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Research Article

Isolation and Characterization of Rhizospheric Bacteria Associated with Canna Plant for Production of Maltooligosaccharide Amylase

Rina Dwi Agustiani[1,3], Oedjijono[1]*, Nanik Rahmani[2], Nuraeni Ekowati[1]
1)Faculty of Biology, University of Jenderal Soedirman, Jalan dr. Soeparno 63 Purwokerto 53122, Indonesia.
2)Center for Applied Microbiology Research, Research Institute for Life and Environmental Sciences, National Research and Innovation Agency (BRIN), Cibinong, Bogor 16911, Indonesia.
3)Department of Biology, Faculty of Science and Technology, International Women University, Bandung 40173, Indonesia.
* Corresponding author, email: oedjijono@unsoed.ac.id

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ABSTRACT
The objectives of the study were to isolate amylolytic bacteria from the rhizosphere and plant tissue of *Canna edulis* Ker., as well as litter; to know oligosaccharide compounds produced from starch hydrolyzed by the bacterial enzymes, and to identify the amylolytic bacteria based on phenetic and 16S rRNA gene sequences. From the rhizosphere, Canna plant tissue, and litters obtained thirty-two amylolytic bacterial isolates. Eight isolates (TH6, TH7, T5, T10, D2, D3, A3, S1) produced high clear zone diameters ranging from 18-30 mm; especially an isolate T10, which was consistent in producing a total clear zone diameter of 20 mm. The hydrolysate of starch hydrolysed by the T10 amylase resulted in three oligosaccharide compounds maltotriose, maltotetraose, and maltopentose. The amylase activity of isolate T10 was optimal at a temperature of 40°C and pH at 0.801 U/mL. The isolate T10 was identified as a species member of *Bacillus toyonensis* based on phenotypic characterization and 16S rDNA gene sequencing analysis with a similarity value of 99.93%.

INTRODUCTION
Oligosaccharides are members of an essential group of carbohydrates. Macromolecules with short-chain polysaccharide sugars of 2 to 20 saccharide units. Functional oligosaccharides such as galactooligosaccharide (GOS), fructooligosaccharide (FOS), and maltooligosaccharide (MOS) are well-known prebiotics owing to their ability to selectively stimulate beneficial bacteria in the intestines, particularly bifidobacterial species (Zhao et al. 2017). Developing oligosaccharide products is one of the businesses with high economic value. Plants such as Canna contain much starch, one of the crucial ingredients (substrate) to produce oligosaccharides enzymatically. Canna plants (*Canna edulis* Ker.) contain high levels of carbohydrates, mainly starch (93.3%), which consists of amylose (33.48%) and amylopectin (59.82%) (El-Fallal et al. 2012). Starch is hydrolysed into smaller oligosaccharides by α-amylase, one of the most important commercial enzymes (Jang et al. 2020).

The starch-processing industry has exploited amylase as a substitute for acid hydrolysis in producing starch hydrolysis. Amylase acts as a biocatalyst for the hydrolysis of starch into simpler carbohydrates, such

as glucose, maltose, and dextrin (Divakaran et al. 2011; Abdalla et al. 2021). Amylolytic bacteria are producers of amylase that can be used as biocatalysts in the starch hydrolysis process (Ding et al. 2021) to produce various maltooligosaccharide products, such as maltotriose, maltotetraose, maltopentaose, and maltohexaose (Pan et al. 2017).

Canna plants and its surrounding, including the rhizosphere and plant tissues, can be sources of isolating amylolytic bacteria. The high starch content in canna tubers makes them a suitable substrate for growing various bacteria, especially amylolytic bacteria. The bacteria isolated from starch-rich sources generally have the potential to produce amylase with high activity (Hellmuth & van den Brink 2013). In addition, the rhizosphere is known as the most diverse microbial habitat concerning species richness and community size. The interaction between plant roots and microorganisms is intensive around the rhizosphere, because the plants secrete exudates containing carbohydrates, amino acids, and other nutrients utilized by bacteria for growth. On the contrary, rhizospheric bacteria can produce proteins and enzymes that are important for the biological function of host plants (Afifah et al. 2018).

Bacteria, fungi, plants, and animals play an important role in utilizing polysaccharides. Members of the genus *Bacillus* were known to produce various enzymes, such as amylase that have been used in many industries, such as fermentation, textiles, paper, medicine, and sugar (Gupta et al. 2003). They are derived mainly from *Bacillus licheniformis* and *B. amyloliquefaciens*. Moradi et al. (2014) found several bacterial isolates producing high amylolytic enzymes, which were subsequently identified as *Bacillus cereus*, *B. amyloliquefaciens*, *B. licheniformis*, and *Paenibacillus lautus*. Luo et al. (2021) isolated *Bacillus toyonensis* P18, a group of Gram-positive bacteria belonging to the *Bacillus cereus* group and often used as probiotics or biocontrol agents. The bacterium has also been known to be treated as a probiotic for preventing microbial diseases in crops or improving the immune response of animals (Santos et al. 2018).

The objectives of the study were to isolate amylolytic bacteria from rhizosphere and plant tissue of Canna, as well as litter; to know oligosaccharide compounds produced from hydrolysate of starch hydrolysed by the bacterial enzymes; and to identify the selected amylolytic bacteria based on 16S rRNA gene sequences.

MATERIALS AND METHODS

Sample Collection and Location of Sampling

Samples were taken from the rhizosphere and parts of Canna plant (*C. edulis* Ker.) including tubers, stems, leaves, tissue, as well as litter growing in two places, namely in the forest and the community gardens around the Perhutani Forest West Banyumas, Central Java, Indonesia. The coordinates of the former are S 07°20.846 'E 109°06.410 and the latter is S 07 °20.812 'E 109°05.92 (Figure 1).

Isolation, Screening, and Morphological Characterization of Amylolytic Bacteria

Plant tissues and litter were cleaned with running water, then cut into 1 cm long pieces and separated according to the plant part. The sample pieces were immersed in 70% alcohol for 1 minute, then in 1% sodium hypochlorite solution for 3 minutes, after which they were soaked again using 70% alcohol for 1 minute, and rinsed with sterile distilled water three times (Duan et al. 2021, with modification). The sterile samples were placed on sterile tissue papers and then crushed using a mortar and one gram of each sample was diluted with 9 mL of sterile distilled water,

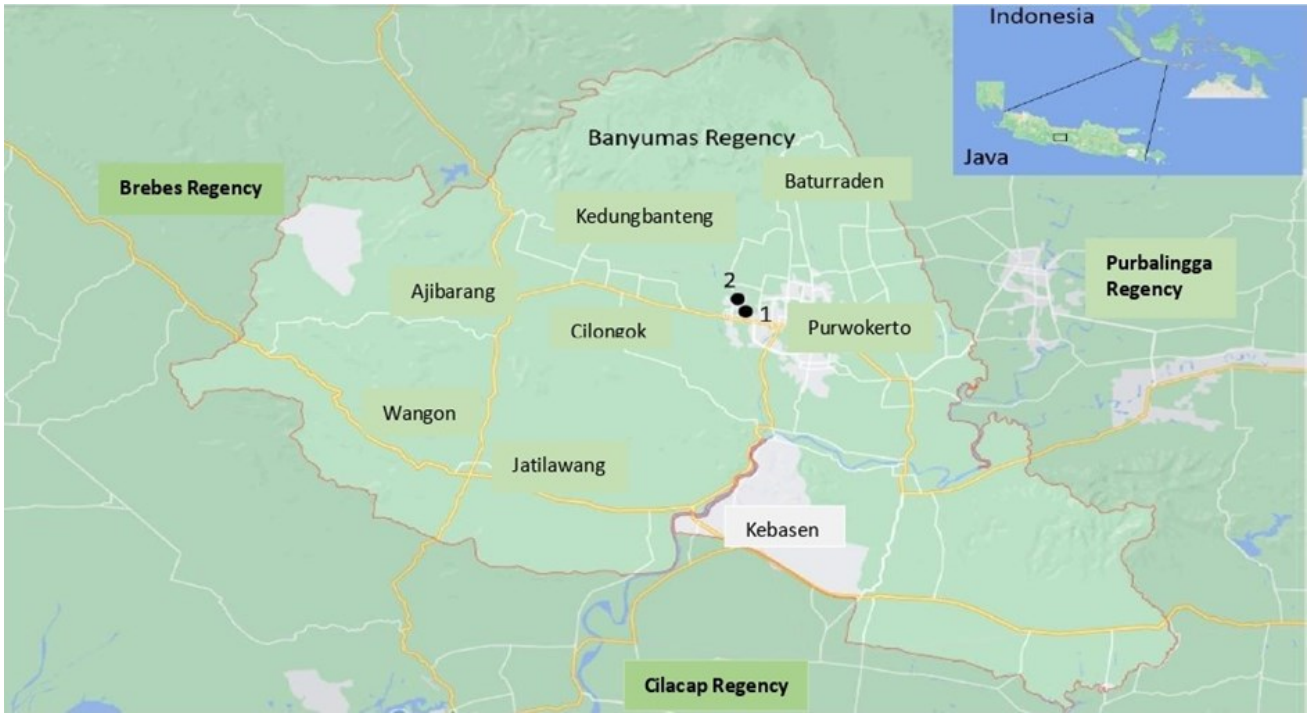

Figure 1. A map of Banyumas Regency and sampling sites: (1) the Perhutani Forest KPH West Banyumas, Central Java, (2) Community gardens around the Perhutani Forest KPH West Banyumas, Central Java.

and then serial dilutions were made up to 10^{-7}.

One gram of Canna rhizospheric soil was put into a 20 mL of nutrient broth (NB) medium containing 1% soluble starch (Merck) in a 100 mL Erlenmeyer flask. The solution was then homogenized in an agitation speed shaker machine at 150 rpm and incubated for 24 hours at 30 ° C. The amount of 1 mL of the solution was diluted with 9 mL of sterile distilled water, and then serial dilutions were made up to 10^{-7}.

One mL from each series of dilutions was inoculated onto nutrient Agar (NA) medium containing 1% soluble starch using a pour plate method. The plates were then incubated for 24 hours at 30 °C. Each growing bacterial colony was then inoculated onto an NA medium containing 1% soluble starch and purified using a streak quadrant method.

The number of 0.5L of bacterial cultures aged 24 hours at 30°C growing on NB medium containing 1% soluble starch was spot inoculated onto NA medium containing 1% commercial soluble starch, then incubated for 72 hours at 30°C. The growing colonies were flooded with iodine solution, and the clear zones formed around the colonies were observed and measured in their diameter (Vijayalakshmi et al. 2012). The isolates having high diameter clear zones were selected and tested for their enzyme production. The colonial and cell morphology of the isolated bacteria were characterized using conventional methods (Smibert & Krieg 1981).

Phenetic and Phylogenetic Characterizations of the Selected Bacterium

Phenetic characterizations of the selected bacterium (producing high diameter clear zones and maltooligosaccharide enzyme) including colony morphology, cell morphology, and biochemistry, were conducted by conventional methods (Smibert & Krieg 1981). Biochemical tests were also conducted using the API 50CHB kit.

The 16S rDNA gene was amplified by polymerase chain reaction (PCR) technique using a pair of primers (9F: 5'GAGTTTGATCCTCCTGGCTCAG-3') 1510R: 5'GGCTACCTTGTTACGA-3')

(Yopi et al. 2017). The obtained bands were stained and visualized by UV Transilluminator. The sequence was confirmed via 1st BASE Sequencing, Malaysia. The 16S rDNA nucleotide sequences were analyzed by nucleotide BLAST (Basic Local Alignment Search Tool) search in the Gene Bank of National Center for Biotechnology Information (NCBI) or BLAST for amino acid analysis (Zhuang et al. 2012). The phylogenetic tree was constructed using a neighbour-joining algorithm in MEGA 6.0 software (De-Moraes-Russo & Selvatti 2018).

Analysis of The Hydrolysis Products by A Thin-Layer Chromatography Method (Rahmani et al. 2013)

An amount of 2 mL of each 24 h old bacterial culture (four selected isolates) was inoculated into a 200 mL flask containing 20 mL of NB medium plus 1% (w/v) starch solution, pH 6 (50 mM acetate buffer) and incubated at 30 °C for 24 h. The culture was sampling every 24 hour and then centrifugated, and the supernatant obtained was tested for its amylolytic activity.

The hydrolytic activity of amylase in a substrate solution was carried out at 30°C in 50 mM acetate buffer, pH 6, containing 0.5% of commercial starch. The enzyme-substrate ratio (v/v) was 1:1 and the reaction times were in hours (0, 1, 2, 3, 4, 24). Reactions were carried out in 2 mL Eppendorf containing 1 mL of reaction mixture in a Deep Well Maximizer (Bioshaker M-BR-022UP, Taitec Japan).

A Thin Layer Chromatography (TLC) of maltooligosaccharide products was carried out on silica gel 60F_{254} plates (Merck Art 20-20 cm) and eluent using a solvent mixture of n-butanol:acid:water (12:6:6, v/v/v). Spots formed were visualized by spraying the sugar colours (0.5 g α-diphenylamine, 25 mL acetone, 2.5 mL phosphate acid, 0.5 mL aniline). All samples were applied in equal quantities (4 µL). Glucose (Sigma-Aldrich, U.S.A), maltose (M2), maltotriose (M3), maltotetraose (M4), maltopentaose (M5), maltohexaose (M6), and maltoheptaose (M7) (Megazyme) were used as standards.

Crude Enzyme Production and Amylase Activity at Different Fermentation Time

An amount of 2 mL of the 24 h old bacterial cultures (isolate T10) was inoculated into a 200 mL flask containing 20 mL of NB medium plus 1% (w/v) starch solution, pH 6 (50 mM acetate buffer), and incubated at 30 °C for five days. The culture was sampled every 24 hours and then centrifuged and the supernatant obtained was tested for its amylase activity.

The enzyme reaction was conducted as above when measuring amylase activity using a DNS method (Miller 1959). The absorbance of the solution was measured using a spectrophotometer at a wavelength of 540 nm. The enzyme activity (U/mL) was calculated based on the equation:

$$\text{enzyme activity} = \frac{c \times d \times 1000}{t \times mw} \text{ U/mL}$$

Where c: amylase concentration; d: dilution; t: incubation time; mw: molecular weight.

A standard curve used D-Glucose at various concentrations. One unit of amylase activity is defined as the amount of enzyme that liberates one µmol of D-Glucose per minute under the experimental condition given.

Effect of pH and Temperature on Enzyme Activity of the Selected Isolate

The optimal pH of the enzyme activity was done at pH ranges of 3.0-10.0 under standard assay conditions. Various buffers (0.05M) used were sodi-

um acetate (pH 3.0-6.0), sodium phosphate (6.0-8.0), Tris-HCL (pH 7.0-9.0), and Glycine-NaOH (pH 8.0-10.0). The enzyme reactions were incubated at 40°C for 30 min in the presence of 0.5% (w/v) starch solution (Merck)). The effect of temperatures on enzyme activity was conducted at temperatures ranging from 30-90 °C in 50 mM acetate buffer at optimum pH for 30 min. Amylase activity was assayed by DNS method (Miller 1959).

RESULTS AND DISCUSSION

Isolation and An Amylolytic Assay of Bacteria Isolated from The Rhizosphere and Plant Tissue of Canna, and Litter

The results of the study found 32 bacterial isolates growing on NA medium supplemented with 1% soluble starch, with details: 11 isolates were from the rhizosphere of the Canna growing in the forest, 12 isolates from the rhizosphere of the Canna growing in the people's gardens around the forest, four isolates from the leave tissue of the Canna growing in the people's gardens around the forest, three isolates from the roots of the Canna growing in the people's gardens around the forest, and two isolates were from the litters of the Canna in the gardens of the residents around the forest (Table 1).

The ability of the bacteria to grow and produce clear zones in the medium indicates that those bacteria were capable of producing amylase. The more amylase is released, the wider clear zones are produced due to the degradation of amylum in the medium, resulting in enhancing the amylolytic index (Ginting et al. 2021). The research results showed that eight isolates of TH6, TH7, T5, T10, D2, D3, A3, and S1 showed high total clear zone diameters (mm) of 18, 18, 18, 20, 18, 30, 18, and 18, respectively (Table 1). The consistency of the bacterial isolates, resulting in the total clear zone diameter, was shown by the isolates TH6, T10, D3, A3, and S1, while the other isolates tended to reduce or lose their amylolytic activity (Figure 2). Based on the ability of isolates to produce a clear zone diameter ≥ 18 mm and consideration of source representatives, five isolates (TH6, T10, D3, A3, and S1) were selected for further testing, namely their ability to hydrolyze starch. Hasanah et al. (2020) reported that bacterial isolates having an amylolytic index of more than 9 mm were potentials to produce amylase. According to Ochoa-Solano & Olmos –Soto (2006), bacterial isolates produce clear zones two or three times the diameter of the colony are potential enzyme producers.

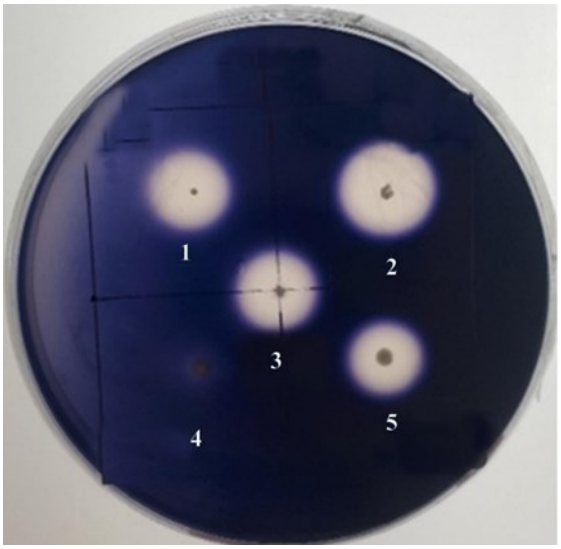

Figure 2. The amylolytic zones produced by bacterial isolates of: (1) T10, (2) D3, (3) A3, (4) TH6, and (5) S1 on a NA medium + 1% soluble starch.

Table 1. Sources, number, and total clear zone diameter of amylolytic bacteria isolated from the rhizosphere, plant tissues of Canna, and litter.

Source of bacterial isolates	Isolate code	Total clear zone diameter (mm)
Rhizospheres of the Canna plants growing in the forest	TH1	16
	TH2	15
	TH3	17
	TH4	16
	TH5	16
	TH6	18
	TH7	18
	TH8	17
	TH9	17
	TH10	16
	TH11	16
Rhizospheres of the Canna plants growing in the people's gardens around the forest	T1	15
	T2	16
	T3	16
	T4	16
	T5	18
	T6	17
	T7	15
	T8	15
	T9	15
	T10	20
	T11	17
	T12	16
Leaves of the Canna plant tissue growing in the people's gardens around the forest	D1	16
	D2	18
	D3	30
	D4	15
Roots of the Canna plant tissue growing in the people's gardens around the forest	A1	17
	A2	16
	A3	18
Litters of the Canna plant from the gardens of the residents around the forest	S1	18
	S2	16

A high number of amylolytic bacteria isolated from the rhizosphere of Canna was by Vaseekaran et al. (2010), who stated that bacteria isolated from starch-rich materials have better potential to produce amylase. Vijayalakshmi et al. (2012) found *Bacillus subtilis* KC3 isolated from the rhizosphere of *Euphorbia hirta* produced a maximum halo zone of 23 mm on a Starch Agar medium. Gebreyohannes (2015) reported that 16 bacterial isolates from soils could produce clear zones of 3-22 mm on starch agar plates. Ginting et al. (2021) found thermophilic bacteria of *Bacillus* sp. L3 and *B. caldotenax* L9 from a marine hydrothermal produces high amylolytic indexes of 3.04 and 3.52, respectively. The clear zone formed results from breaking starch compounds into simple compounds; the wider the clear zone formed, the higher the amylolytic activity (Zubaidah et al. 2019).

The characteristics of colonial morphology of the 34 isolates were rough, dry, bright, and pink; cells were Gram-positive, rod shape, motile, and had endospores. The endospore position of isolates D1-D4 and S1-S2 was in terminal, while isolates of TH1-TH11, T1-T12, and D1-D4 had endospores in the centre. All isolates were able to hydrolyse starch and produce lecithinase (Table 2). Those characteristics indicated that the bacteria were members of the genus *Bacillus*. According to Logan & De

Table 2. Morphological and physiological properties of the bacteria isolated from Canna plants and their surrounding.

Characteristics	Isolate code				
	TH1-TH11	T1-T12	D1-D4	A1-A3	S1-S2
Colonial morphology on NA Agar	Rough, dry, bright, and Pink	Rough, dry, bright and pink	Rough, dry, bright and pink	Rough, dry, bright and pink	Rough, dry, bright, and pink
Gram reaction	+	+	+	+	+
Cell shape	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+
Presence of spore	+	+	+	+	+
Position of spore	Centre	Centre	Terminal	Centre	Terminal
Starch hydrolysis	+	+	+	+	+
Lecithinase production	+	+	+	+	+

Vos (2009), the main characteristics of the genus *Bacillus* are cells rod-shaped, straight or slightly curved, occurring singly and in pairs, some in chains, form endospores, Gram-positive or Gram-negative, motile, aerobes or facultative anaerobes, and mostly isolated from soil.

Identification of The Selected Isolate of T10 Based on Phenetic and Phylogenetic Characteristics

Based on the ability of the selected isolate to produce malto-oligosachharides of maltotriose, maltotetraose, and maltopentaose (Method 3.3); further characterization of the isolate T10 was conducted. The isolate had colonial morphology of irregular with undulate edges, opaque, cream-coloured, and had a granular texture. The cells formed endospores, facultatively anaerobic, Gram-positive, rod-shaped, motile, and occurring singly or in chains (Table 2, Table 3, Figure 3). These characteristics include biochemistry, physiology, and nutrition, indicating that isolate T10 was similar to those typical of the species *Bacillus cereus*. This species is a species complex within the genus *Bacillus*, with members including *B. anthracis*, *B. thuringiensis*, *B. mycoides*, and *B. toyonensis* (Luo et al. 2021).

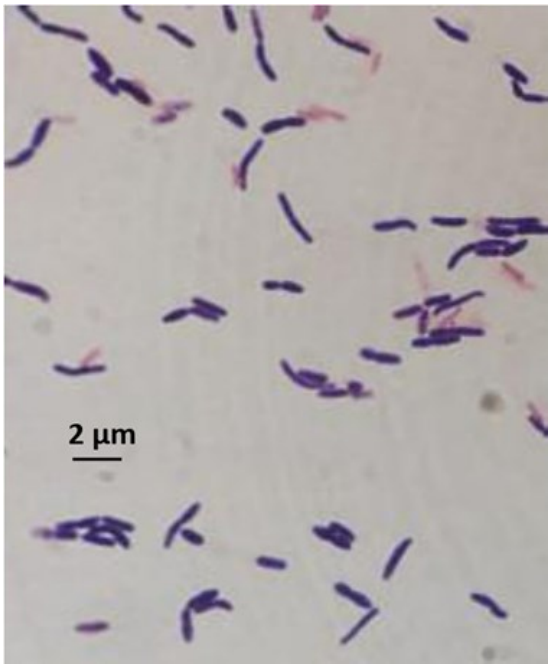

Figure 3. The appearance of bacterial cells isolate T10 under a microscope with a magnification of 1000x. The cells appear single or in chains.

Table 3. Phenotypic characterization of the isolate T10.

Characteristics	Isolate T10
Cell length (µm)	3.00 - 4.00
Egg-yolk lecithinase	+
Anaerobic growth	+
Rhizoid colony	-
Parasporal crystal	-
Growth temperature range (°C)	10 - 45
Optimal growth temperature (°C)	35
Salinity tolerance range (%NaCl)	≤ 4
API 50CHB	
Glycerol	-
D-Ribose	+
D-Mannose	+
Methyl-αD-glucopyranoside	+
Amygdalin	+w
Arbutin	+
Salicin	+
Cellobiose	-
D-saccharose	+
D-trehalose	+
Starch	+
Glycogen	+
D-turanose	+

The electrophoresis visualization of the PCR product showed that the DNA of T10 produced a single band with a size of 1500 kb (Figure 4). The results of comparing the 16S rRNA gene sequence of isolate T10 and nucleotide sequences in the GeneBank (http://blast.ncbi.mlm.nih.gov/) showed that the bacterium is closely related to species members of the genus *Bacillus*. The BLAST analysis showed that isolate T10 had a similarity of 99.3% with either *Bacillus toyonensis* SPa09NA, *B. toyonensis* PZ-48, or *B. toyonensis* SMP1. The phylogenetic tree was constructed using Neighbor-Joining, Model Maximum Composite Likelihood, and 1000x Bootstrapping. A dendrogram resulted from MEGA10 program showed that isolate T10 joined *B. toyonensis* SX04NA, *B. toyonensis* Spa09NA, *B. toyonensis* SMP1, *B. toyonensis* PZ-48, *B. toyonensis* BCT-7112, and *B. toyonensis* l3aM to form a separate cluster (Figure 5). Hence, isolate T10 was identified as the species member of *B. toyonensis* based on the phenetic and phylogenetic characteristics.

B. toyonensis strain BCT-7112^T was first isolated in 1966 in Japan from a survey designed to obtain naturally occurring microorganisms as pure cultures in the laboratory for use as probiotics in animal nutrition (Jiménez et al. 2013). This strain was first identified as *B. cereus* var. *toyoi*, and it has been used as the active ingredient of the preparation TOYOCERIN, an additive for animal nutrition (e.g. swine, poultry, cattle, rabbits and aquaculture). Agamennone et al. (2019) isolated *B. toyonensis* strain VU-DES13 from the gut of the soil-dwelling springtail *Folsomia candida*, which was highly resistant to penicillin and inhibited the growth of a variety of pathogenic microorganisms. Its secondary metabolite clusters produce siderophores, bacteriocins, and nonribosomal peptide synthetases. Wang et al. (2021) reported that *Bacillus toyonensis* XIN-YC13 produced a novel antibiotic, toyoncin, with antimicrobial activity against *B. cereus* and *Listeria monocytogenes*. This antibiotic exerts bactericidal activity and induces cell membrane damage.

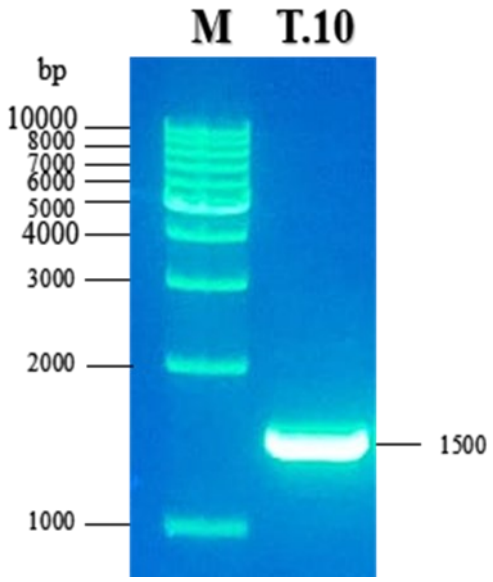

Figure 4. An electropherogram of the amplified 16S rRNA gene of isolate T10 with a size of 1500 bp. Marker (M): 1 kb DNA ladder.

Analysis of Hydrolysed Products by The Selected Bacterial Amylases Using a TLC Method

Starch hydrolysis products were assayed by oligosaccharide profile analysis on the amylase-hydrolyzed samples from the fourth isolates (T10, D3, A3, and S1) qualitatively. The results of TLC analysis showed that isolate T10 produced three bands, namely maltotriose (M3), maltotetraose (M4), and maltopentaose (M5), isolate S1 produced two bands, namely maltotriose (M3) and maltotetraose (M4), while two amylases of the isolates D3 and A3 were unable to hydrolyze starch (Figure 6).

Based on the TLC chromatogram, the starch degraded by T10 amylase resulted in malto-oligosaccharides of maltotriose, maltotetraose, and maltopentaose. Amylases can break down starch polymer bonds into shorter oligosaccharides or simple sugar molecules (Putri et al. 2012). The results showed that amylolytic bacteria with high amylolytic indexes (AI) did not correlate with their ability to degrade amylum. The isolate T10, with its total diameter lower than isolate D3, showed a higher ability to break down starch polymer bonds into shorter or oligosaccharides. The results of this study proved that a high AI value is only sometimes accompanied by the high ability of the amylase to break down starch polymer bonds. The ability of the T10 amylase to produce the maltooligosaccharides was similar to the amylase of *Bacillus circulans* GRS 313 isolated from soil that also produced maltotriose, maltotetraose, and maltopentaose (Dey et al. 2002). On the contrary, Rahmani et al. (2013) found maltose and maltotriose produced by amylase of *Brevibacterium* sp. using black potato starch as substrate, while amylase of *Bacillus subtilis* strain SDP1 isolated from rhizosphere of Acacia produces maltotriose and maltotetraose (Ozturk et al. 2014). Furthermore, Abdul-Manas et al. (2014) reported that amylase of an alkaliphilic *Bacillus lehensis* G1 could degrade oligosaccharides by producing maltooligosaccharides with a higher degree of polymerization than maltoheptaose observed on thin-layer chromatography and high-performance liquid chromatography analyses.

Crude Enzyme Production of a Selected Isolate and Measurement of its Amylase Activity at Different Culture Incubations

Based on the ability of the fourth selected amylolytic bacteria to produce different types of hydrolysed product, isolate T10 was further assayed for

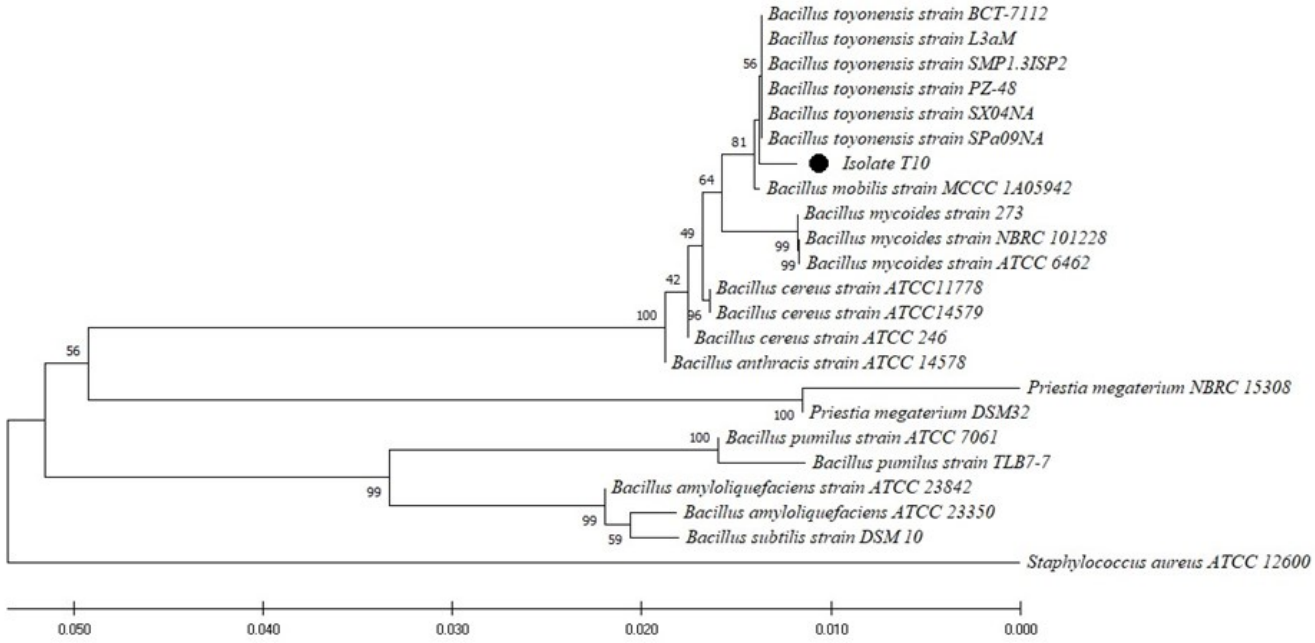

Figure 5. A phylogenetic tree showing the relationship between strain T10 isolated from rhizospheres of Canna (*C. edulis*) and several species members of the genus *Bacillus* on the basis of 16S rRNA gene sequence reconstructed based on Neighbor-Joining, Model Maximum Composite Likelihood, and 1000x Bootstrapping. The analysis used a MEGA10 program and *Staphylococcus aureus* ATCC 12600 as an outgroup.

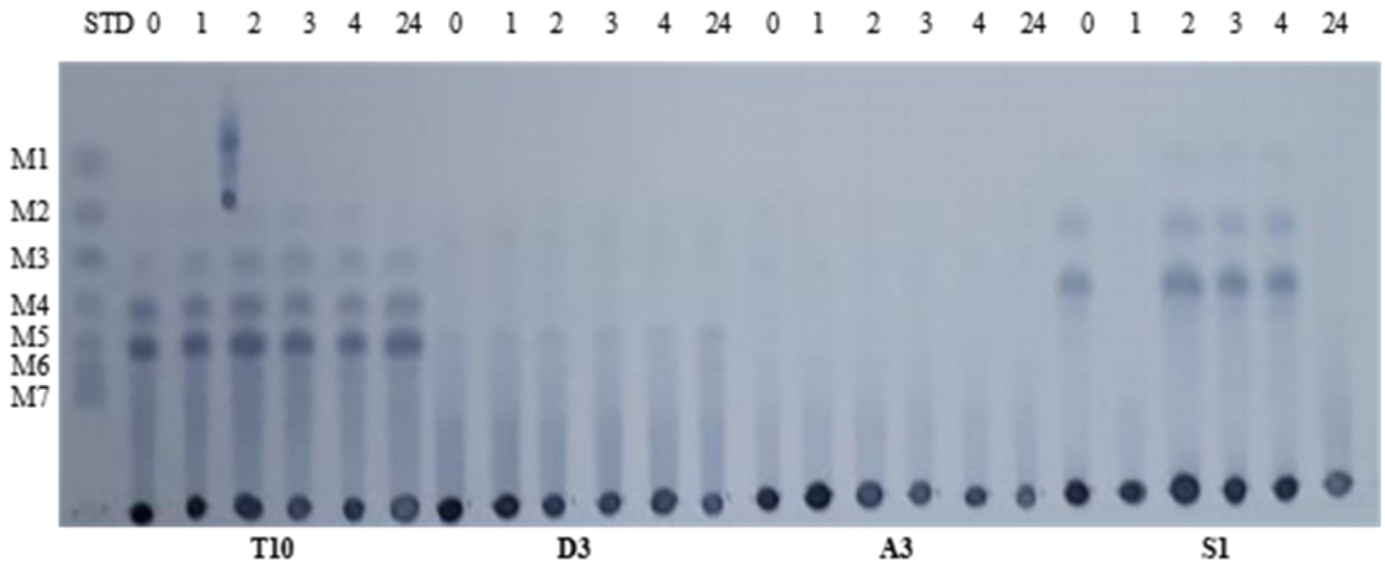

Figure 6. The product profile of starch hydrolyzed by amylase of the amylolytic bacteria (T10, D3, A3, and S1) using a TLC method with reaction times (hours): of 0, 1, 2, 3, 4, and 24 at 30°C. The Standards (STD): monosaccharide (M1), maltose (M2), maltotriose (M3), maltotetraose (M4), maltopentaose (M5), maltohexaose (M6) and maltohepta (M7).

its optimal amylase activity at different incubation times. The results showed that incubation times affected the amylase activity of isolate T10 carried out in a 0.5% starch solution at 30°C in 50 mM acetate buffer of pH 6. The amylase activity of T10 was optimal during incubation 1-3 days ranging from 0.546-0.717 U/mL and the highest amylase activity was found at 24 hr incubation of 0.717 U/mL (Figure 7). The results also showed that amylase activity decreased after 72 h of incubation. The amylase activity value at day 0 is quite high, this might be due to the measurement of the enzyme activity using the DNS method, in which reduc-

ing sugar formed from a carbon source (starch) is used by bacteria for the initial stages of growth; then, the bacteria will use the carbon source for the production of enzymes.

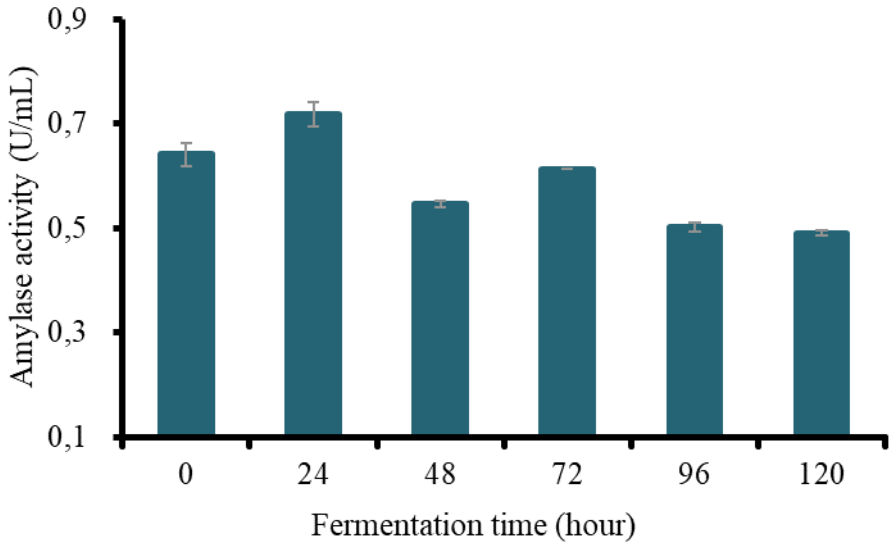

Figure 7. Amylase activity of T10 at different incubation times.

The amylase activity of *Bacillus cereus* KN isolated from Ranu Ngebel and incubated for three days was 0.016 U/mL, while strain G20 isolated from Ranu Grati was lower at about 0.0001 U/mL (Nisa et al. 2021). Luang et al. (2019) found *Bacillus* sp. 3.5AL2 isolated from soils of the unexplored Nasinuan Forest, Thailand and incubated for three days exhibiting amylase activity of 1.97 U/mg protein at the optimal conditions of 60°C and pH 7.0 after 30 min incubation with 1% starch in 0.05 M phosphate buffer. Gebreyohannes (2015) reported that the amylase activity of *Bacillus* spp. decreased after 48 h incubations due to the suppression and accumulation of other byproducts in the fermentation medium and also depletion of nutrients.

Enzyme Characterization: The Effect of pH and Temperature Against Enzyme Activity of The Selected Isolate

The effects of pH's on the amylase activity of isolate T10 showed that optimum conditions were in sodium acetate buffer pH 6 with an amylase activity of 0.262 U/mL and in sodium phosphate buffer pH 7 with an amylase activity of 0.341 U/mL (Figure 8). The optimal pH of isolate T10 was by Naidu et al. (2019) for *Paenibacillus* sp. D9 that its optimal pH for amylase activity is in the neutral range (pH 6-8). The increase in pH beyond these values resulted in a decline in enzyme activity. Any change in pH causes a change in the enzyme's active site (Lim & Oslan 2021). Bajpai et al. (2015) reported that the optimal pH for amylase activity of *Haloferax* sp. HA10 was at pH 7.0. According to Asgher et al. (2007), each enzyme has an optimal pH to work most actively, and the optimal pH of amylase is varied from pH 3.8 to 9.5 depending on the type of enzyme and the source. Behal et al. (2016) reported an amylase produced by *Bacillus* sp. AB04 had optimal activity at pH 8. Moreover, the enzyme is stable in neutral to alkaline (pH 7-10).

The amylase activity of isolate T10 was observed at temperatures ranging from 30-90°C at pH 7.0. Amylase activity of the T10 isolate tended to be optimum at 40°C with an activity value of 0.801 U/mL (Figure 9). A similar finding was also reported by Sivaramakrishnan et al. (2006) for several species of *Bacillus* sp., *B. subtilis*, *B. stearothermophilus*, *B.*

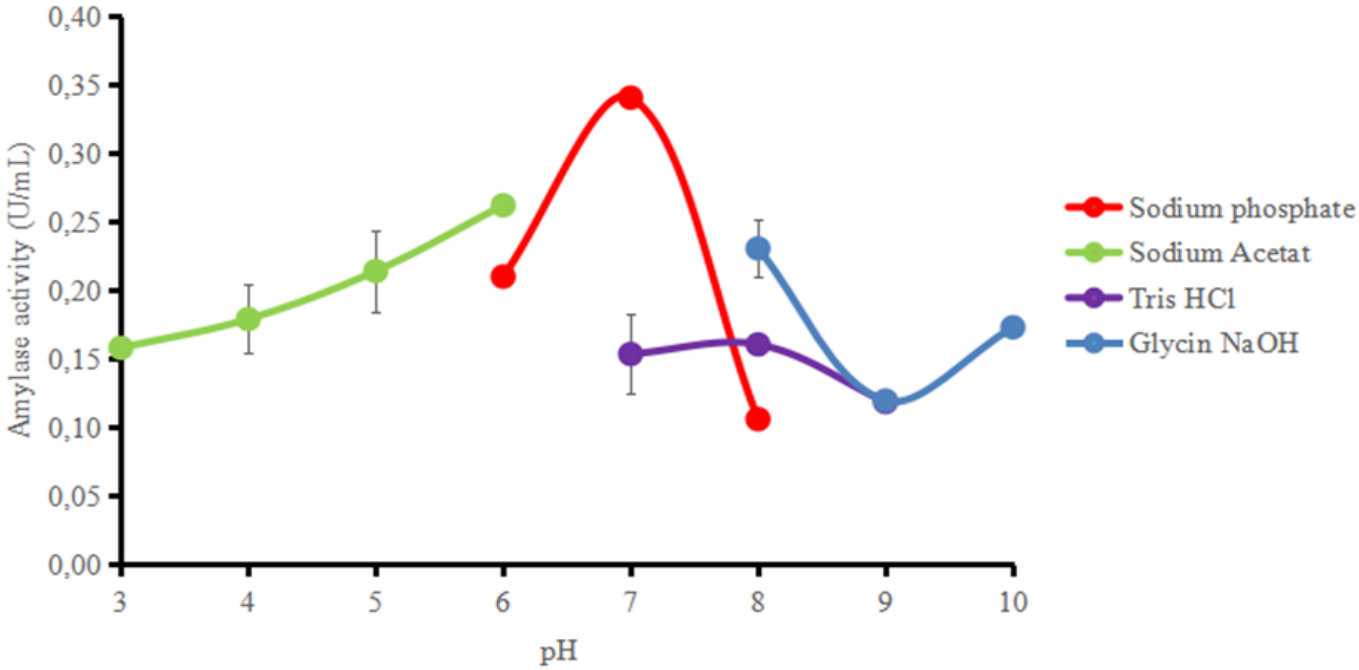

Figure 8. Amylase activity of T10 at different pH and buffers.

licheniformis, and *B. amiloliquefaciens* have optimum temperatures of 37-60°C. Gebreyohannes (2015) found that the maximum amylase activity of *Bacillus* spp. was 40°C and *Streptomyces* spp. at 37°C, used 4% starch concentration at a neutral pH and an incubated for 48 h. The crude enzyme of *Bacillus* sp. AB04 showed maximum activity at pH 8 with an optimum temperature of 40° C with more than 75% activity in range of 50 - 80° C (Behal et al. 2016). The results showed that either pH or temperature significantly affected the enzyme activity of the T10 amylase which was optimum at pH 7.0 and a temperature of 40°C.

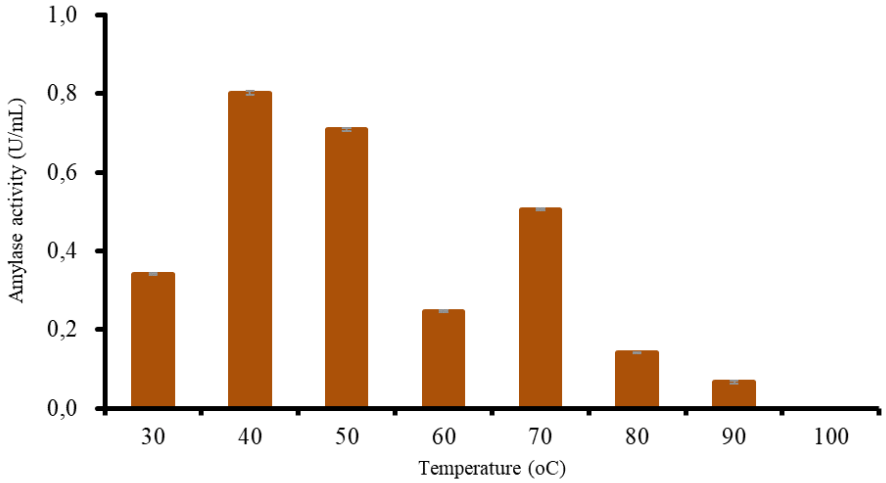

Figure 9. Amylase activity of isolate T10 at different temperatures.

The differences in the pH and temperature characteristics of enzyme activity indicated that enzymes are specific, depending on the species that produces them. A decrease or increase in temperature can affect the secretion of extracellular enzymes by changing the physiology of the cell membrane (Rahmani et al. 2018). The optimum temperature is the temperature that causes chemical reactions at the most incredible speed (Subagiyo et al. 2017). The results showed that after reaching the optimum condition, it was seen that the activity of the T10 amylase de-

creased. High temperatures can cause enzymatic reactions to decrease because enzyme proteins undergo conformational changes so that protein molecules will experience denaturation (Yufinta et al. 2018).

The production of a specific maltooligosaccharide in high yield through the enzymatic hydrolysis of starch is of considerable commercial interest. This has been achieved on an industrial scale after discovering a suitable maltooligosaccharide-forming amylase (MFA_{ses}). Moreover, several studies have tried to improve existing methods by increasing the yields of M3 and M5. These studies have included efforts to find new wild-type strains producing $MFA_{ses,}$ construct novel systems to achieve large-scale MFA_{ses} expression, and immobilize MFA_{ses} for stability and productivity (Ben-Ali et al. 2006). MFA_{ses} from *Bacillus toyonensis*, a novel M5-amylase, seems promising for the manufacture of high M5 syrups from starch and may apply to starch processing technologies due to their particular activity, unique substrate specificity, and endo-type action pattern (Pan et al. 2017).

CONCLUSIONS

It can be concluded that amounts of 32 amylolytic bacteria were isolated from rhizosphere and plant tissue of *Canna edulis*, as well as litter; the selected amylolytic bacterial isolate of T10 was capable of hydrolysing starch by producing maltotriose (M3), maltotetraose (M4) and maltopentaose (M5); and the identity of the selected isolate T10 belonged to a species member of *B. toyonensis* based on phenotypic and phylogenetic characterizations.

AUTHORS CONTRIBUTION

RNA designed, collected, and analysed the research data, O, NR and NE supervised all the process, and re-wrote the manuscript.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest in this research.

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Isolation and Characterization of Rhizospheric Bacteria Associated with Canna Plant for Production of Maltooligosaccharide Amylase

by Oedjijono Oedjijono

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Research Article

Isolation and Characterization of Rhizospheric Bacteria Associated with Canna Plant for Production of Maltooligosaccharide Amylase

Ria Dwi Agustiani[1,3], Oedjijono[1]*, Nanik Rahmani[2], Nuraeni Ekowati[1]
1)Faculty of Biology, University of Jenderal Soedirman, Jalan dr. Soeparno 63 Purwokerto 53122, Indonesia.
2)Center for Applied Microbiology Research, Research Institute for Life and Environmental Sciences, National Research and Innovation Agency (BRIN), Cibinong, Bogor 16911, Indonesia.
3)Department of Biology, Faculty of Science and Technology, International Women University, Bandung 40173, Indonesia.
* Corresponding author, email: oedjijono@unsoed.ac.id

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ABSTRACT

The objectives of the study were to isolate amylolytic bacteria from the rhizosphere and plant tissue of *Canna edulis* Ker., as well as litter; to know oligosaccharide compounds produced from starch hydrolyzed by the bacterial enzymes, and to identify the amylolytic bacteria based on phenetic and 16S rRNA gene sequences. From the rhizosphere, Canna plant tissue, and litters obtained thirty-two amylolytic bacterial isolates. Eight isolates (TH6, TH7, T5, T10, D2, D3, A3, S1) produced high clear zone diameters ranging from 18-30 mm; especially an isolate T10, which was consistent in producing a total clear zone diameter of 20 mm. The hydrolysate of starch hydrolysed by the T10 amylase resulted in three oligosaccharide compounds maltotriose, maltotetraose, and maltopentose. The amylase activity of isolate T10 was optimal at a temperature of 40°C and pH at 0.801 U/mL. The isolate T10 was identified as a species member of *Bacillus toyonensis* based on phenotyphic characterization and 16S rDNA gene sequencing analysis with a similarity value of 99.93%.

INTRODUCTION

Oligosaccharides are members of an essential group of carbohydrates. Macromolecules with short-chain polysaccharide sugars of 2 to 20 saccharide units. Functional oligosaccharides such as galactooligosaccharide (GOS), fructooligosaccharide (FOS), and maltooligosaccharide (MOS) are well-known prebiotics owing to their ability to selectively stimulate beneficial bacteria in the intestines, particularly bifidobacterial species (Zhao et al. 2017). Developing oligosaccharide products is one of the businesses with high economic value. Plants such as Canna contain much starch, one of the crucial ingredients (substrate) to produce oligosaccharides enzymatically. Canna plants (*Canna edulis* Ker.) contain high levels of carbohydrates, mainly starch (93.3%), which consists of amylose (33.48%) and amylopectin (59.82%) (El-Fadl et al. 2012). Starch is hydrolysed into smaller oligosaccharides by α-amylase, one of the most important commercial enzymes (Jang et al. 2020).

The starch-processing industry has exploited amylase as a substitute for acid hydrolysis in producing starch hydrolysis. Amylase acts as a biocatalyst for the hydrolysis of starch into simpler carbohydrates, such

as glucose, maltose, and dextrin (Divakaran et al. 2011; Abdalla et al. 2021). Amylolytic bacteria are producers of amylase that can be used as biocatalysts in the starch hydrolysis process (Ding et al. 2021) to produce various maltooligosaccharide products, such as maltotriose, maltotetraose, maltopentaose, and maltohexaose (Pan et al. 2017).

Canna plants and its surrounding, including the rhizosphere and plant tissues, can be sources of isolating amylolytic bacteria. The high starch content in canna tubers makes them a suitable substrate for growing various bacteria, especially amylolytic bacteria. The bacteria isolated from starch-rich sources generally have the potential to produce amylase with high activity (Hellmuth & van den Brink 2013). In addition, the rhizosphere is known as the most diverse microbial habitat concerning species richness and community size. The interaction between plant roots and microorganisms is intensive around the rhizosphere, because the plants secrete exudates containing carbohydrates, amino acids, and other nutrients utilized by bacteria for growth. On the contrary, rhizospheric bacteria can produce proteins and enzymes that are important for the biological function of host plants (Afifah et al. 2018).

Bacteria, fungi, plants, and animals play an important role in utilizing polysaccharides. Members of the genus *Bacillus* were known to produce various enzymes, such as amylase that have been used in many industries, such as fermentation, textiles, paper, medicine, and sugar (Gupta et al. 2003). They are derived mainly from *Bacillus licheniformis* and *B. amyloliquefaciens*. Moradi et al. (2014) found several bacterial isolates producing high amylolytic enzymes, which were subsequently identified as *Bacillus cereus*, *B. amyloliquefaciens*, *B. licheniformis*, and *Paenibacillus lautus*. Luo et al. (2021) isolated *Bacillus toyonensis* P18, a group of Gram-positive bacteria belonging to the *Bacillus cereus* group and often used as probiotics or biocontrol agents. The bacterium has also been known to be treated as a probiotic for preventing microbial diseases in crops or improving the immune response of animals (Santos et al. 2018).

The objectives of the study were to isolate amylolytic bacteria from rhizosphere and plant tissue of Canna, as well as litter; to know oligosaccharide compounds produced from hydrolysate of starch hydrolysed by the bacterial enzymes; and to identify the selected amylolytic bacteria based on 16S rRNA gene sequences.

MATERIALS AND METHODS

Sample Collection and Location of Sampling

Samples were taken from the rhizosphere and parts of Canna plant (*C. edulis* Ker.) including tubers, stems, leaves, tissue, as well as litter growing in two places, namely in the forest and the community gardens around the Perhutani Forest West Banyumas, Central Java, Indonesia. The coordinates of the former are S 07°20.846 'E 109°06.410 and the latter is S 07 °20.812 'E 109°05.92 (Figure 1).

Isolation, Screening, and Morphological Characterization of Amylolytic Bacteria

Plant tissues and litter were cleaned with running water, then cut into 1 cm long pieces and separated according to the plant part. The sample pieces were immersed in 70% alcohol for 1 minute, then in 1% sodium hypochlorite solution for 3 minutes, after which they were soaked again using 70% alcohol for 1 minute, and rinsed with sterile distilled water three times (Duan et al. 2021, with modification). The sterile samples were placed on sterile tissue papers and then crushed using a mortar and one gram of each sample was diluted with 9 mL of sterile distilled water,

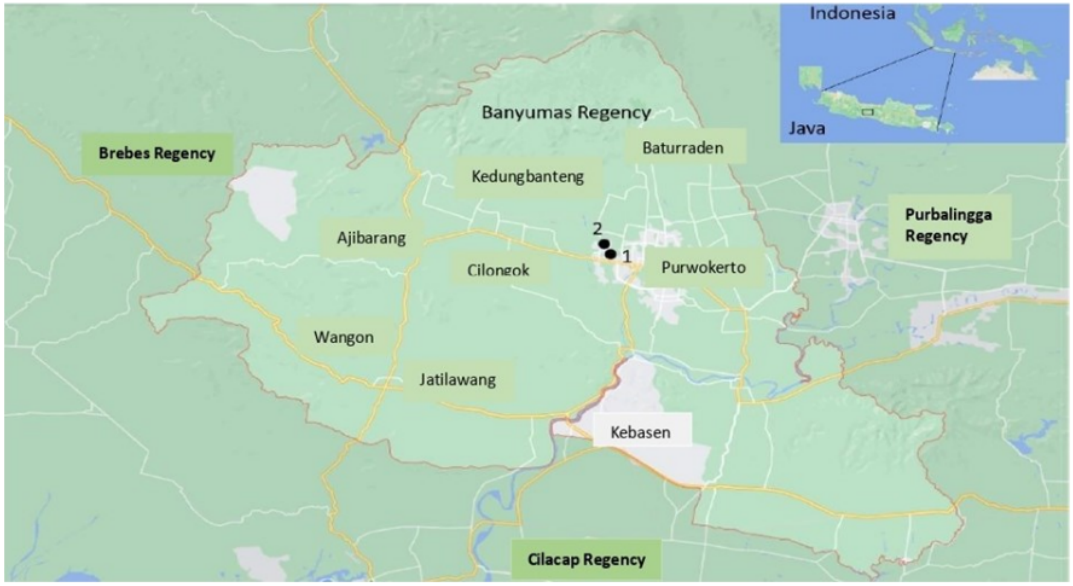

Figure 1. A map of Banyumas Regency and sampling sites: (1) the Perhutani Forest KPH West Banyumas, Central Java, (2) Community gardens around the Perhutani Forest KPH West Banyumas, Central Java.

and then serial dilutions were made up to 10^{-7}.

One gram of Canna rhizospheric soil was put into a 20 mL of nutrient broth (NB) medium containing 1% soluble starch (Merck) in a 100 mL Erlenmeyer flask. The solution was then homogenized in an agitation speed shaker machine at 150 rpm and incubated for 24 hours at 30 °C. The amount of 1 mL of the solution was diluted with 9 mL of sterile distilled water, and then serial dilutions were made up to 10^{-7}.

One mL from each series of dilutions was inoculated onto nutrient Agar (NA) medium containing 1% soluble starch using a pour plate method. The plates were then incubated for 24 hours at 30 °C. Each growing bacterial colony was then inoculated onto an NA medium containing 1% soluble starch and purified using a streak quadrant method.

The number of 0.5L of bacterial cultures aged 24 hours at 30°C growing on NB medium containing 1% soluble starch was spot inoculated onto NA medium containing 1% commercial soluble starch, then incubated for 72 hours at 30°C. The growing colonies were flooded with iodine solution, and the clear zones formed around the colonies were observed and measured in their diameter (Vijayalakshmi et al. 2012). The isolates having high diameter clear zones were selected and tested for their enzyme production. The colonial and cell morphology of the isolated bacteria were characterized using conventional methods (Smibert & Krieg 1981).

Phenetic and Phylogenetic Characterizations of the Selected Bacterium

Phenetic characterizations of the selected bacterium (producing high diameter clear zones and maltooligosaccharide enzyme) including colony morphology, cell morphology, and biochemistry, were conducted by conventional methods (Smibert & Krieg 1981). Biochemical tests were also conducted using the API 50CHB kit.

The 16S rDNA gene was amplified by polymerase chain reaction (PCR) technique using a pair of primers (9F: 5'GAGTTTGATCCTCCTGGCTCAG-3') 1510R: 5'GGCTACCTTGTTACGA-3')

(Yopi et al. 2017). The obtained bands were stained and visualized by UV Transilluminator. The sequence was confirmed via 1st BASE Sequencing, Malaysia. The 16S rDNA nucleotide sequences were analyzed by nucleotide BLAST (Basic Local Alignment Search Tool) search in the Gene Bank of National Center for Biotechnology Information (NCBI) or BLAST for amino acid analysis (Zhuang et al. 2012). The phylogenetic tree was constructed using a neighbour-joining algorithm in MEGA 6.0 software (De-Moraes-Russo & Selvatti 2018).

Analysis of The Hydrolysis Products by A Thin-Layer Chromatography Method (Rahmani et al. 2013)

An amount of 2 mL of each 24 h old bacterial culture (four selected isolates) was inoculated into a 200 mL flask containing 20 mL of NB medium plus 1% (w/v) starch solution, pH 6 (50 mM acetate buffer) and incubated at 30 °C for 24 h. The culture was sampling every 24 hour and then centrifugated, and the supernatant obtained was tested for its amylolytic activity.

The hydrolytic activity of amylase in a substrate solution was carried out at 30°C in 50 mM acetate buffer, pH 6, containing 0.5% of commercial starch. The enzyme-substrate ratio (v/v) was 1:1 and the reaction times were in hours (0, 1, 2, 3, 4, 24). Reactions were carried out in 2 mL Eppendorf containing 1 mL of reaction mixture in a Deep Well Maximizer (Bioshaker M-BR-022UP, Taitec Japan).

A Thin Layer Chromatography (TLC) of maltooligosaccharide products was carried out on silica gel $60F_{254}$ plates (Merck Art 20-20 cm) and eluent using a solvent mixture of n-butanol:acid:water (12:6:6, v/v/v). Spots formed were visualized by spraying the sugar colours (0.5 g α-diphenylamine, 25 mL acetone, 2.5 mL phosphate acid, 0.5 mL aniline). All samples were applied equal quantities (4 µL). Glucose (Sigma-Aldrich, U.S.A), maltose (M2), maltotriose (M3), maltotetraose (M4), maltopentaose (M5), maltohexaose (M6), and maltoheptaose (M7) (Megazyme) were used as standards.

Crude Enzyme Production and Amylase Activity at Different Fermentation Time

An amount of 2 mL of the 24 h old bacterial cultures (isolate T10) was inoculated into a 200 mL flask containing 20 mL of NB medium plus 1% (w/v) starch solution, pH 6 (50 mM acetate buffer), and incubated at 30 °C for five days. The culture was sampled every 24 hours and then centrifuged and the supernatant obtained was tested for its amylase activity.

The enzyme reaction was conducted as above when measuring amylase activity using a DNS method (Miller 1959). The absorbance of the solution was measured using a spectrophotometer at a wavelength of 540 nm. The enzyme activity (U/mL) was calculated based on the equation:

$$\text{enzyme activity} = \frac{c \times d \times 1000}{t \times mw} \text{ U/mL}$$

Where c: amylase concentration; d: dilution; t: incubation time; mw: molecular weight.

A standard curve used D-Glucose at various concentrations. One unit of amylase activity is defined as the amount of enzyme that liberates one µmol of D-Glucose per minute under the experimental condition given.

Effect of pH and Temperature on Enzyme Activity of the Selected Isolate

The optimal pH of the enzyme activity was done at pH ranges of 3.0-10.0 under standard assay conditions. Various buffers (0.05M) used were sodi-

um acetate (pH 3.0-6.0), sodium phosphate (6.0-8.0), Tris-HCL (pH 7.0-9.0), and Glycine-NaOH (pH 8.0-10.0). The enzyme reactions were incubated at 40°C for 30 min in the presence of 0.5% (w/v) starch solution (Merck)). The effect of temperatures on enzyme activity was conducted at temperatures ranging from 30-90 °C in 50 mM acetate buffer at optimum pH for 30 min. Amylase activity was assayed by DNS method (Miller 1959).

RESULTS AND DISCUSSION

Isolation and An Amylolytic Assay of Bacteria Isolated from The Rhizosphere and Plant Tissue of Canna, and Litter

The results of the study found 32 bacterial isolates growing on NA medium supplemented with 1% soluble starch, with details: 11 isolates were from the rhizosphere of the Canna growing in the forest, 12 isolates from the rhizosphere of the Canna growing in the people's gardens around the forest, four isolates from the leave tissue of the Canna growing in the people's gardens around the forest, three isolates from the roots of the Canna growing in the people's gardens around the forest, and two isolates were from the litters of the Canna in the gardens of the residents around the forest (Table 1).

The ability of the bacteria to grow and produce clear zones in the medium indicates that those bacteria were capable of producing amylase. The more amylase is released, the wider clear zones are produced due to the degradation of amylum in the medium, resulting in enhancing the amylolytic index (Ginting et al. 2021). The research results showed that eight isolates of TH6, TH7, T5, T10, D2, D3, A3, and S1 showed high total clear zone diameters (mm) of 18, 18, 18, 20, 18, 30, 18, and 18, respectively (Table 1). The consistency of the bacterial isolates, resulting in the total clear zone diameter, was shown by the isolates TH6, T10, D3, A3, and S1, while the other isolates tended to reduce or lose their amylolytic activity (Figure 2). Based on the ability of isolates to produce a clear zone diameter ≥ 18 mm and consideration of source representatives, five isolates (TH6, T10, D3, A3, and S1) were selected for further testing, namely their ability to hydrolyze starch. Hasanah et al. (2020) reported that bacterial isolates having an amylolytic index of more than 9 mm were potentials to produce amylase. According to Ochoa-Solano & Olmos –Soto (2006), bacterial isolates produce clear zones two or three times the diameter of the colony are potential enzyme producers.

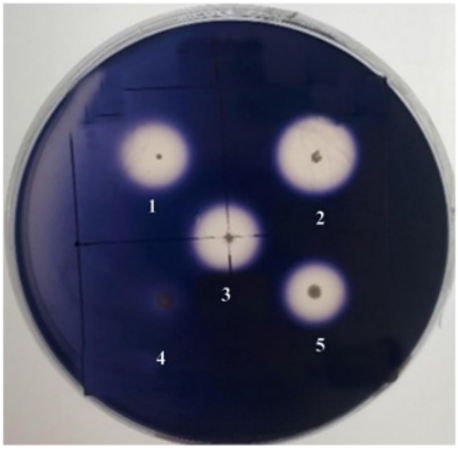

Figure 2. The amylolytic zones produced by bacterial isolates of: (1) T10, (2) D3, (3) A3, (4) TH6, and (5) S1 on a NA medium + 1% soluble starch.

Table 1. Sources, number, and total clear zone diameter of amylolytic bacteria isolated from the rhizosphere, plant tissues of Canna, and litter.

Source of bacterial isolates	Isolate code	Total clear zone diameter (mm)
Rhizospheres of the Canna plants growing in the forest	TH1	16
	TH2	15
	TH3	17
	TH4	16
	TH5	16
	TH6	18
	TH7	18
	TH8	17
	TH9	17
	TH10	16
	TH11	16
Rhizospheres of the Canna plants growing in the people's gardens around the forest	T1	15
	T2	16
	T3	16
	T4	16
	T5	18
	T6	17
	T7	15
	T8	15
	T9	15
	T10	20
	T11	17
	T12	16
Leaves of the Canna plant tissue growing in the people's gardens around the forest	D1	16
	D2	18
	D3	30
	D4	15
Roots of the Canna plant tissue growing in the people's gardens around the forest	A1	17
	A2	16
	A3	18
Litters of the Canna plant from the gardens of the residents around the forest	S1	18
	S2	16

A high number of amylolytic bacteria isolated from the rhizosphere of Canna was by Vaseekaran et al. (2010), who stated that bacteria isolated from starch-rich materials have better potential to produce amylase. Vijayalakshmi et al. (2012) found *Bacillus subtilis* KC3 isolated from the rhizosphere of *Euphorbia hirta* produced a maximum halo zone of 23 mm on a Starch Agar medium. Gebreyohannes (2015) reported that 16 bacterial isolates from soils could produce clear zones of 3-22 mm on starch agar plates. Ginting et al. (2021) found thermophilic bacteria of *Bacillus* sp. L3 and *B. caldotenax* L9 from a marine hydrothermal produces high amylolytic indexes of 3.04 and 3.52, respectively. The clear zone formed results from breaking starch compounds into simple compounds; the wider the clear zone formed, the higher the amylolytic activity (Zubaidah et al. 2019).

The characteristics of colonial morphology of the 34 isolates were rough, dry, bright, and pink; cells were Gram-positive, rod shape, motile, and had endospores. The endospore position of isolates D1-D4 and S1-S2 was in terminal, while isolates of TH1-TH11, T1-T12, and D1-D4 had endospores in the centre. All isolates were able to hydrolyse starch and produce lecithinase (Table 2). Those characteristics indicated that the bacteria were members of the genus *Bacillus*. According to Logan & De

Table 2. Morphological and physiological properties of the bacteria isolated from Canna plants and their surrounding.

Characteristics	Isolate code				
	TH1-TH11	T1-T12	D1-D4	A1-A3	S1-S2
Colonial morphology on NA Agar	Rough, dry, bright, and Pink	Rough, dry, bright and pink	Rough, dry, bright and pink	Rough, dry, bright and pink	Rough, dry, bright, and pink
Gram reaction	+	+	+	+	+
Cell shape	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+
Presence of spore	+	+	+	+	+
Position of spore	Centre	Centre	Terminal	Centre	Terminal
Starch hydrolysis	+	+	+	+	+
Lecithinase production	+	+	+	+	+

Vos (2009), the main characteristics of the genus *Bacillus* are cells rod-shaped, straight or slightly curved, occurring singly and in pairs, some in chains, form endospores, Gram-positive or Gram-negative, motile, aerobes or facultative anaerobes, and mostly isolated from soil.

Identification of The Selected Isolate of T10 Based on Phenetic and Phylogenetic Characteristics

Based on the ability of the selected isolate to produce malto-oligosachharides of maltotriose, maltotetraose, and maltopentaose (Method 3.3); further characterization of the isolate T10 was conducted. The isolate had colonial morphology of irregular with undulate edges, opaque, cream-coloured, and had a granular texture. The cells formed endospores, facultatively anaerobic, Gram-positive, rod-shaped, motile, and occurring singly or in chains (Table 2, Table 3, Figure 3). These characteristics include biochemistry, physiology, and nutrition, indicating that isolate T10 was similar to those typical of the species *Bacillus cereus*. This species is a species complex within the genus *Bacillus*, with members including *B. anthracis*, *B. thuringiensis*, *B. mycoides*, and *B. toyonensis* (Luo et al. 2021).

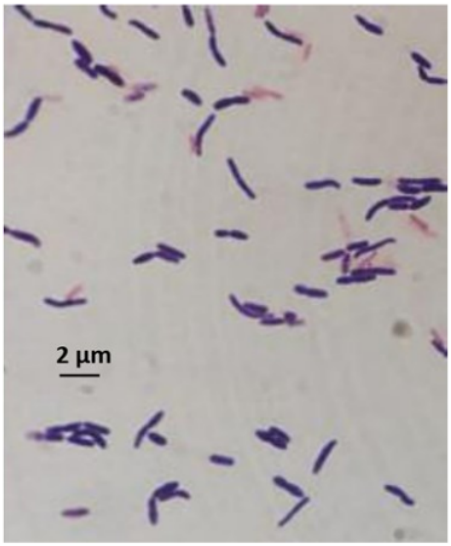

Figure 3. The appearance of bacterial cells isolate T10 under a microscope with a magnification of 1000x. The cells appear single or in chains.

Table 3. Phenotypic characterization of the isolate T10.

Characteristics	Isolate T10
Cell length (µm)	3.00 - 4.00
Egg-yolk lecithinase	+
Anaerobic growth	+
Rhizoid colony	-
Parasporal crystal	-
Growth temperature range (°C)	10 - 45
Optimal growth temperature (°C)	35
Salinity tolerance range (%NaCl)	≤ 4
API 50CHB	
Glycerol	-
D-Ribose	+
D-Mannose	+
Methyl-αD-glucopyranoside	+
Amygdalin	+w
Arbutin	+
Salicin	+
Cellobiose	-
D-saccharose	+
D-trehalose	+
Starch	+
Glycogen	+
D-turanose	+

The electrophoresis visualization of the PCR product showed that the DNA of T10 produced a single band with a size of 1500 kb (Figure 4). The results of comparing the 16S rRNA gene sequence of isolate T10 and nucleotide sequences in the GeneBank (http://blast.ncbi.mlm.nih.gov/) showed that the bacterium is closely related to species members of the genus *Bacillus*. The BLAST analysis showed that isolate T10 had a similarity of 99.3% with either *Bacillus toyonensis* SPa09NA, *B. toyonensis* PZ-48, or *B. toyonensis* SMP1. The phylogenetic tree was constructed using Neighbor-Joining, Model Maximum Composite Likelihood, and 1000x Bootstrapping. A dendrogram resulted from MEGA10 program showed that isolate T10 joined *B. toyonensis* SX04NA, *B. toyonensis* Spa09NA, *B. toyonensis* SMP1, *B. toyonensis* PZ-48, *B. toyonensis* BCT-7112, and *B. toyonensis* l3aM to form a separate cluster (Figure 5). Hence, isolate T10 was identified as the species member of *B. toyonensis* based on the phenetic and phylogenetic characteristics.

B. toyonensis strain BCT-7112^T was first isolated in 1966 in Japan from a survey designed to obtain naturally occurring microorganisms as pure cultures in the laboratory for use as probiotics in animal nutrition (Jiménez et al. 2013). The strain was first identified as *B. cereus* var. *toyoi*, and it has been used as the active ingredient of the preparation TOYOCERIN, an additive for animal nutrition (e.g. swine, poultry, cattle, rabbits and aquaculture). Agamennone et al. (2019) isolated *B. toyonensis* strain VU-DES13 from the gut of the soil-dwelling springtail *Folsomia candida*, which was highly resistant to penicillin and inhibited the growth of a variety of pathogenic microorganisms. Its secondary metabolite clusters produce siderophores, bacteriocins, and nonribosomal peptide synthetases. Wang et al. (2021) reported that *Bacillus toyonensis* XIN-YC13 produced a novel antibiotic, toyoncin, with antimicrobial activity against *B. cereus* and *Listeria monocytogenes*. This antibiotic exerts bactericidal activity and induces cell membrane damage.

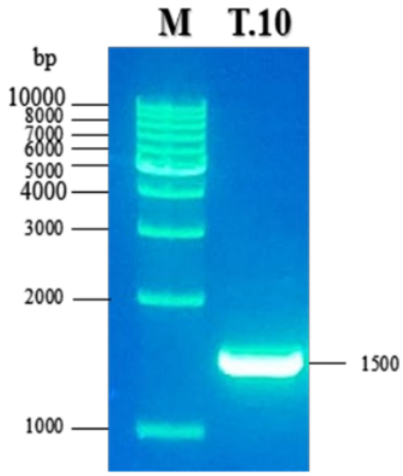

Figure 4. An electropherogram of the amplified 16S rRNA gene of isolate T10 with a size of 1500 bp. Marker (M): 1 kb DNA ladder.

Analysis of Hydrolysed Products by The Selected Bacterial Amylases Using a TLC Method

Starch hydrolysis products were assayed by oligosaccharide profile analysis on the amylase-hydrolyzed samples from the fourth isolates (T10, D3, A3, and S1) qualitatively. The results of TLC analysis showed that isolate T10 produced three bands, namely maltotriose (M3), maltotetraose (M4), and maltopentaose (M5), isolate S1 produced two bands, namely maltotriose (M3) and maltotetraose (M4), while two amylases of the isolates D3 and A3 were unable to hydrolyze starch (Figure 6).

Based on the TLC chromatogram, the starch degraded by T10 amylase resulted in malto-oligosaccharides of maltotriose, maltotetraose, and maltopentaose. Amylases can break down starch polymer bonds into shorter oligosaccharides or simple sugar molecules (Putri et al. 2012). The results showed that amylolytic bacteria with high amylolytic indexes (AI) did not correlate with their ability to degrade amylum. The isolate T10, with its total diameter lower than isolate D3, showed a higher ability to break down starch polymer bonds into shorter or oligosaccharides. The results of this study proved that a high AI value is only sometimes accompanied by the high ability of the amylase to break down starch polymer bonds. The ability of the T10 amylase to produce the maltooligosaccharides was similar to the amylase of *Bacillus circulans* GRS 313 isolated from soil that also produced maltotriose, maltotetraose, and maltopentaose (Dey et al. 2002). On the contrary, Rahmani et al. (2013) found maltose and maltotriose produced by amylase of *Bifidobacterium* sp. using black potato starch as substrate, while amylase of *Bacillus subtilis* strain SDP1 isolated from rhizosphere of Acacia produces maltotriose and maltotetraose (Ozturk et al. 2014). Furthermore, Abdul-Manas et al. (2014) reported that amylase of an alkaliphilic *Bacillus lehensis* G could degrade oligosaccharides by producing maltooligosaccharides with a higher degree of polymerization than maltoheptaose observed on thin-layer chromatography and high-performance liquid chromatography analyses.

Crude Enzyme Production of a Selected Isolate and Measurement of Amylase Activity at Different Culture Incubations

Based on the ability of the fourth selected amylolytic bacteria to produce different types of hydrolysed product, isolate T10 was further assayed for

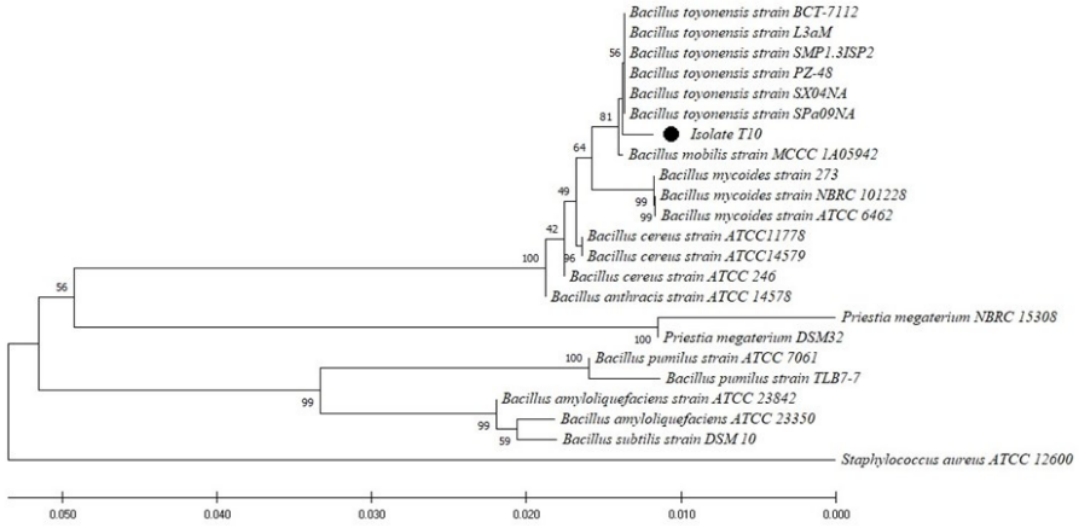

Figure 5. A phylogenetic tree showing the relationship between strain T10 isolated from rhizospheres of Canna (*C. edulis*) and several species members of the genus *Bacillus* on the basis of 16S rRNA gene sequence reconstructed based on Neighbor-Joining, Model Maximum Composite Likelihood, and 1000x Bootstrapping. The analysis used a MEGA10 program and *Staphylococcus aureus* ATCC 12600 as an outgroup.

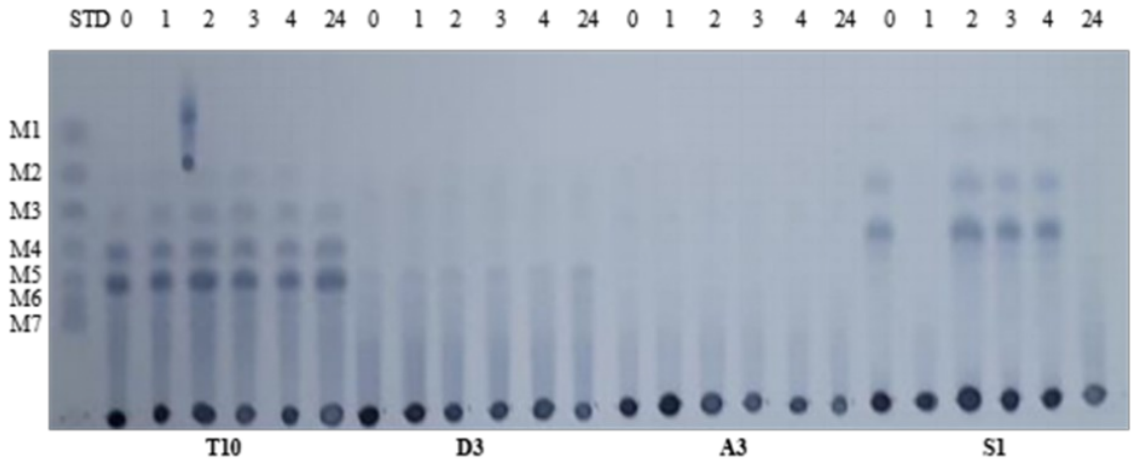

Figure 6. The product profile of starch hydrolyzed by amylase of the amylolytic bacteria (T10, D3, A3, and S1) using a TLC method with reaction times (hours): of 0, 1, 2, 3, 4, and 24 at 30°C. The Standards (STD): monosaccharide (M1), maltose (M2), maltotriose (M3), maltotetraose (M4), maltopentaose (M5), maltohexaose (M6) and maltohepta (M7).

its optimal amylase activity at different incubation times. The results showed that incubation times affected the amylase activity of isolate T10 carried out in a 0.5% starch solution at 30°C in 50 mM acetate buffer of pH 6. The amylase activity of T10 was optimal during incubation 1-3 days ranging from 0.546-0.717 U/mL and the highest amylase activity was found at 24 hr incubation of 0.717 U/mL (Figure 7). The results also showed that amylase activity decreased after 72 h of incubation. The amylase activity value at day 0 is quite high, this might be due to the measurement of the enzyme activity using the DNS method, in which reduc-

ing sugar formed from a carbon source (starch) is used by bacteria for the initial stages of growth; then, the bacteria will use the carbon source for the production of enzymes.

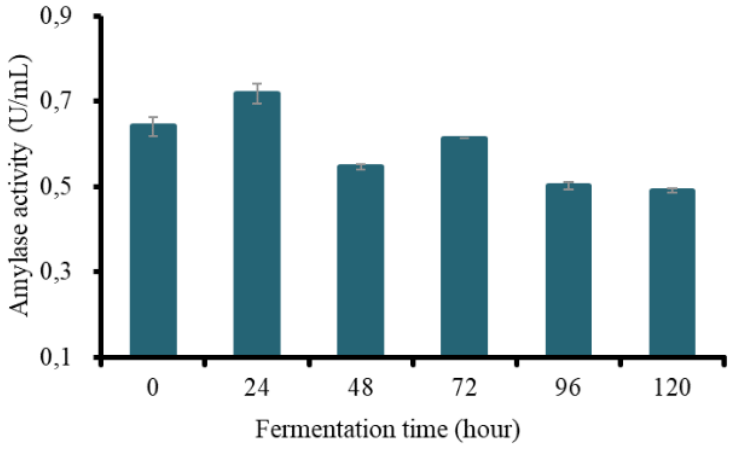

Figure 7. Amylase activity of T10 at different incubation times.

The amylase activity of *Bacillus cereus* KN isolated from Ranu Ngebel and incubated for three days was 0.016 U/mL, while strain G20 isolated from Ranu Grati was lower at about 0.0001 U/mL (Nisa et al. 2021). Luang et al. (2019) found *Bacillus* sp. 3.5AL2 isolated from soils of the unexplored Nasinuan Forest, Thailand and incubated for three days exhibiting amylase activity of 1.97 U/mg protein at the optimal conditions of 60°C and pH 7.0 after 30 min incubation with 1% starch in 0.05 M phosphate buffer. Gebreyohannes (2015) reported that the amylase activity of *Bacillus* spp. decreased after 48 h incubations due to the suppression and accumulation of other byproducts in the fermentation medium and also depletion of nutrients.

Enzyme Characterization: The Effect of pH and Temperature against Enzyme Activity of The Selected Isolate

The effects of pH's on the amylase activity of isolate T10 showed that optimum conditions were in sodium acetate buffer pH 6 with an amylase activity of 0.262 U/mL and in sodium phosphate buffer pH 7 with an amylase activity of 0.341 U/mL (Figure 8). The optimal pH of isolate T10 was by Naidu et al. (2019) for *Paenibacillus* sp. D9 that its optimal pH for amylase activity is in the neutral range (pH 6-8). The increase in pH beyond these values resulted in a decline in enzyme activity. Any change in pH causes a change in the enzyme's active site (Lim & Oslan 2021). Bajpai et al. (2015) reported that the optimal pH for amylase activity of *Haloferax* sp. HA10 was at pH 7.0. According to Asgher et al. (2007), each enzyme has an optimal pH to work most actively, and the optimal pH of amylase is varied from pH 3.8 to 9.5 depending on the type of enzyme and the source. Behal et al. (2016) reported an amylase produced by *Bacillus* sp. AB04 had optimal activity at pH 8. Moreover, the enzyme is stable in neutral to alkaline (pH 7-10).

The amylase activity of isolate T10 was observed at temperatures ranging from 30-90°C pH 7.0. Amylase activity of the T10 isolate tended to optimum at 40°C with an activity value of 0.801 U/mL (Figure 9). A similar finding was also reported by Sivaramakrishnan et al. (2006) for several species of *Bacillus* sp., *B. subtilis*, *B. stearothermophilus*, *B.*

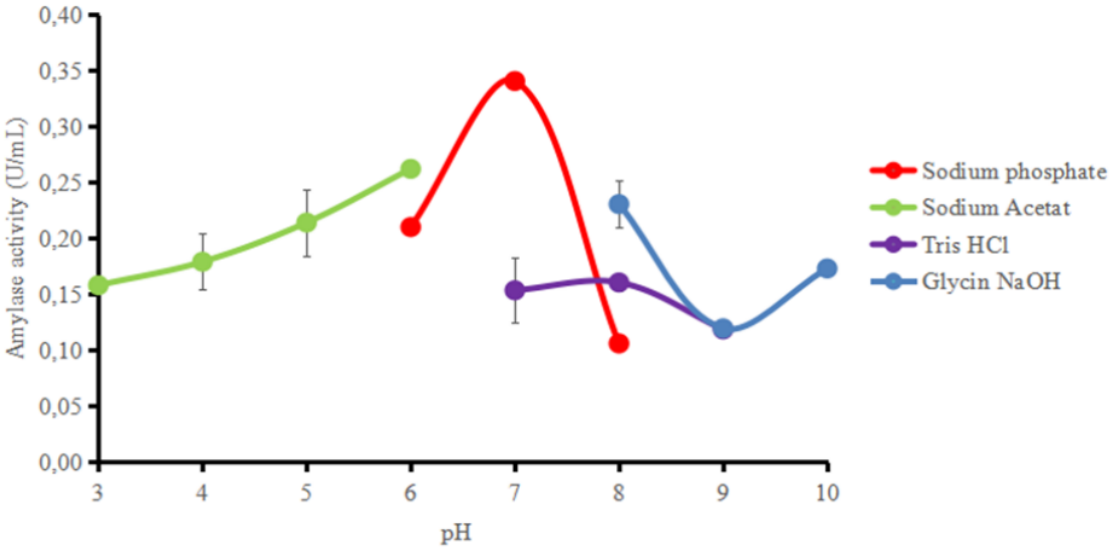

Figure 8. Amylase activity of T10 at different pH and buffers.

licheniformis, and *B. amiloliquefaciens* have optimum temperatures of 37-60°C. Gebreyohannes (2015) found that the maximum amylase activity of *Bacillus* spp. was 40°C and *Streptomyces* spp. at 37°C, used 4% starch concentration at a neutral pH and an incubated for 48 h. The crude enzyme of *Bacillus* sp. AB04 showed maximum activity at pH 8 with an optimum temperature of 40° C with more than 75% activity in range of 50 - 80° C (Behal et al. 2016). The results showed that either pH or temperature significantly affected the enzyme activity of the T10 amylase which was optimum at pH 7.0 and a temperature of 40°C.

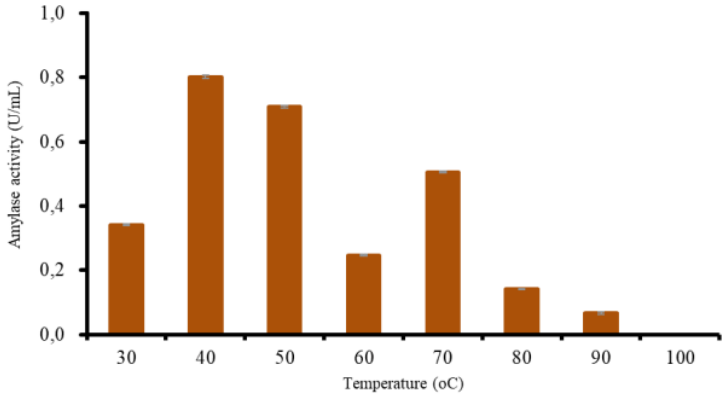

Figure 9. Amylase activity of isolate T10 at different temperatures.

The differences in the pH and temperature characteristics of enzyme activity indicated that enzymes are specific, depending on the species that produces them. A decrease or increase in temperature can affect the secretion of extracellular enzymes by changing the physiology of the cell membrane (Rahmani et al. 2018). The optimum temperature is the temperature that causes chemical reactions at the most incredible speed (Subagiyo et al. 2017). The results showed that after reaching the optimum condition, it was seen that the activity of the T10 amylase de-

creased. High temperatures can cause enzymatic reactions to decrease because enzyme proteins undergo conformational changes so that protein molecules will experience denaturation (Yufinta et al. 2018).

The production of a specific maltooligosaccharide in high yield through the enzymatic hydrolysis of starch is of considerable commercial interest. This has been achieved on an industrial scale after discovering a suitable maltooligosaccharide-forming amylase (MFA_{ses}). Moreover, several studies have tried to improve existing methods by increasing the yields of M3 and M5. These studies have included efforts to find new wild-type strains producing MFA_{ses}, construct novel systems to achieve large-scale MFA_{ses} expression, and immobilize MFA_{ses} for stability and productivity (Ben-Ali et al. 2006). MFA_{ses} from *Bacillus toyonensis*, a novel M5-amylase, seems promising for the manufacture of high M5 syrups from starch and may apply to starch processing technologies due to their particular activity, unique substrate specificity, and endo-type action pattern (Pan et al. 2017).

CONCLUSIONS

It can be concluded that amounts of 32 amylolytic bacteria were isolated from rhizosphere and plant tissue of *Canna edulis*, as well as litter; the selected amylolytic bacterial isolate of T10 was capable of hydrolysing starch by producing maltotriose (M3), maltotetraose (M4) and maltopentaose (M5); and the identity of the selected isolate T10 belonged to a species member of *B. toyonensis* based on phenotypic and phylogenetic characterizations.

AUTHORS CONTRIBUTION

RNA designed, collected, and analysed the research data, O, NR and NE supervised all the process, and re-wrote the manuscript.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest in this research.

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