1000x magnification, C: Unipolar buds of isolate YAS 2 with butiliform-oval cell shape, at 400x magnification, D: Unipolar buds of isolate YAS 3 with ogival-oval cell shape, at 1000x magnification, E: shmoo structure of YAS 1, 1000x magnification, F: cell mating of YAS 1, 1000x magnification).

Table 4. Macroscopic characterization results of sago pulp mold isolates

Colony characteristic	KAS 1 Isolate	KAS 2 Isolate
Surface colony colour	White hyphae and greenish-yellow spores	White hyphae and beige spores
Reverse colony colour	Yellowish white	Yellow
Shape	Circular	Circular
Texture	Cottony	Powdery
Margin	Filamentous	Entire
Zonation	Exist	Exist
Radial Line	Exist	Exist

The growth of the mold isolates also showed several characteristics, observed both macroscopically and microscopically. Macroscopic characterization of mold isolates includes surface colony color, reverse colony color, shape, texture, margin, zonation, and radial lines, as shown in Table 4.

The microscopic characterization of the mold isolates observed focused on the reproductive structure, as shown in Figure 2. Both isolates showed branching conidiophores. The branched conidiophores observed on both KAS isolate cells can be classified as Gliocladium based on the structural similarities described by Barnett and Hunter (1998). This finding was supported by Asben and Irawadi (2013), who successfully isolated several fungi, including the genus Gliocladium, from sago pulp waste. Gliocladium is a genus distributed worldwide and generally isolated from plant litter (Kidd et al., 2016).

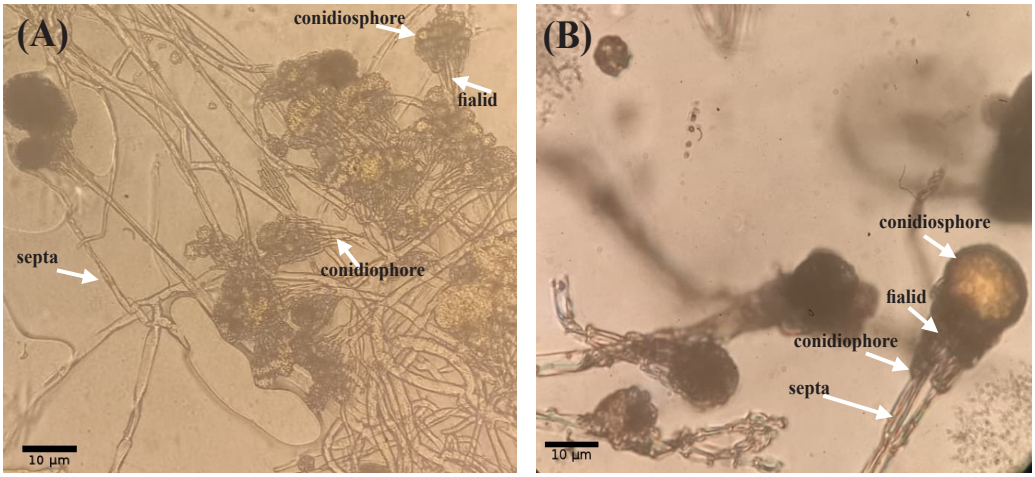

Figure 2. Microscopic characterization results of mold isolates (A: KAS 1 isolate, conidiophore structure, at 1000x magnification, B: KAS 2 isolate, conidiophore structure, at 1000x magnification)

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Saccharification Ability Performed by BAS Isolates

As mentioned before, four potential BAS isolates are BAS 1B, 2A, 2B, and 3A. Saccharification is a process that uses microorganisms to hydrolyse cellulose into glucose using cellulase enzymes (Putra and Sanjaya, 2020). In this study, saccharification ability was evaluated using two parameters: the cellulolytic index (CI), determined by the clear zone formed on solid CMC media, and reducing sugar production measured by Benedict's test in modified liquid CMC media.

The cellulolytic index of those isolates was indicated by the formation of a halo or clear zone on solid CMC medium after Congo red staining, as shown in Figure 3. The cellulolytic index values were calculated as the halo zone diameter divided by the colony diameter.

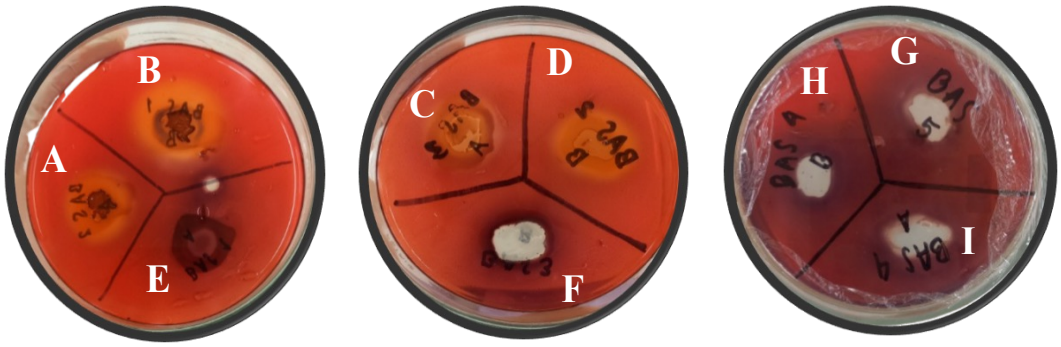

Figure 3. Clear zones form on solid CMC medium as evidence of saccharification by bacterial isolates (A: clear zone of BAS 2A isolate, B: clear zone of BAS 1B isolate, C: clear zone of BAS 3A isolate, D: clear zone of BAS 2B isolate, E-I: no clear zone form)

Table 5 showed that isolate BAS 1B had the highest cellulolytic index value (1.78) among all isolates. Isolates with a cellulolytic index >2.0 are categorized as high, 1.0–2.0 as moderate, and ≤1.0 as low, based on the classification proposed by Choi et al. (2005). According to the data in Table 5, BAS 1B and 2A demonstrate a moderate cellulolytic index, BAS 3A falls near the threshold for moderate, and BAS 2B is classified as a low cellulolytic index.

Further saccharification ability was validated by using Benedict's test, which confirms the four potential isolates. The pre-treated sago pulp, which served as the primary carbon source in the modified liquid CMC medium, was utilized by the four potential isolates, which transformed it into reducing sugar. Figure 4 shows color changes from blue to yellowish-green and greenish-yellow compared to a control positive (red–glucose solution) and a control negative (blue–distilled water).

These color shifts can be further interpreted by comparing them to a standard glucose color gradient (Pratiwi and Hufri, 2020), where increasing glucose concentrations typically result in a transition from blue (low or no glucose) through greenish tones to red (high glucose), indicating varying levels of glucose presence in the tested samples. Overall, this result indicated the presence of reducing sugars at 0.5-1.5%, suggesting that pre-treatment of sago pulp was successful in degrading it into simple sugars such as glucose.

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Table 5. Cellulolytic index of potential sago pulp bacterial isolates

BAS Isolate	Diameter (cm)*		Cellulolytic Index*
	Halo/clear zone	Colony	
BAS 1B	2.85	1.025	1.78
BAS 2A	2.52	0.950	1.65
BAS 2B	2.50	1.350	0.85
BAS 3A	2.32	1.125	1.06

*) All values represent single-point observation (n=1), no replication; therefore standard deviation could not be assessed.

Figure 4. Saccharification test results using Benedict's reagent

Lipid Accumulation Ability

Yeast and mold isolates were used to accumulate lipid under nitrogen-limited conditions. The result showed that all isolates were able to accumulate lipid. This accumulation was qualitatively assessed using Sudan Black B staining, following the standardized protocol (Tampitak et al., 2015), with a modification (without counterstaining), as shown in Figures 5 and 6. The stained preparations were observed under a light microscope at 1000× magnification with oil immersion. Lipid droplets appeared as light blue to dark blue or black inclusions in the cytoplasm.

All five isolates (YAS 1-3 and KAS 1-2) and the positive control (S. cerevisiae grown in NLM liquid medium), that were grown in NLM, showed intracellular lipids stained from light to dark blue in Figure 5A, 5B, 5C, 5D, 6A, & 6C, in contrast to the negative control in Figure 6B (S. cerevisiae grown in PDA solid media), which showed no lipid staining. Gravimetric analysis was conducted to quantify lipid accumulation using the Bligh and Dyer method, adjusted for wet biomass as described in a previous study by Stepanus (2023). Table 6 shows the wet-weight biomass and the weight of the extraction yield after solvent evaporation.

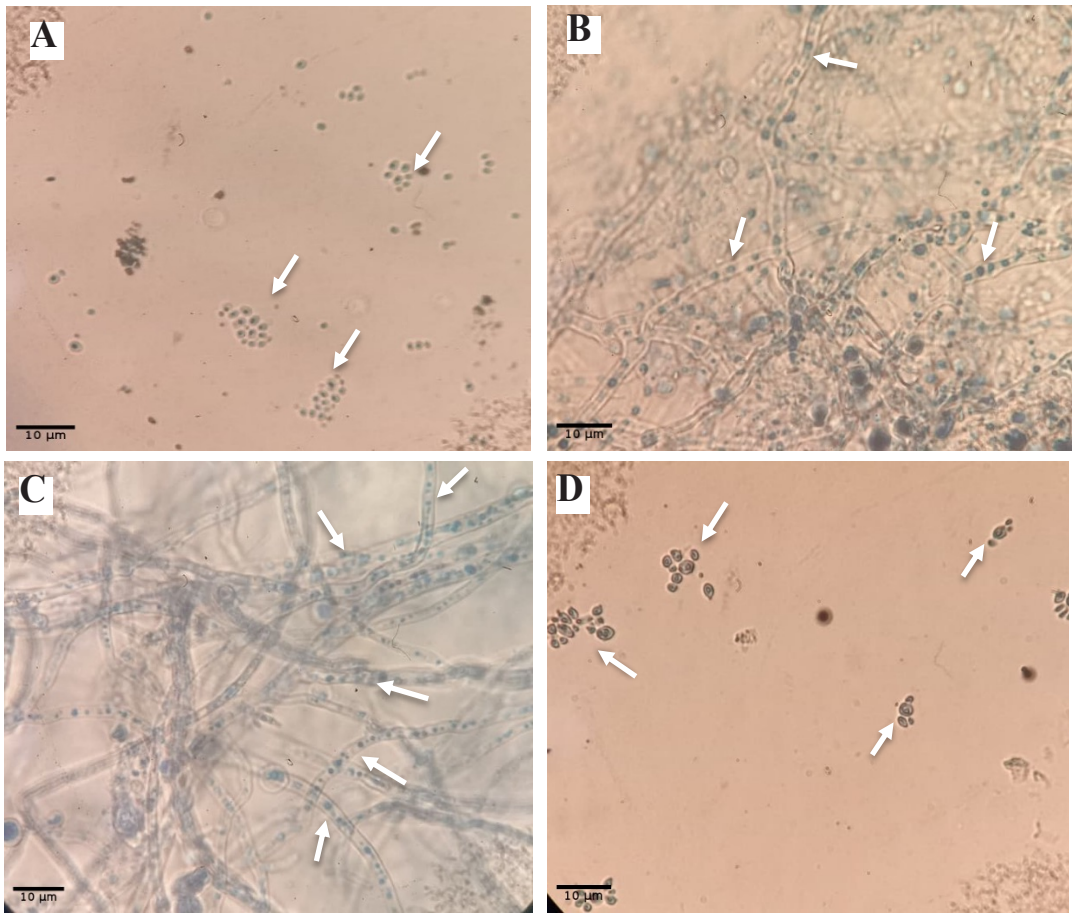

Figure 5. Staining of accumulated lipids with Sudan Black B with A: lipids stained in cells of YAS 3 isolate at 1000x magnification, B: lipids stained in cells of KAS 1 isolate at 1000x magnification, C: lipids stained in cells of KAS 2 isolate at 1000x magnification, D: lipids stained in cells of YAS 2 isolate at 1000x magnification.

Table 6. Weighing of wet biomass and the extraction yield of each isolate

Isolates	wet weight biomass (g)*	Extraction weight (g)*
KAS 1	7.8111	0.0375
KAS 2	5.6475	0.0352
YAS 1	2.9654	0.0169
YAS 2	1.7034	0.0108
YAS 3	3.7141	0.0123
Positive control	2.4013	0.01768

*) All values represent single-point observation (n=1), no replication; therefore, standard deviation could not be assessed

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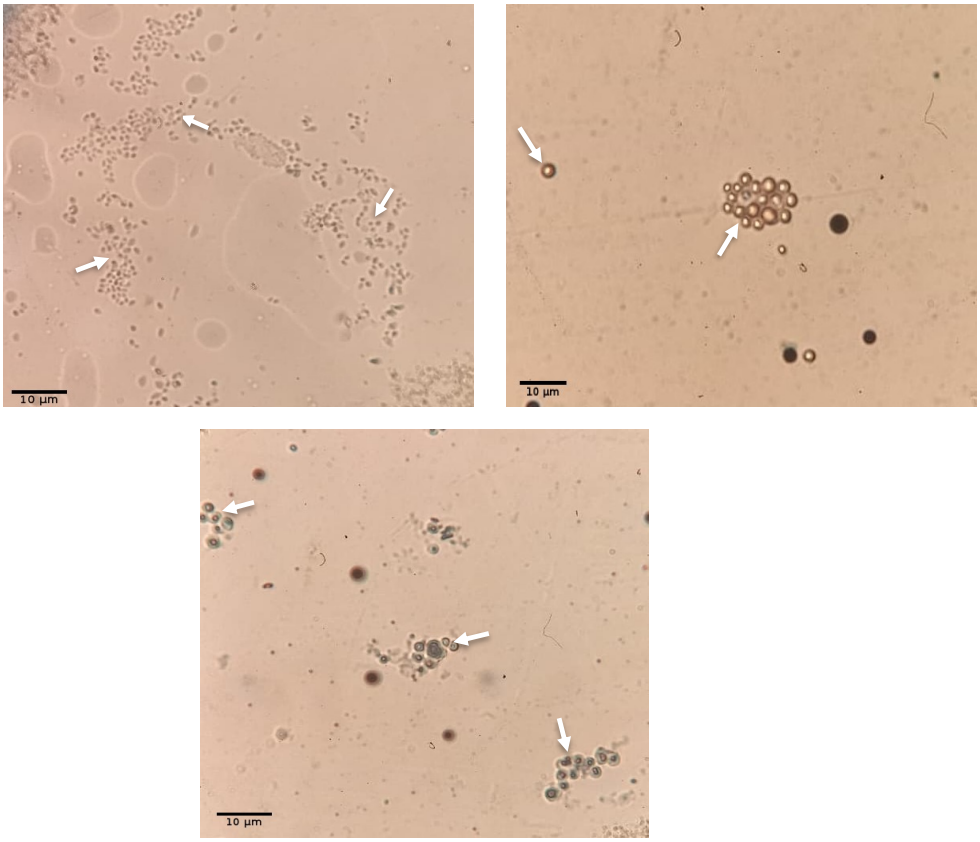

Figure 6. Staining of accumulated lipids with Sudan Black B with A: lipids stained in cells of YAS 1 isolate at 1000x magnification, B: negative control, without lipid accumulation on PDA media - no lipids stained in cells, C: positive control on NLM media - lipids stained in cells.

Each isolate of wet biomass was used to estimate lipid accumulation using the yield-on-a-wet-basis formula described previously. YAS 2 and KAS 2 isolates exhibit the highest lipid accumulation among the isolate groups, with 0.73% in the positive control (Figure 7).

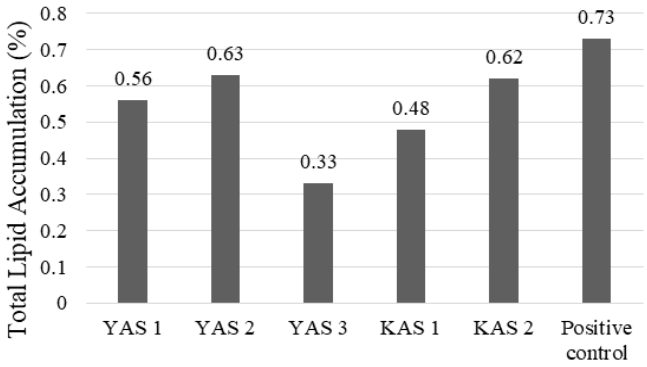

Figure 7. Descriptive values of total lipid accumulation for yeast isolates, mold isolates, and the positive control. All data represent single-point observation (n=1); therefore, error bars and statistical comparisons are not included.

Lipid accumulation in this study was calculated on a wet-weight basis, with grams of extracted lipids per gram of wet biomass reported as a percentage (% w/w). This study focused solely on calculating lipid accumulation from wet biomass. Therefore, the data obtained are exploratory and are only used to compare the relative ability of lipid accumulation between isolates in the group under the same incubation conditions, but are not used as a reference to determine the oleaginous nature of each isolate, because the determination standard refers to lipid accumulation ≥ 20% of the dry weight of cell biomass (Cho & Park, 2018). Among all isolates, YAS 2 and KAS 2 have the highest lipid accumulation profiles in wet biomass and can be prioritised as potential agents for biodiesel research. Although the isolates demonstrated promising saccharification and lipid accumulation ability, this result should be considered as preliminary screening.

This study was limited to lipid extraction from microorganisms, without further steps such as transesterification, purification, biodiesel quality testing, or blending with fossil diesel; it can also be used directly without blending (Roy et al., 2014). Therefore, future studies should include quantitative validation and scale-up before any industrial application is considered. While the prospect of developing microorganism-based biodiesel remains promising in the long term, further robust investigations are essential to evaluate its feasibility and potential contribution to sustainable energy and climate change mitigation.

CONCLUSION

In conclusion, This study identified four bacterial isolates (BAS 1B, 2A, 2B, and 3A) with saccharification ability, with BAS 1B exhibiting the highest cellulolytic index on Congo red–CMC agar. All bacterial isolates produced detectable reducing sugars (0.5–1.5%) in the Benedict test. Among yeast and mold isolates, YAS 2 and KAS 2 demonstrated the highest intracellular lipid accumulation based on Sudan Black B staining and wet-biomass gravimetric analysis. As all measurements in this preliminary screening were conducted as single-point observations ($n = 1$), the results are exploratory and should be interpreted cautiously. Nevertheless, isolates BAS 1B, YAS 2, and KAS 2 show potential as microbial candidates for further biodiesel-related applications using lignocellulosic waste. Future research incorporating statistical replications, quantitative validation, and scale-up studies is required before any industrial application can be considered.

AUTHOR CONTRIBUTION

A.CH.L. conceived the study, performed the experiments, and wrote the initial draft. **C.A.S**. contributed to methodology design, data interpretation, and manuscript review. **E.T.A.** supervised the research, validated the data, and contributed to the final editing of the manuscript. All authors read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Mr. Yohanis Maspaitella, Mrs. Yani Manusama, and the government of Rutong Village for providing sago pulp waste and for their support and permission to access local sago processing sites. The authors are grateful to the laboratory technicians of the Microbiology Laboratory for their

technical assistance and dedication during the experimental work and to all funders for funding this research.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this study.

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