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Original article

A new source of anti-hyperglycemic agent: endophyte bacteria isolated from stems of sea ferns (A*crostichum aureum* l.) on Rupat Island, Riau Province, Indonesia

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Abstract

Endophytic bacteria are microorganisms that are symbiotic with plants without causing adverse effects to the plants. Generally, the compounds produced by the host plant align with those produced by the endophytic bacteria. Secondary metabolites of endophytic bacteria are reported as antibacterial, anti-cancer, enzyme production, and pharmaceutical agents. This study aims to isolate, screen and test the activity of secondary metabolites of endophytic bacteria from the stems of sea fern (*Acrostichum aureum* L.), producing α-amylase inhibitors. This study obtained 20 isolates of endophytic bacteria isolated using an NA medium. Three isolates of endophytic bacteria were selected from the screening results using MRSA and MRSA media with the addition of $CaCO_3$, namely isolates BRS 2.3, BRS 5.2, and BRF 5.1. The α-amylase inhibitor activity of isolate BRF 5.1 was 8.05% on 1% starch substrate and 23.76% on 2% starch with a percent inhibition of 30.495% for antioxidants. This preliminary investigation will be useful in identifying and using functional products containing anti-hyperglycemic benefits.

Keywords: *Acrostichum aureum* L., antioxidant, endophytic bacteria, inhibitor α-amylase
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Introduction

Sumatra Island is home to 35% wetlands, making it the leading region in Indonesia for wetland coverage. Among its provinces, Riau has the most extensive wetlands, accounting for 56.1% or approximately 4.04 million hectares (Mubekti 2011). The biodiversity found in these wetlands includes species such as Nipah (*Nypa fruticans*), mangroves (*Rhizophora stylosa*) (Hardiansyah & Noorhidayati), and Sea fern (*Acrostichum aureum* L.) (Sofiyanti et al. 2020). Sea ferns are known to produce various phytochemical compounds, including flavonoids, steroids, saponins, phenols, and terpenoids (Badhsheeba & Vadivel 2020). Flavonoids, in particular, serve important functions as antioxidants, helping to combat free radicals in the body (Ultari et al. 2021), while also reducing blood sugar levels by acting as inhibitors of α-amylase and α-glucosidase (Zulcafli et al. 2020).

Phytochemical compounds in plants can also be sourced from endophytic bacteria. Due to the long-term symbiotic relationship between these bacteria and their host plants, the bacteria can produce metabolites similar to those of the plants (Chigurupati et al. 2019). Linda et al. (2022) reported the presence of alkaloids and saponins as phytochemicals in the leaves of sea fern (*Acrostichum aureum* L), which are also found in the metabolites produced by endophytic bacteria. Utilizing endophytic bacteria can provide significant advantages, as their shorter life cycle allows for large-scale production of secondary metabolites through fermentation (Pujiyanto et al. 2018).

Natural products derived from endophytic bacteria have been highlighted for the development of new drugs because long-term use of synthetic drugs can lead to complications such as hyponatremia, edema, and gastrointestinal issues (Krentz & Bailey, 2005). The bioactive compounds in endophytic bacteria can offer therapeutic benefits. Numerous studies in the health sector have researched the potential of endophytic bacteria, demonstrating their effectiveness as antibacterial agents (Linda et al. 2023), anticancer agents (Sulistiyani & Kusumawati, 2019), antioxidants (Chigurupati et al. 2019), and inhibitors of α-glucosidase (Frediansyah et al. 2018) and α-amylase (Linda et al. 2023; Elvani 2023). Endophytic bacteria of sea fern stems from Rupat Island Riau Province are potentially having bioactivities. This research aims to isolated, screening and test the activity of secondary metabolites of endophytic bacteria from the stems of sea fern (*Acrostichum aureum* L.), producing α-amylase inhibitors to discover new drugs from natural sources that are safe and exhibit minimal side effects.

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Methods

Research Location and Isolation

Sampling of *Acrostichum aureum* L. was conducted in Rupat District, Bengkalis Regency, Riau Province, Indonesia: Location 1: 1,73831˚ N, 101,45654˚ E; Location 2: 1,71572˚ N, 101,49480˚E; Location 3: 1,71317˚ N, 101,49480˚ E; Location 4 :1,69838˚ N, 101,61820˚ E and Location 5: 1,72621˚ N, 101,47952˚ E. The maps can be seen in Figure 1.

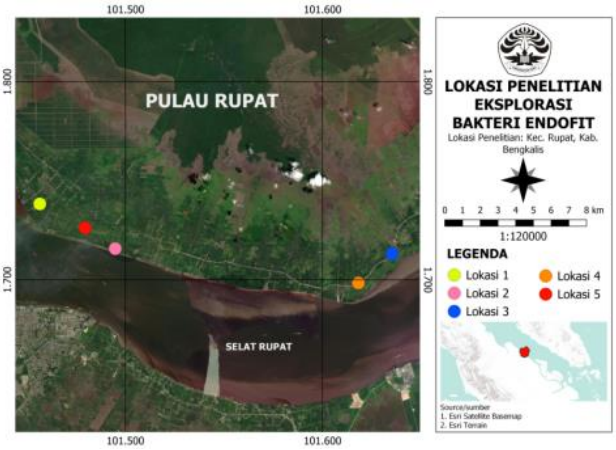

Figure 1. Location of Rupat District, Bengkalis Regency, Riau Province, Indonesia

The stems of *A. aureum* were used to isolate endophytic bacteria which were then purified using a Nutrient Agar (NA) medium. Stems of sea fern (*A. aureum*) were washed under running water until clean and cut using a sterile knife (4 x 4 cm). The sample's surface was sterilized using 70% ethanol for 75 seconds and then dried with sterile tissue. Furthermore, the sample was soaked in 5.25% sodium hypochlorite (NaOCl) solution for 6 minutes and then rinsed three times with sterile water. Stems of sea fern samples were dried using sterile tissue. Samples with the size of 2 x 2 cm were inoculated on NA medium and incubated for 24 hours at room temperature. As a control, the last rinse water, as much as 1 ml, was grown into NA using the pour plate technique and then incubated for 24 hours. The last rinse water of sterilized leaves surfaces is used as a control to ensure the endophytic bacteria that grow on the agar plate are the endophytic bacteria. The bacteria colonies that grew around the stem were purified with NA medium.

Characterization of Bacteria

Macroscopic morphological characterization includes observations of colour, shape, edges, elevation and colony size. Microscopic characterization involved observing bacterial cell shapes, while physiological characterization involved Gram staining.

Screening of Endophytic Bacteria as Lactic Acid Bacteria

dophytic bacteria isolates were grown on a deMan Rogose Sharpe Agar (MRSA) selective medium. Positive test results were characterized by colony growth after 24 hours of incubation. Further tests, endophytic bacteria isolates were also grown on an MRSA selective medium with the addition of $CaCO_3$. Positive test results were characterized by forming a clear zone around the colony (Taha et al. 2019)

Antioxidant Activity Test

For the antioxidant test, three possible endophytic bacteria were selected. A 5 ml (10^8 CFU/ml) culture of endophytic bacteria was added to 495 ml of Nutrient Broth (NB) and incubated for 72 hours at 120 rpm. A centrifuge at 3500 rpm was used to extract the crude extract of endophytic bacteria's secondary metabolites for 15 minutes. After extracting the supernatant using a 1:1 ethyl acetate solvent, it was macerated at 4˚C for a full day. The layers formed were separated using a separating funnel to take the top layer and then evaporated using a rotary evaporator at 90 rpm at 40˚C to obtain extracts containing secondary metabolites of endophytic bacteria. The dried extract was used for an antioxidant activity test (Pudjas et al. 2022).

The antioxidant test was adapted from the techniques described by Kuntari et al (2017). After adding 2 ml of methanol p.a. to dry samples of extracted secondary metabolites with concentration variations of 31.25, 62.5, 125, 250, 500, and 1000 ppm, 1 ml of a 100 ppm DPPH solution was added. It was then homogenized with a vortex. The absorbance had to be measured at a maximum wavelength of 517 nm using a spectrophotometer. The formula that follows was used to determine the percentage of inhibition based on the absorbance of the test and control samples:

$$I\% = \frac{A_{Control} - A_{Sample}}{A_{Control}} \; x\; 100$$

Inhibitor α-Amylase

Three endophytic bacteria isolates that produced clear zones on the MRSA with the addition of $CaCO_3$ medium were used for the α-Amylase inhibitor test. A total of 10 ml (10^8 CFU/ml) of endophytic bacteria culture was inoculated in 90 ml Nutrient Broth (NB) incubated at 150 rpm for 72 hours. The crude extract of secondary metabolites of endophytic bacteria was centrifuged at 3500 rpm for 15 minutes. The supernatant was used for the α-amylase inhibitor test (Linda et al. 2023). The α-amylase inhibitor test refers to the method of Pujiyanto et al. (2018). The test formulation is shown in Table 1. The positive control used acarbose. Each treatment was made with five replicates. The absorbance of each sample solution was measured with a spectrophotometer at a wavelength of 540 nm. The formula calculated the percentage of inhibitors:

$$\text{I\%} = \frac{\text{C} - \text{S}}{\text{C}} \; x\; 100$$

Table 1. Formula and test inhibitor α-amylase

Reagen	Volume (ml)		
	Control (C)	Sample (S)	*Acarbose* (A)
Sample	-	0,5	-
Acarbose	-	-	0,5
Distilled water	0,5	-	-
Enzyme	0,5	0,5	0,5
	Incubation of 10 minute of temp 25ºC		
Substrate	1	1	1
	Incubation of 10 minute of temp 25ºC		
DNS	2	2	2
	Boiling (100ºC) of 5 minutes		

Data analysis

Characterization data of endophytic bacteria isolates were analyzed descriptively. The test results of antioxidant activity and α-amylase inhibitor of endophytic bacteria from the stem of *A. aureum* were analyzed and presented in graphs and tables.

Results

Morphological Characterization of Endophytic Bacteria

Isolation of bacteria from the stem sample of sea fern (*A. aureum*) is characterized by bacteria growth around the stem placed in a Petri dish. Isolation results obtained 20 endophytic bacteria isolates and colony characterization (Table 2). Characterization of colony morphology includes color, shape, size, elevation, and edge of the colony, thus obtaining varied results. All isolated with ledges is entire. One of the characteristics used for bacteria identification is colony color.

Characterization Physiological and Biochemical of Endophytic Bacteria

Twenty successfully isolated *A. aureum* endophytic bacteria isolates were characterized for physiology and biochemistry (Table 2). Gram staining test results obtained six Gram-positive isolates characterized by purple cells and 14 Gram-negative isolates characterized by pink cells (Figure 2). The KOH 3% test results obtained six isolates that are Gram-positive and characterized by unformed mucus and 14 isolates that are Gram-negative and characterized by mucus produced.

Table 2. Characterization macroscopy, microscopy and biochemical of bacteria

Code Isolates	Morphology Colony					Gram staining	Microscopy Cell
	Color	Shape	Size	Elevation	Edge		
BRS 1.1	Yellowish White	Circular	Small	Convex	Entire	-	Bacilli-Short
BRS 2.1	Creamy White	Circular	Small	Raised	Entire	+	Bacilli
BRS 2.2	Transparent Yellow	Circular	Moderate	Raised	Entire	-	Bacilli
BRS 2.3	Milky White	Circular	Small	Convex	Entire	+	Coccus
BRS 2.4	Creamy White	Circular	Small	Flat	Entire	-	Bacilli
BRS 2.5	Transparent Yellow	Circular	Small	Convex	Entire	-	Bacilli-Short
BRF 2.1	Transparent	Spindle	Small	Flat	Entire	-	Bacilli
BRF 2.2	Milky White	Circular	Moderate	Convex	Entire	-	Coccus
BRS 3.1	Milky White	Circular	Moderate	Raised	Entire	+	Bacilli
BRS 3.2	Transparent Yellow	Circular	Small	Convex	Entire	-	Bacilli-Short
BRF 3.1	Milky White	Circular	Small	Convex	Entire	-	Coccus
BRF 3.2	Creamy White	Circular	Moderate	Convex	Entire	+	Bacilli
BRF 3.3	Yellowish White	Circular	Small	Raised	Entire	-	Bacilli-Short
BRS 4.1	Transparent	Circular	Small	Convex	Entire	-	Coccus
BRS 4.2	Milky White	Circular	Small	Flat	Entire	+	Bacilli-Short
BRS 5.1	Milky White	Circular	Small	Convex	Entire	+	Bacilli-Short
BRS 5.2	Milky White	Circular	Moderate	Convex	Entire	-	Coccus
BRS 5.3	Transparent Yellow	Circular	Small	Raised	Entire	-	Bacilli-Short
BRF 5.1	Yellowish White	Circular	Small	Raised	Entire	-	Coccus
BRF 5.2	Milky White	Circular	Small	Raised	Entire	+	Bacilli

Screening of Endophytic Bacteria as Lactic Acid Bacteria

The results of the selection of bacteria endophytes using MRSA medium with the addition of $CaCO_3$ 0.25% obtained three isolates capable of forming a clear zone, namely isolate BRS 2.3, isolate BRS 5.2, and isolate BRF 5.1 (Figure 3. A, B, and C). On MRSA media with the addition of $CaCO_3$ 0.5%, two isolates formed a clear zone, namely BRS 2.3, isolate BRS 5.2 and BRF 5.1. Three isolates capable of forming a clear zone are shown in Table 3.

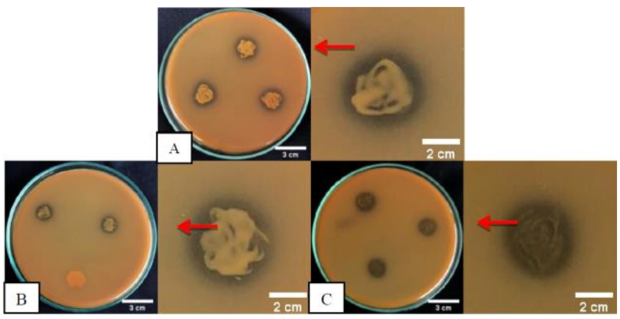

Figure 3. Clear zone results of endophytic bacteria on MRSA + CaCO3 0.25% medium. (A) isolate BRS 2.3, (B) isolate BRS 5.2 and (C) isolate BRF 5.1

Table 3. The results of selection bacteria endophyte using MRSA medium with the addition of $CaCO_3$ obtained three isolates capable of forming a clear zone

Isolates	Media MRSA + $CaCO_3$ clear zone (mm)
BRS 1.1	-
BRS 2.1	-
BRS 2.2	-
BRS 2.3	15.65±0.56
BRS 2.4	-
BRS 2.5	-
BRF 2.1	-
BRF 2.2	-
BRS 3.1	-
BRS 3.2	-
BRF 3.1	-
BRF 3.2	-
BRF 3.3	-
BRS 4.1	-
BRS 4.2	-
BRS 5.1	-
BRS 5.2	10.95±0.57
BRS 5.3	-
BRF 5.1	11.98±0.80
BRF 5.2	-

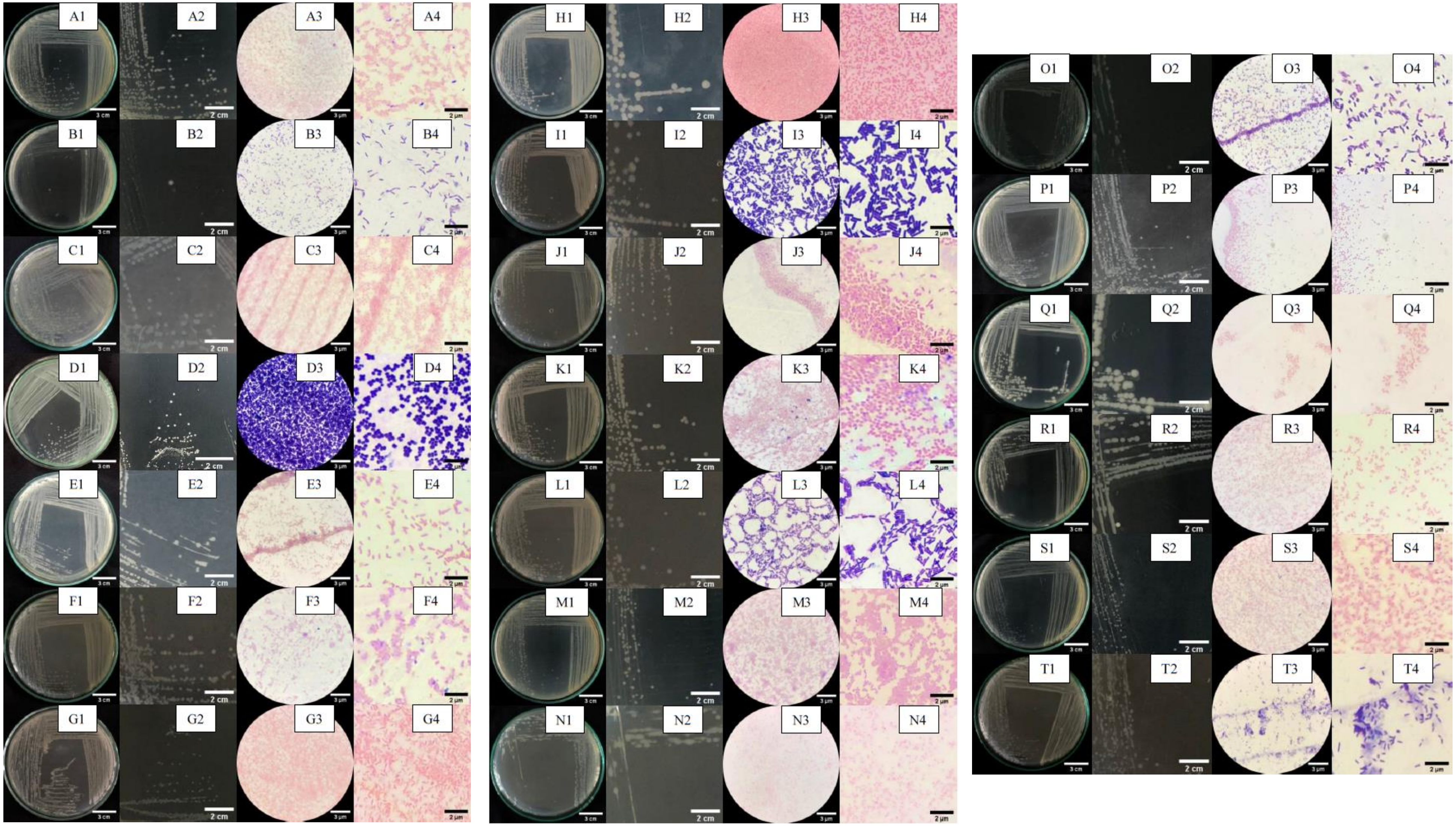

Figure 2. Characterization of endophytic bacteria isolates isolated from the stems of Sea ferns. (A. isolate BRS 1.1, B. isolate BRS 2.1, C. isolate BRS 2.2, D. isolate BRS 2.3, E. isolate BRS 2.4, F. isolate BRS 2.5, G. isolate BRF 2. 1, H. BRF isolate 2.2, I. BRS isolate 3.1, J. BRS isolate 3.2, K. BRF isolate 3.1, L. BRF isolate 3.2, M. BRF isolate 3.3, N. BRS isolate 4.1, O. BRS isolate 4.2, P. BRS isolate 5.1, Q. BRS isolate 5.2, R. BRS isolate 5.3, S. BRF isolate 5.1, T. BRF isolate 5.2) and (1. colony morphology, 2. colony morphology details, 3. bacteria cell morphology after Gram staining, 4. bacteria cell shape).

Table 4. Antioxidant activity of endophytic bacteria from sea fern by DPPH method

Concentration (μg/mL)	% Inhibition			IC_{50} (μg/mL)		
	BRS 2.3	BRS 5.2	BRF 5.1	BRS 2.3	BRS 5.2	BRF 5.1
1000	51.619±0.012	14.794±0.007	30.495±0.009	>1000 ppm	>1000 ppm	>1000 ppm
500	31.238±0.021	14.044±0.002	19.405±0.007			
250	23.619±0.013	2.996±0.014	11.485±0.016			
125	24.761±0.010	2.808±0.004	9.306±0.003			
62.50	5.523±0.006	1.685±0.009	8.910±0.006			
31.25	3.238±0.007	2.996±0.014	7.326±0.012			

Antioxidant Activity

Three bacteria isolates that can hydrolyzed 0.25% MRSA + $CaCO_3$ media, namely isolates BRS 2.3, BRS 5.2 and BRF 5.1, were extracted for their secondary metabolites and antioxidant activity was measured. Secondary metabolites of bacteria endophytes extracted with ethyl acetate (1:1) obtained dry extracts of the three isolates as much as 5.7 mg, 68.3 mg and 21.8 mg, respectively. An antioxidant activity test was conducted using the DPPH method. The results of the antioxidant activity test are shown in (Table 4). The results of this study indicated the beneficial effects of three bacterial isolates in raising the antioxidant.

Inhibitor α-Amylase

The results of α-amylase inhibitor testing of endophytic bacteria isolate BRF 5.1 inhibited the work of α-amylase enzyme with a percentage inhibition of 8.05% on 1% starch substrate and 23.76% on 2% starch. Bacteria isolates BRS 2.3 and BRS 5.2 could not inhibit the action of the α-amylase enzyme (Table 5).

Table 5. α-amylase inhibitor activity of endophytic bacteria

Code Isolates	Inhibition (%)	
	Starch (1%)	Starch (2%)
BRS 2.3	-	-
BRS 5.2	-	-
BRF 5.1	8.05%	23.76%
Acarbose (Control Positive)	12.31%	41.10%

Discussion

The results of the morphological characterization of colonies from 20 isolates of endophytic bacteria (Figure 2) have varying colours. Yellowish white (3 isolates), creamy white (3 isolates), translucent-yellow (4 isolates), milky white (8 isolates), and translucent (2 isolates). Colony shape is circular (19 isolates) and spindle (1 isolate). Colony size is small (15 isolates) and moderate (5 isolates). The elevation colony obtained in this study is convex (10 isolates), raised (7 isolates), and flat (3 isolates). The colony edges of all endophytic bacteria isolates are entire. In this study, three cell forms were obtained, namely bacilli (6 isolates), short bacilli (8 isolates) and cocci (6 isolates).

Bacterial colony colour is influenced by the pigments they produce (Ahmad et al. 2012). Research by Linda et al. (2023) endophytic bacteria from the leaves of sea fern were reported to contain dull-white, yellow and light-yellow pigments. Variations in bacteria colony colour pigments have been reported by several studies, namely canthaxin (dark red) (Duy et al. 202l), astaxanthin (pink-red), pycocyanin (blue-green) and violacein (purple) (Usman et al. 2018). According to Thawornwiriyanun et al. (2012), zeaxanthin is the pigment affecting bacteria colonies' yellow, golden yellow, beige, and brown colours. Some bacteria can produce diverse pigments, such as those from the genus *Serratia* (Venil et al. 2021) and *Bacillus, Choromobacterium* (Usman et al. 2018).

Gram-positive bacteria have more peptidoglycan in the cell wall, which is about 40-80% of the dry weight of the cell wall. The cell wall of Gram-negative bacteria is thinner because it contains a higher percentage of lipids (Trivedi et al. 2010).

The clear zone produced on MRSA + $CaCO_3$ media, according to Suteja et al. (2022), is due to lactic acid bacteria isolates producing acids that react with $CaCO_3$ so that a clear zone is formed around the bacteria colonies. Calcium in the medium becomes soluble. Generally, the $CaCO_3$ compound is used to isolate lactic acid bacteria because of its ability to react with lactic acid and form a new compound, namely calcium lactate ($C_6H_{10}CaO_6$), which gives a clear color to the media (Haro et al. 2020).

Numerous results are consistent with an increase in antioxidant levels caused by bacteria. Antioxidant enzymes produced by bacteria, namely superoxide dismutase and glutathione peroxidase, are essential in eradicating and warding free radicals (Manguntungi et al. 2020). The use of antioxidants in patients with diabetes mellitus is known to be effective in reducing the appearance of complications that arise. Antioxidants benefit the pathological process of diabetes mellitus due to oxidative stress conditions. Some compounds that contain antioxidants include lycopene, beta-carotene, polyphenols, and catechins (Zhang et al. 2015). Antioxidants can slow down, inhibit, or prevent the oxidation process, eliminate free radicals, and reduce oxidative stress (Amaral et al. 2020).

Percent inhibition of BRS 2.3 isolate was 51.619%, BRS 5.2 isolate was 14.794%, and BRF 5.1 was 30.495%. Percent inhibition describes the ability of antioxidant compounds in the sample to capture free radicals at the concentration of the test solution (Khaira 2010). All three isolates showed IC50 values >1000 ppm. The IC50 value means that endophytic bacteria isolate from the stem of sea fern (*A. aureum* L.) do not have antioxidant activity. The type of solvent, solvent formulation and the origin of the isolate used influence antioxidant activity. According to Hidajat (2005), the sample has a fragile antioxidant activity if the IC50 value is > 200 ppm. Research by Chigurupati et al. (2021) reported the antioxidant activity of endophytic bacteria from durian (*Durio zibethinus*) extracted using ethyl acetate organic solvent with a ratio of 1:3 obtained a percent inhibition of 91.43% and an IC50 value of 161 ppm. Research by Kemung *et al*., (2020) reported the antioxidant activity of endophytic bacteria from mangrove plants extracted using methanol solvent with an IC50 value of 24.71 ppm. It is necessary to optimize the length of fermentation time to obtain antioxidant activity.

The alpha-glucosidase inhibitor test showed negative results for isolates BRS 2.3, BRS 5.1 and BRS 5.2. The contrast result reported by Linda et al. (2023) stated that isolate D.SB 5.2 from endophytic bacteria from the leaf of sea fern (*A. aureum*) – Bengkalis island has activity as α-amylase enzyme inhibitor with 18.13% inhibition value. Linda et al. (2024) isolate A.T 2 and A.A 3 from endophytic bacteria from the root of sea fern (*A. aureum*)-Rupat island has activity as α-amylase enzyme inhibitor with 17% and 8 % inhibition on 1% starch substrate, respectively. Different results can be influenced by differences organs used and the location of sea fern sampling. Chigurupati et al. (2021) also reported that the endophytic bacteria from durian (*Durio zibethinus*) isolate DZLM as a potential α-amylase enzyme inhibitor with an inhibition value of 90.9%.

According to Ishnava & Motisariya (2018), antidiabetic activity occurs due to active compounds such as alkaloids, flavonoids, saponins, tannins, and sterols. Research by Raja & Ravindranadh (2014) reported sea spikes to have phytochemical compounds in saponins, alkaloids, flavonoids, and steroids. A study by Linda et al. (2022) reported that endophytic bacteria from sterile leaves of sea spikes (*A. aureum*) contain phytochemical compounds of alkaloids and saponins. The content of phytochemical compounds present in sea ferns and their symbiosis with endophytic bacteria gives plants and endophytic bacteria the potential to produce α-amylase inhibitors.

Conclusion

Total 20 endophytic bacteria isolated from stems of sea ferns (Acrostichum *aureum* L.) on Rupat Island, Riau Province. Colony morphology physiological and biochemical tests provided essential data on 20 endophytic bacteria isolates. The α-amylase inhibitor activity obtained from isolate BRF 5.1 was 8.05% on 1% starch substrate and 23.76% on 2% starch. BRF 5.1 isolate can be developed as a pharmaceutical material for anti-hyperglycemia.

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