



[JTBB] Notice for Initial Review Result External Inbox x

Salwa Shabria Wafi wafisalwa@gmail.com via ugm.ac.id
to Rina, me, Nanik, Nuraeni

Tue, Oct 25, 2022, 10:00

Dear Authors,

Thank you for submitting your manuscript entitled "Maltooligosaccharides-Forming Amylase of Canna Rhizosphere Bacteria: Isolation, Identification, and Characterization" to Journal of Tropical of Biodiversity and Biotechnology. After an initial review by our editorial team, your manuscript is considered suitable to be processed in the peer-review process. However, due to the current loads of the manuscripts in the under-review process, your manuscript is currently under queue to be assigned to the editor in charge. The estimation duration for your manuscript to be assigned and enter the under-review process is 4 weeks from this announcement.

We really appreciate your interest to submit your manuscript to our journal and we will try to get back to you as soon as possible. We hope for your kind understanding in this matter, if you have any questions or concerns, please do not hesitate to contact us.

Best regards,
Editorial Team of JTBB.

Journal of Tropical Biodiversity and Biotechnology
<http://jtbb.or.id>
ISSN 2540-9573 (print)
ISSN 2540-9581 (online)

Oedjijono 1 <oedjijono@unsoed.ac.id>
to Rina

Tue, Oct 25, 2022, 3:00

Reply Forward

Connect to chat



[JTBB] Editor Decision External Inbox x



Liya Audinah liyaaudinah15@gmail.com via ugm.ac.id to me, Rina, Nanik, Nuraeni

Mon, Dec 19, 2022, 4:30

Dear Dr. Oedjijono, M. Sc.,

Thank you for your revision. However, some revisions can not detect by us. We invite you to do the revision and return it to us. When revising your manuscript, please consider all issues mentioned in the reviewer's comments carefully. PLEASE OUTLINE EVERY CHANGE MADE IN RESPONSE TO THEIR COMMENTS and provide suitable rebuttals for any comments not addressed.

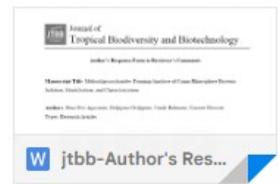
Please send answers to the reviewers' comments in a separate file and activate your track changes.

If you have any questions, please feel free to contact us.

Sincerely yours,
Liya Audinah
Faculty of Biology, Universitas Gadjah Mada
liyaaudinah15@gmail.com

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One attachment • Scanned by Gmail





[JTBB] Editor Decision External Inbox x



Liya Audinah liyaaudinah15@gmail.com via ugm.ac.id
to me, Rina, Nanik, Nuraeni

Mon, Dec 12, 2022, 10:00

Dear Oedjijono,

Thank you for submitting your work, titled "Maltooligosaccharides-Forming Amylase of Canna Rhizosphere Bacteria: Isolation, Identification, and Characterization", to Journal of Tropical Biodiversity and Biotechnology. After reviewing your submission, we will consider publishing your manuscript.

However, before we can proceed to publish the manuscript, we invite you to respond to the reviewers' comments and revise your manuscript carefully. Please activate the track changes mode in MS Word to track your revisions. We enclosed the reviewer comments for you to learn.

Please send us answers to the reviewers' comments in a separate file.

We expect to receive your revision within two (2) weeks. If you fail to turn your revision in within the designated time, we may have to decline your manuscript without notification.

If you have any questions, please feel free to contact us.

Sincerely yours,
Liya Audinah
Faculty of Biology, Universitas Gadjah Mada
liyaaudinah15@gmail.com

Reviewer A:
This paper provides sufficient results and novelty for publication. However, some revisions need to be made in order to improve the quality of the manuscript. The manuscript organization needs to be rearranged, especially for the methodology. I think it should be more brief and concise. The methodology missed some details. Some incorrect comprehensions, typos, sentences, and inconsistencies in some terms were found in the manuscript (authors may find the review details in the specific comments part). The manuscript also needs linguistic editing and paraphrasing.

5,860
25
+

The manuscript also needs linguistic editing and paraphrasing.

5,860
25
+

1. The title used in this manuscript is still unclear; I suggest the title could be rephrased as "Isolation and characterization of rhizospheric bacteria associated with *Canna* sp. for production of malto-oligosaccharide amylase."
2. The background of why the research is specifically focused on malto-oligosaccharides amylase instead of other oligosaccharides needs to be explained in the manuscript.
3. Which species of *Canna* plant were used in this study? "*Canna edulis*" or "*Canna* sp."? It should be specific and consistently used throughout the manuscript because sometimes the authors wrote "*Canna* sp.", but in another part, they wrote "*Canna edulis*". Also pay attention to how to write the correct scientific name (this is very important in scientific writing; it should be italicized!)
4. In the introduction (paragraph 5), the author(s) explain that members of *Bacillus* were known to produce some enzymes. However, in the next sentence, the authors mentioned "*Aspergillus oryzae*" with the impression that it is a part of the *Bacillus* species. Please correct this sentence since *Aspergillus* is not a bacterial! It is a species of fungi anyway. The reader will be confused by this sentence!
5. In the methodology (isolation, screening of amylolytic bacteria), the authors cited (Niu, 2017) as their reference. However, we could not find such a methodology for isolation in the literature cited.
6. The methodology missed some details, such as:
 - a. In Section 2.2, the authors crushed plant tissues and litter samples in the mortar after washing them with water. Is it possible to crush the wet samples without drying them first?
 - b. The authors specified a five-day incubation period for the crude enzyme production at different time intervals (section 2.4). What variations in time intervals did the authors make? Please correct it, since it caused a misunderstanding.
7. In the results and discussion:
 - a. To facilitate interpretation, results are better presented in graphs rather than tables.
 - b. The authors should explain why the D3 isolate had a higher amilolytic clear zone than the other isolate, but in the qualitative TLC analysis it couldn't hydrolyze starch.
 - c. Results for the electrophotogram (Figure 5): Please give details for the size of each band of the marker
8. Some formatting inconsistencies have been observed in some references that need correction.



rather than tables.

b. The authors should explain why the D3 isolate had a higher amilolytic clear zone than the other isolate, but in the qualitative TLC analysis it couldn't hydrolyze starch.

c. Results for the electrophotogram (Figure 5): Please give details for the size of each band of the marker

8. Some formatting inconsistencies have been observed in some references that need correction.

Reviewer B:

This research explores the diversity of amylase-producing bacteria from the rhizosphere of the Canna, evaluate their amylolytic abilities, looks for isolates with the most potential to hydrolyse starch, detects degradation products, determines the characteristics of the amylase enzyme from selected isolates, and identifies the names of bacterial species with the most potential for hydrolysing starch.

The data has not been displayed properly so researchers need to explore how to present data from other references.

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Oedjijono 1 <oedjijono@unsoed.ac.id>
 to Nanik

Dec 26, 2022, 12:

Reply Forward



Author's Response External Inbox x



Oedjijono 1 <oedjijono@unsoed.ac.id>
to Liya

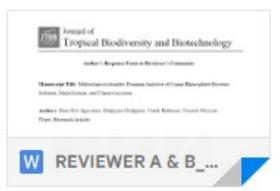
Mon, Dec 26, 2022, 9:

Dear the Editor of **JTBB**

Here, we submit our revised article entitled 'Isolation and characterisation of rhizospheric bacteria associated with Canna plant for production of maltooligosaccharide amylase'.

Thank you, for your attention and collaboration.

4 Attachments • Scanned by Gmail



Liya Audinah <liyaaudinah15@gmail.com>
to me

Mon, Dec 26, 2022, 9:

Dear Dr. Oedjijoni, M. Sc.,

Thank you for your revision.

Best regards,
Liya Audinah



- You are welcome.
- Noted with thanks.
- Thanks a lot.

- Reply
- Forward

[JTBB] Editor Decision External Inbox x



Dr Miftahul Iلمي <m.ilmii@ugm.ac.id>
to me, Rina, Nanik, Nuraeni

Mon, Jan 30, 10:00

Dear Oedji Oedjijono Oedjijono:

Thank you for submitting your work, titled "Maltooligosaccharides-Forming Amylase of Canna Rhizosphere Bacteria: Isolation, Identification, and Characterization", to Journal of Tropical Biodiversity and Biotechnology. After reviewing your submission, we decided to accept your manuscript for publication.

Shortly after this, our copyeditor will contact you to review the final edited version of your manuscript. Please keep in mind that we will not publish the manuscript before you approve the final version.

If you have any questions, please feel free to contact us.

Sincerely yours,
Dr Miftahul Iلمي
Faculty of Biology, Universitas Gadjah Mada
m.ilmii@ugm.ac.id

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ISSN 2540-9573 (print)
ISSN 2540-9581 (online)



Oedjijono 1 <oedjijono@unsoed.ac.id>
to Dr

Mon, Jan 30, 11:00

Thank you for the information, I'm waiting for further contact.

Sincerely yours,

Oedjijono



[JTBB] Editor Decision_similaritycheck External Inbox x



Liya Audinah liyaaudinah15@gmail.com via ugm.ac.id

Thu, Feb 2, 10:00

to me, Rina, Nanik, Nuraeni

Dear Dr. Oedjijono, M.Sc.,

Thank you for submitting your manuscript. However, before we process it for editing, please paraphrase some sentences based on the result of the similarity check. We suggest for the similarity be less than 20%.

If you have any questions, please feel free to contact us. Thank you.

Sincerely yours,
Liya Audinah
Faculty of Biology, Universitas Gadjah Mada
liyaaudinah15@gmail.com

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Oedjijono 1 <oedjijono@unsoed.ac.id>

Fri, Feb 3, 9:00

to Liya, Rina, Nanik, Nuraeni

Thank you, I will do that.



Oedjijono 1

Dear Editors, We hereby send my revised manuscript no. 78346 (attached), which has been checked for similarity by using Turnitin. Thank you. Sincerely yours, Oe



2



Oedjijono 1

On Tue, Mar 7, 2023 at 11:52 AM Oedjijono 1 <oedjijono@unsoed.ac.id> wrote: Dear Editors, I hereby send back the revised manuscript from three reviewers. Sincer



[JTBB] Copyediting Completed External Inbox x

 **Salwa Shabria Wafi** wafisalwa@gmail.com via ugm.ac.id
to me

Mon, Mar 13, 5:4

Oedji Oedjijono Oedjijono:

We have now copyedited your submission "Maltooligosaccharides-Forming Amylase of Canna Rhizosphere Bacteria: Isolation, Identification, and Characterization" for Journal of Tropical Biodiversity and Biotechnology. To review the proposed changes and respond to Author Queries, please follow these steps:

1. Log into the journal using URL below with your username and password (use Forgot link if needed).
2. Click on the file at 1. Initial Copyedit File to download and open copyedited version.
3. Review the copyediting, making changes using Track Changes in Word, and answer queries.
4. Save file to desktop and upload it in 2. Author Copyedit.
5. Click the email icon under COMPLETE and send email to the editor.

This is the last opportunity that you have to make substantial changes. You will be asked at a later stage to proofread the galleys, but at that point only minor typographical and layout errors can be corrected.

Manuscript URL: <https://jurnal.ugm.ac.id/jtbb/author/submissionEditing/78346>
Username: oedji_123

If you are unable to undertake this work at this time or have any questions, please contact me.

Hereby, I also attach the Copyright Transfer Agreement as well as invoice of the payment for the article. We would like to ask you to sign the Copyright Transfer Agreement as a sign that you have agreed on transferring the copyright of your manuscript to JTBB upon publication. After signing it, please send the scanned of the signed document along with the payment proof of the invoice to my email. Please also note that the payment of the invoice should be completed within seven days after receiving this email and we are pleased to inform you that we will process your manuscript as soon as possible afterwards.



[JTBB] Copyediting Review Completed External Inbox x



Oedjijono Oedjijono

Salwa Shabria Wafi: I have now reviewed the copyediting of the manuscript, "Maltooligosaccharides-Forming Amylase of Canna Rhizosphere Bacteria: Isolation, Iden



Oedjijono 1

Thanks a lot.



Salwa Wafi <wafisalwa@gmail.com>

to me

Mon, Mar 20, 12:00

Dear Mr. Oejjiono,

We would like to inform you that we have one additional comment that we need you to do. In figure 2, please revise the label (1,2,3,4,5) placement so that it can cover the real label of the figure (A3-P, S1-P, D3-P, etc.). Here I attach

Thank you very much for your cooperation.

Best regards,
Salwa Shabria Wafi
Admin of **JTBB**.

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Oedjijono 1



[JTBB] Proofread Layout External Inbox x



Salwa Shabria Wafi wafisalwa@gmail.com via ugm.ac.id
to Rina, me, Nanik, Nuraeni

Tue, Mar 28, 3:...

Dear Author(s),

We would like to inform you that we have finished the draft layout of your article entitled "Isolation and Characterization of Rhizospheric Bacteria Associated with Canna Plant for Production of Maltooligosaccharide Amylase". Please kindly check the draft thoroughly and let us know if there are any changes needed to be made.

Furthermore, considering we have been indexed by Scopus, all of your data that appear in the layout such as name and affiliation will appear on your Scopus ID. Therefore, we encourage you to recheck the spelling of authors' names and the affiliation, as well as the similarity of authors' names and affiliation in the layout. Please take note that we will not accept revision once it is published and appeared in Scopus database.

Best regards,

Salwa Shabria Wafi
Admin of JTBB.

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Author's Response Form to Reviewer's Comments

Manuscript Title: Maltooligosaccharides-Forming Amylase of Canna Rhizosphere Bacteria: Isolation, Identification, and Characterization

Authors: Rina Dwi Agustiani, Oedjijono Oedjijono, Nanik Rahmani, Nuraeni Ekowati

Types: Research Articles

No.	Reviewer's Comments	Author's Response	Line
Reviewer A			
	<p>This paper provides sufficient results and novelty for publication. However, some revisions need to be made in order to improve the quality of the manuscript. The manuscript organization needs to be rearranged, especially for the methodology. I think it should be more brief and concise. The methodology missed some details. Some incorrect comprehensions, typos, sentences, and inconsistencies in some terms were found in the manuscript (authors may find the review details in the specific comments part). The manuscript also needs linguistic editing and paraphrasing.</p>		

	<p>The title used in this manuscript is still unclear; I suggest the title could be rephrased as "Isolation and characterization of rhizospheric bacteria associated with <i>Canna</i> sp. for production of malto-oligosaccharide amylase."</p>		
	<p>The background of why the research is specifically focused on malto-oligosaccharides amylase instead of other oligosaccharides needs to be explained in the manuscript.</p>		
	<p>Which species of <i>Canna</i> plant were used in this study? <i>Canna edulis</i> or <i>Canna</i> sp.? It should be specific and consistently used throughout the manuscript because sometimes the authors wrote <i>Canna</i> sp., but in another part, they wrote <i>Canna edulis</i>. Also pay attention to how to write the correct scientific name (this is very important in scientific writing; it should be italicized!) In the introduction (paragraph 5), the author(s) explain that members of <i>Bacillus</i> were known to produce some enzymes. However, in the next sentence, the authors mentioned <i>Aspergillus oryzae</i> with the impression that it is a part of the <i>Bacillus</i> species. Please correct this sentence since</p>		

	<p>Aspergillus is not a bacteria! It is a species of fungi anyway. The reader will be confused by this sentence! In the methodology (isolation, screening of amylolytic bacteria), the authors cited (Niu, 2017) as their reference. However, we could not find such a methodology for isolation in the literature cited.</p>		
	<p>The methodology missed some details, such as:</p> <p>a. In Section 2.2, the authors crushed plant tissues and litter samples in the mortar after washing them with water. Is it possible to crush the wet samples without drying them first?</p> <p>b. The authors specified a five-day incubation period for the crude enzyme production at different time intervals (section 2.4). What variations in time intervals did the authors make? Please correct it, since it caused a misunderstanding.</p>		
	<p>In the results and discussion:</p> <p>a. To facilitate interpretation, results are better presented in graphs rather than tables.</p> <p>b. The authors should explain why the D3 isolate had a higher amylolytic clear zone than the other isolate, but</p>		

	<p>in the qualitative TLC analysis it couldn't hydrolyze starch.</p> <p>c. Results for the electrophotogram (Figure 5): Please give details for the size of each band of the marker</p> <p>Some formatting inconsistencies have been observed in some references that need correction.</p>		

Reviewer B

	<p>This research explores the diversity of amylase-producing bacteria from the rhizosphere of the Canna, evaluate their amylolytic abilities, looks for isolates with the most potential to hydrolyse starch, detects degradation products, determines the characteristics of the amylase enzyme from selected isolates, and identifies the names of bacterial species with the most potential for hydrolysing starch.</p>		
	<p>The data has not been displayed properly so researchers need to explore how to present data from other references.</p>		

Author's Response Form to Reviewer's Comments

Manuscript Title: Maltooligosaccharides-Forming Amylase of Canna Rhizosphere Bacteria: Isolation, Identification, and Characterization

Authors: Rina Dwi Agustiani, Oedjijono Oedjijono, Nanik Rahmani, Nuraeni Ekowati
Types: Research Articles

No.	Reviewer's Comments	Author's Response	Line
Reviewer A			
	<p>This paper provides sufficient results and novelty for publication. However, some revisions need to be made in order to improve the quality of the manuscript. The manuscript organization needs to be rearranged, especially for the methodology. I think it should be more brief and concise. The methodology missed some details. Some incorrect comprehensions, typos, sentences, and inconsistencies in some terms were found in the manuscript (authors may find the review details in the specific comments part). The manuscript also needs linguistic editing and paraphrasing.</p>	<p>The organization of the section Materials and methods, we rearranged as the following:</p> <ol style="list-style-type: none"> 2.1. Sample collection and location of sampling 2.2. Isolation, screening, and morphological characterization of amyolytic bacteria 2.3. Phenetic and phylogenetic characterizations of the selected bacterium 2.4. Effect of pH and temperature toward enzyme activity of the selected isolate 2.5. Crude enzyme production and amylase activity at different fermentation time 2.6. Analysis of the hydrolysis products by a thin-layer chromatography method (Rahmani et al. 2013) 	<p>Line 71-165</p>

	<p>The title used in this manuscript is still unclear; I suggest the title could be rephrased as "Isolation and characterization of rhizospheric bacteria associated with <i>Canna</i> sp. for production of malto-oligosaccharide amylase."</p>	<p>We already change the title to become: "Isolation and characterization of rhizospheric bacteria associated with <i>Canna</i> sp. for production of malto-oligosaccharide amylase."</p>	<p>Line 1-2</p>
	<p>The background of why the research is specifically focused on malto-oligosaccharides amylase instead of other oligosaccharides needs to be explained in the manuscript.</p>	<p>The type of oligosaccharide produced by enzymatic hydrolysis depend on the type of carbohydrate polymer and enzymes type which we used in study</p> <p>We used starch and amylase for this research so hydrolysis product of starch by amylase is maltooligosaccharide.</p>	<p>Line 23-67</p>
	<p>Which species of <i>Canna</i> plant were used in this study? <i>Canna edulis</i> or <i>Canna</i> sp.? It should be specific and consistently used throughout the manuscript because sometimes the authors wrote <i>Canna</i> sp., but in another part, they wrote <i>Canna edulis</i>. Also pay attention to how to write the correct scientific name (this is very important in scientific writing; it should be italicized!)</p> <p>In the introduction (paragraph 5), the author(s) explain that members of <i>Bacillus</i> were known to produce some enzymes. However, in the next sentence, the authors</p>	<p>According to a plant systematic expert, the <i>Canna</i> plant used in this research is <i>Canna indica</i></p> <p>In the Introduction</p> <p>We already delete the word of <i>Aspergillus oryzae</i></p>	<p>Line 7, 72, 274, 397</p> <p>Line 58</p>

	<p>mentioned <i>Aspergillus oryzae</i> with the impression that it is a part of the Bacillus species. Please correct this sentence since Aspergillus is not a bacteria! It is a species of fungi anyway. The reader will be confused by this sentence!</p> <p>In the methodology (isolation, screening of amyolytic bacteria), the authors cited (Niu, 2017) as their reference. However, we could not find such a methodology for isolation in the literature cited.</p>	<p>In the methodology</p> <p>We already change the literature of Niu, 2017 to become Duan et al. 2021</p>	<p>Line 88</p>
	<p>The methodology missed some details, such as:</p> <p>a. In Section 2.2, the authors crushed plant tissues and litter samples in the mortar after washing them with water. Is it possible to crush the wet samples without drying them first?</p> <p>b. The authors specified a five-day incubation period for the crude enzyme production at different time intervals (section 2.4). What variations in time intervals did the authors make? Please correct it, since it caused a misunderstanding.</p>	<p>a. Yes, we missed one step.... Actually after washing, the samples then put on sterile tissue papers</p> <p>b. We already revision the sentences to become: The culture was sampling every 24 hour and then centrifuge, and the supernatant obtained was tested for its amylase activity.</p>	<p>Line 88-89</p> <p>Line 128-129</p>
	<p>In the results and discussion:</p> <p>a. To facilitate interpretation, results are better presented in graphs rather than tables.</p>	<p>a. We already presented the results in graphs rather than tables, such as:</p> <p>Table 3 to become Figure 7,</p> <p>Table 4 to become Figure 8, and</p> <p>Table 5 to become Figure 9.</p>	<p>Line 326</p> <p>Line 354</p> <p>Line 370</p>

	<p>b. The authors should explain why the D3 isolate had a higher amilolytic clear zone than the other isolate, but in the qualitative TLC analysis it couldn't hydrolyze starch.</p> <p>c. Results for the electrophotogram (Figure 5): Please give details for the size of each band of the marker</p> <p>Some formatting inconsistencies have been observed in some references that need correction.</p>	<p>We also change the image caption in Figure 6.</p> <p>The isolate D3 had a higher amilolytic clear zone that means this isolate had higher activity of amylase. But we need check this isolate produce of alpha or other type of amylase. Alpha amylase could hydrolysis of the starch to become various type of maltooligosacharide.</p> <p>b. We already add the size of each band of the marker in the electrophotogram Figure 4.</p> <p>We already revision some references following authors guidelines from the journal.</p>	<p>Line 287</p> <p>Line 269</p>

Reviewer B

	<p>This research explores the diversity of amylase-producing bacteria from the rhizosphere of the Canna, evaluate their amylolytic abilities, looks for isolates with the most potential to hydrolyse starch, detects degradation products, determines the characteristics of the amylase enzyme from selected isolates, and identifies the names of bacterial species with the most potential for hydrolysing starch.</p>		
--	--	--	--

	<p>The data has not been displayed properly so researchers need to explore how to present data from other references.</p>	<p>We already present the data from some table to become figure to make the data has been displayed properly. Table 3 to become Figure 7, Table 4 to become Figure 8, and Table 5 to become Figure 9.</p> <p>We also improve the Figure 6 to make more beautiful figure</p>	<p>Line 326 Line 354 Line 370</p> <p>Line 287</p>

Abstract

Line	Suggestion	Author's Response
Line 7	Writing <i>Canna</i> sp. denoting the genus should be written in italics	We already corrected in Line 7
Line 10	Is the C in <i>canna</i> written in lowercase or uppercase?	We already corrected in Line 11

Introduction

Line	Suggestion	Author's Response (Blue color)
Line 55	<i>Bacillus</i> must be written in italics. Please check and correct the entire manuscript	We already revised in Line 55, 216, 217, 233, 245, 274

Material dan method

Line	Suggestion	Author's Response
Line 77	Please improve the quality of figure 1	We already revised in Line 77-78
Line 91	Was the soil sample not suspended first in sterile saline solution and then grown on NB + 1% soluble starch media?	Yes, we did as explained in Line 84-85
Line 111	Why use a precultural estimate of 1-3 mL, what is the exact amount used in this study? Are there any data on culture turbidity or initial abundance of added cells?	Yes that's right, it should be 2 mL (the volume of the 1-3 mL bacterial culture is stated in the research proposal procedure). Line 126
Line 129	What is the exact amount of T10 culture added to media? This is important for data reproducibility	It is similar with the above procedure. Line 144

Result

Line	Suggestion	Author's Response (Blue color)
Line 169-175	Add "bacterial isolate". How many cultures were added in this study? This information is important for data reproducibility	We already revised in Line 170
Line 185	Please add reference that isolate which has clear zone ≥ 18 mm classified as good activity	We already added the references as explained in Line 188-191
Line 189	Please mind to the consistency of writing capital letters in table titles Please improve the display of Table 1	We already revised in Line 194 (Table 1)
Line 191	Please write clearly the sample code in the image	We already revised in Line 196

Line 199	There is no need to write enzyme after the word amylase. Please check and correct throughout the manuscript	We already revised as in Line 39, 202, 293, 299, 350
Line 201	The results of the clear zone in the study of Vijayalakshmi et al. 2018 should be stated in mm	We already revised as in Line 204
Line 215	The statement that <i>Bacillus</i> might have a Gram-negative form, please check again.	Line 216-219. The statement is right, it was cited from Logan & De Vos (2009) page 21.
Line 217	Please complete the sentences in this line. What is meant by an environment that is polluted directly and indirectly by soil?	The sentence: or from environments that may have been contaminated directly or indirectly by soil was deleted. Line 219
Line 239	After the sentence amylyotic index should be added the abbreviation (AI)	We already inserted the abbreviation (AI) in Line 296
Line 265	In table 3 the data shown was fluctuating. Why did the amylase activity show high activity on day 0? At what time after the initial inoculation, the enzyme measurements were conducted?	The additional explanation was explained in Line 320-323
Line 327	All <i>Bacillus</i> genera are written in italic. Please check and correct the entire manuscript	We already revised as in Line 55, 216, 217, 233, 245, 274
Line 329	Please add a bar in Figure 4	We already done as in Figure 3 (Line 235)
Paragraph 335	Is there any data reporting the potency of <i>B.toyonensis</i> ?	We already added about the potency of <i>B. toyonensis</i> as explained in Line 254-266

Reference

Line	Suggestion	Author's Response
Line 35	There is no reference to the name Jang et al. 2020	We already added as in Line 471
Line 198	There is no reference to the name Vassekaran et al. 2010	We already added as in Line 530
Line 443	References on behalf of Mogan and De Vos published in 2000 or 2009?	We already revised. The correct one is 2009. Line 480
Line 484	Reference on behalf of Sivaramakrishnan et al. published in 2006 or 2016 ?	We already revised. The correct one is 2006. Line 523

1 Isolation and Characterization of Rhizospheric Bacteria Associated with Canna Plant 2 for Production of Malto-oligosaccharides Amylase

3 4 5 **Abstract**

6 The objectives of the study were to isolate amylolytic bacteria from rhizosphere and
7 plant tissue of *Canna indica*, as well as litter; to know oligosaccharide compounds produced
8 from starch hydrolyzed by the bacterial enzymes, and to identify the amylolytic bacteria
9 based on phenetic and 16S rRNA gene sequences. Thirty-two isolates of amylolytic bacteria
10 were obtained from the rhizosphere, *Canna* plant tissue, and litters. Eight isolates (TH6, TH7,
11 T5, T10, D2, D3, A3, S1) produced high clear zone diameters ranging from 18-30 mm;
12 especially an isolate T10 which was consistent in producing a total clear zone diameter of 20
13 mm. The hydrolysate of starch hydrolyzed by the T10 amylase resulted in three
14 oligosaccharide compounds of maltotriose, maltotetraose, and maltopentose. The amylase
15 activity of isolate T10 was optimal at a temperature 40°C and pH 7 in an amount of 0.801
16 U/mL. The isolate T10 was identified as a species member of *Bacillus toyonensis* based on
17 phenotypic characterization and 16S rDNA gene sequencing analysis with a similarity value
18 of 99.93%.

19
20 **Keywords:**

21 Amylolytic bacteria, *Canna*, Malto-oligosaccharides, 16S rDNA gene.

22 23 **1. Introduction**

24 Oligosaccharides are members of an important group of carbohydrate
25 macromolecules with short-chain polysaccharide sugars of 2 to 20 saccharide units.

26 Functional oligosaccharides such as galactooligosaccharide (GOS), fructooligosaccharide
27 (FOS), and malto-oligosaccharide (MOS), are well-known prebiotics owing to their ability to
28 selectively stimulate beneficial bacteria in the intestines, particularly bifidobacterial species
29 (Zhao et al. 2017). The development of oligosaccharide products is one of the businesses that
30 has high economic value. One of the basic ingredients (substrates) to produce
31 oligosaccharides enzymatically is starch, which is found in many plants, such as Canna.
32 Canna plants contain high levels of carbohydrates, especially starch (93.3%), which consists
33 of amylose (33.48%), and amylopectin (59.82%) (El-Fallal et al. 2012). Starch is hydrolyzed
34 into smaller oligosaccharides by α -amylase, which is one of the most important commercial
35 enzymes (Jang et al. 2020).

36 Amylase has been exploited by the starch processing industry as a substitute for acid
37 hydrolysis in the production of starch hydrolysis. Amylase acts as a biocatalyst for the
38 hydrolysis of starch into simpler carbohydrates, such as glucose, maltose, and dextrin
39 (Divakaran et al. 2011; Abdalla et al. 2021). Amylolytic bacteria are producers of [amylase](#)
40 that can be used as biocatalysts in the starch hydrolysis process (Ding et al. 2021) to produce
41 various malto-oligosaccharide products, such as maltotriose, maltotetraose, maltopentaose,
42 and maltohexaose (Pan et al. 2017).

43 Canna plants and its surrounding, including the rhizosphere and plant tissues, can be
44 sources of isolating amylolytic bacteria. The high starch content in canna tubers makes them
45 a good substrate for the growth of various bacteria, especially the amylolytic bacteria. The
46 bacteria isolated from starch-rich sources generally have the potential to produce amylase
47 with high activity (Hellmuth & van den Brink 2013). In addition, the rhizosphere is known as
48 the most diverse microbial habitats with respect to species richness and community size. The
49 interaction between plant roots and microorganisms is intensive around the rhizosphere,
50 because the plants secrete exudates containing carbohydrates, amino acids, and other

51 nutrients utilized by bacteria for the growth. On the contrary, rhizospheric bacteria can
52 produce protein and enzymes that are important for the biological function of the host plants
53 (Afifah et al. 2018).

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

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

69

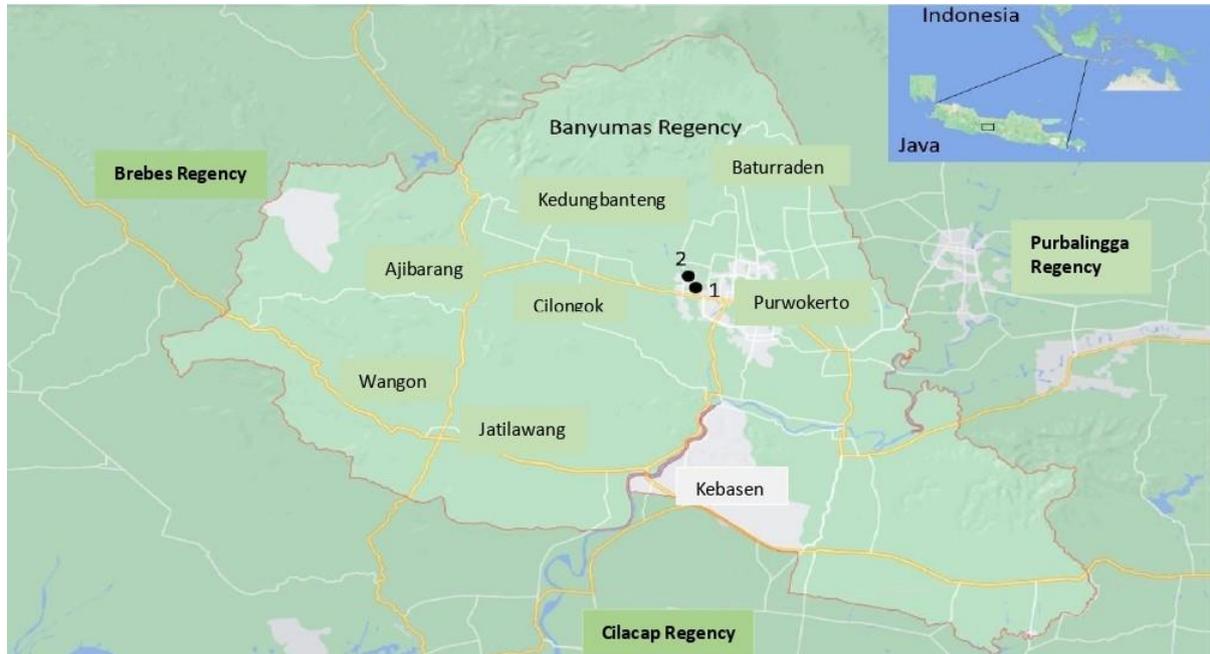
70 **2. Materials and methods**

71 2.1. Sample collection and location of sampling

72 Samples were taken from the rhizosphere and parts of *Canna* plant (*C. indica*)
73 including tubers, stems, leaves, tissue, as well as litter growing in two places, namely in the
74 forest and the community gardens around the Perhutani Forest West Banyumas, Central Java,

75 Indonesia. The coordinates of the former are S 07°20.846 'E 109°06.410 and the later is S 07
76 °20.812 'E 109°05.92 (Figure 1).

77



78

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

82

83 2.2. Isolation, screening, and morphological characterization of amyolytic bacteria

84 Plant tissues and litter were cleaned with running water, then cut into 1 cm long
85 pieces and separated according to the plant part. The sample pieces were immersed in 70%
86 alcohol for 1 minute, then in 1% sodium hypochlorite solution for 3 minutes, after which they
87 were soaked again using 70% alcohol for 1 minute, and rinsed with sterile distilled water
88 three times (Duan et al. 2021, with modification). The sterile samples were placed on sterile
89 tissue papers and then crushed using a mortar and one gram of each sample was diluted with
90 9 mL of sterile distilled water and then serial dilutions were made up to 10^{-7} .

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

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

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

108

109 2.3. Phenetic and phylogenetic characterizations of the selected bacterium

110 Phenetic characterizations of the selected bacterium (producing high diameter clear
111 zones and malto-oligosaccharide enzyme) included colony morphology, cells morphology,
112 and biochemistry, were conducted by conventional methods (Smibert & Krieg 1981).
113 Biochemical tests were also conducted using the API 50CHB kit.

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

116 5'GGCTACCTTGTTACGA-3') (Yopi et al. 2017). The obtained bands were stained and
117 visualized by UV Transilluminator. The sequence was confirmed via 1st BASE Sequencing,
118 Malaysia. The 16S rDNA nucleotide sequences were analyzed by nucleotide BLAST (Basic
119 Local Alignment Search Tool) search in the Gene Bank of National Center for Biotechnology
120 Information (NCBI) or BLAST for amino acid analysis (Zhuang et al. 2012). The
121 phylogenetic tree was constructed using neighbor joining algorithm in MEGA 6.0 software
122 (De Moraes Russo & Selvatti 2018).

123

124 2.4 Analysis of the hydrolysis products by a thin-layer chromatography method (Rahmani et
125 al. 2013)

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

131 The hydrolytic activity of amylase in a substrate solution was carried out at 30°C in
132 50 mM acetate buffer, pH 6, containing 0.5% of commercial starch. The enzyme-substrate
133 ratio (v/v) was 1:1 and the reaction times were in hours (0, 1, 2, 3, 4, 24). Reactions were
134 carried out in 2 mL Eppendorf containing 1 mL of reaction mixture in a Deep Well
135 Maximizer (Bioshaker M-BR-022UP, Taitec Japan).

136 A Thin Layer Chromatography (TLC) of malto-oligosaccharide products was carried
137 out on silica gel 60F₂₅₄ plates (Merck Art 20-20 cm) and eluent using a solvent mixture of n-
138 butanol:acid:water (12:6:6, v/v/v). Spots formed were visualized by spraying the sugar colors
139 (0.5 g α -diphenylamine, 25 mL acetone, 2.5 mL phosphate acid, 0.5 mL aniline). All samples
140 were applied in equal quantities (4 μ L). Glucose (Sigma-Aldrich, U.S.A), maltose (M2),

141 maltotriose (M3), maltotetraose (M4), maltopentaose (M5), maltohexaose (M6), and
142 maltoheptaose (M7) (Megazyme) were used as standards.

143

144 2.5. Crude enzyme production and amylase activity at different fermentation time

145 Amount of 2 mL of the 24 h old bacterial cultures (isolate T10) was inoculated into a
146 200 mL flask containing 20 mL of NB medium plus 1% (w/v) starch solution, pH 6 (50 mM
147 acetate buffer), and incubated at 30 °C for five days. The culture was sampling every 24 hour
148 and then centrifuge and the supernatant obtained was tested for its amylase activity.

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

$$153 = \frac{c \times d \times 1000}{t \times mw} \text{ U/mL}$$

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

155 A standard curve used D-Glucose at various concentrations. One unit of amylase activity is
156 defined as the amount of enzyme that liberates 1 μmol of D-Glucose per minute under the
157 experimental condition given.

158

159 2.6. Effect of pH and temperature toward enzyme activity of the selected isolate

160 The optimal pH of the enzyme activity was done at pH ranges of 3.0-10.0 under
161 standard assay conditions. Various buffers (0.05M) used were sodium acetate (pH 3.0-6.0),
162 sodium phosphate (6.0-8.0), Tris-HCL (pH 7.0-9.0), and Glycine-NaOH (pH 8.0-10.0). The
163 enzyme reactions were incubated at 40°C for 30 min in the presence of 0.5% (w/v) starch
164 solution (Merck)). The effect of temperatures on enzyme activity was conducted at

165 temperatures ranging from 30-90 °C in 50 mM acetate buffer at optimum pH for 30 min.
166 Amylase activity was assayed by DNS method (Miller 1959).

167

168 **3. Results and Discussion**

169 3.1 Isolation and amyolytic assay of bacteria isolated from the rhizosphere and plant tissue
170 of Canna, and litter

171 The results of the study found 32 bacterial isolates growing on NA medium
172 supplemented with 1% soluble starch, with details: 11 isolates were from the rhizosphere of
173 the Canna growing in the forest, 12 isolates from rhizosphere of the Canna growing in the
174 people's gardens around the forest, 4 isolates from the leave tissue of the Canna growing in
175 the people's gardens around the forest, 3 isolates from the roots of the Canna growing in the
176 people's gardens around the forest, and 2 isolates were from the litters of the Canna in the
177 gardens of the residents around the forest (Table 1).

178 The ability of the bacteria to grow and to produce clear zones in the medium,
179 indicating that those bacteria were capable of producing amylase. The more amylase is
180 released, the wider clear zones are produced due to the degradation of amylum in the
181 medium, resulting in the enhancement of the amyolytic index (Ginting et al. 2021). The
182 research results showed that eight isolates of TH6, TH7, T5, T10, D2, D3, A3, and S1
183 showing high total clear zone diameters (mm) of 18, 18, 18, 20, 18, 30, 18, and 18,
184 respectively (Table 1). The consistency of the bacterial isolates in resulting in the total clear
185 zone diameter was shown by the isolates TH6, T10, D3, A3, and S1, while the other isolates
186 tended to reduce or loss their amyolytic activity (Figure 2). Based on the ability of isolates to
187 produce a clear zone diameter \geq 18 mm and consideration of source representatives, four
188 isolates (TH6, T10, D3, A3, and S1) were selected for further testing, namely their ability to
189 hydrolyze starch. Hasanah et al. (2020) reported that bacterial isolates having an amyolytic

190 index of more than 9 mm were potentials for producing amylase. According to Ochoa–
 191 Solano & Olmos–Soto (2006), bacterial isolates producing clear zones two or three times the
 192 diameter of the colony are potential enzyme producers.

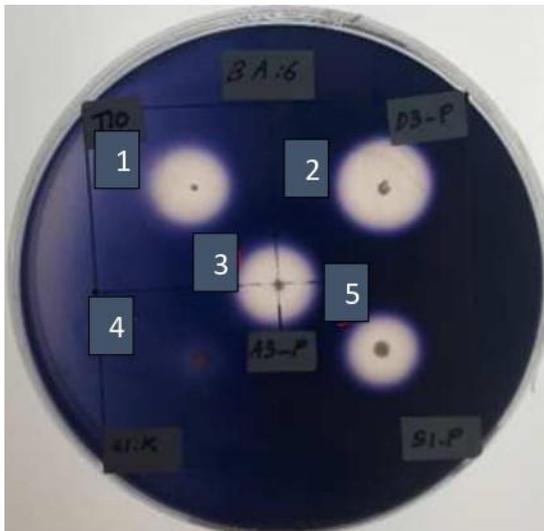
193

194 **Table 1.** Sources, number, and total clear zone diameter of amylolytic bacteria isolated from
 195 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

196



197

198

Figure 2. The amylytic zones produced by bacterial isolates of: (1) T10, (2) D3, (3)

199

A3, (4) TH6, and (5) S1 on a NA medium + 1% soluble starch.

200

201 A high number of amyolytic bacteria isolated from the rhizosphere of Canna was in
202 accordance with Vassekaran et al. (2010) who stated that bacteria isolated from starch rich
203 materials may have better potential to produce *amylase*. Vijayalakshmi et al. (2012) found
204 *Bacillus subtilis* KC3 isolated from the rhizosphere of *Euphorbia hirta* producing maximum
205 halo zone of 23 mm on a Starch Agar medium. Gebreyohannes (2015) reported that 16
206 bacterial isolates from soils were capable of producing clear zones of 3-22 mm on starch agar
207 plates. Ginting et al. (2021) found thermophilic bacteria of *Bacillus* sp. L3 and *B. caldotenax*
208 L9 from a marine hydrothermal producing high amyolytic indexes of 3.04 and 3.52,
209 respectively. The clear zone formed is the result of a reaction from the breakdown of starch
210 compounds into simple compounds; the wider the clear zone formed, the higher the
211 amyolytic activity (Zubaidah et al. 2019).

212 The characteristics of colonial morphology of the 34 isolates were rough, dry, bright,
213 and pink; cells were Gram-positive, rod shape, motile, and had endospores. The endospore
214 position of isolates of D1-D4 and S1-S2 was in terminal, while isolates of TH1-TH11, T1-
215 T12, and D1-D4 had endospores in the center. All of the isolates were able to hydrolyze
216 starch and to produce lecithinase (Table 2). Those characteristics indicated that the bacteria
217 were members of the genus *Bacillus*. According to Logan & De Vos (2009), the main
218 characteristics of the genus *Bacillus* are cells rod-shaped, straight or slightly curved,
219 occurring singly and in pairs, some in chains, form endospores, Gram-positive or Gram-
220 negative, motile, aerobes or facultative anaerobes, and mostly isolated from soil.

221

222 **Table 2.** Morphological and physiological properties of the bacteria isolated from Canna
223 plants and its 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	Center	Center	Terminal	Center	Terminal
Starch hydrolysis	+	+	+	+	+
Lecithinase production	+	+	+	+	+

224

225 3.2. Identification of the selected isolate of T10 based on phenetic and phylogenetic

226 characteristics

227 **Based on the ability of the selected isolate enzyme to produce maltooligosachharides**

228 **of maltotriose, maltotetraose, and maltopentaose (Method 3.3); further characterization of the**

229 **isolate T10 was conducted.** The isolate had colonial morphology of irregular with undulate

230 edges, opaque, cream-colored, and had a granular texture. The cells formed endospores,

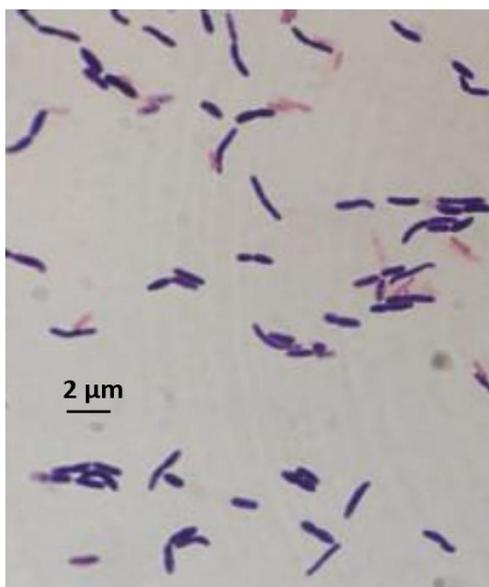
231 facultatively anaerobic, Gram-positive, rod shaped, motile, and occurring singly or in chains

232 **(Table 2, Table 3, Figure 3).** These characteristics including biochemistry, physiology, and

233 nutrition, indicated that isolate T10 was similar to those typical of the species *Bacillus cereus*.

234 This species is a species complex within the genus *Bacillus*, with members including *B.*

235 *anthracis*, *B. thuringiensis*, *B. mycoides*, and *B. toyonensis* (Luo et al. 2021).



236

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

239

240 **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 ($^{\circ}\text{C}$)	10 - 45
Optimal growth temperature ($^{\circ}\text{C}$)	35
Salinity tolerance range (%NaCl)	≤ 4
API 50CHB	
Glycerol	-
D-Ribose	+

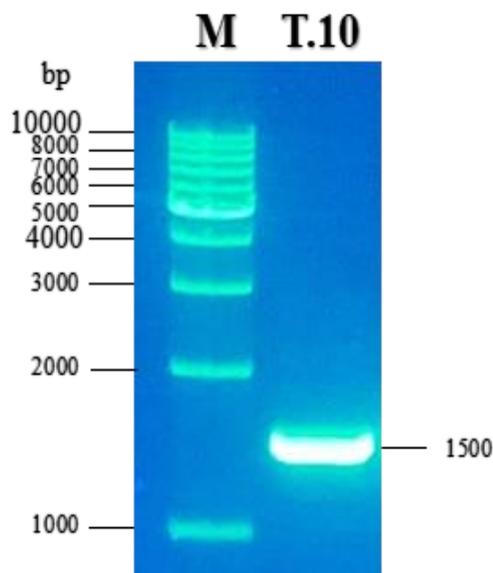
D-Mannose	+
Methyl- α D-glucopyranoside	+
Amygdalin	+w
Arbutin	+
Salicin	+
Cellobiose	-
D-saccharose	+
D-trehalose	+
Starch	+
Glycogen	+
D-turanose	+

241

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

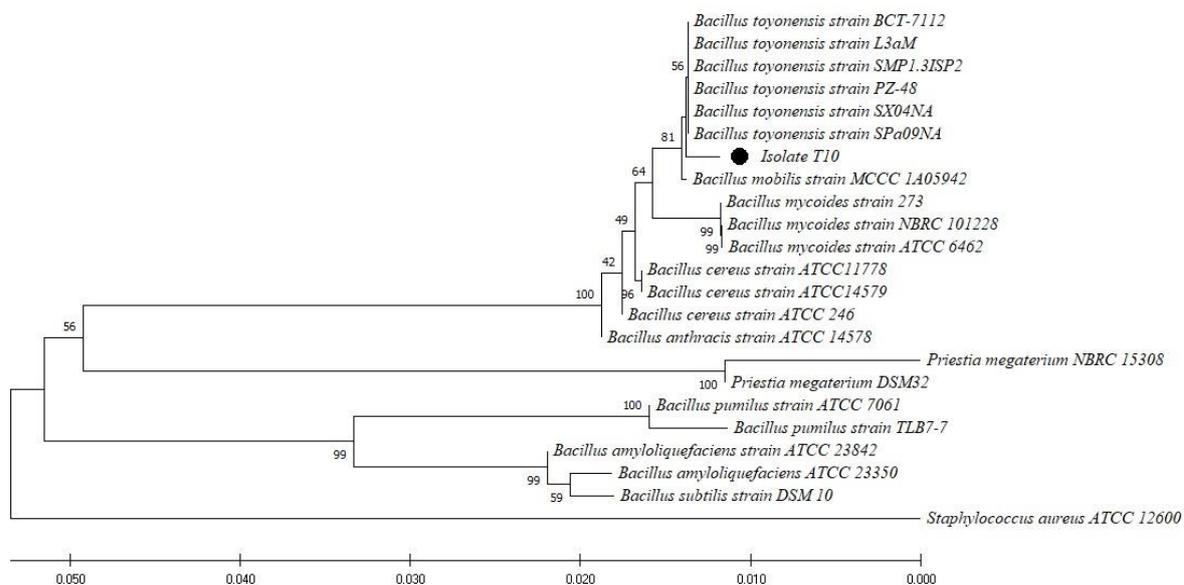
255 *B. toyonensis* strain BCT-7112^T was firstly isolated in 1966 in Japan from a survey
256 designed to obtain naturally occurring microorganisms as pure cultures in the laboratory for
257 use as probiotics in animal nutrition (Jiménez et al. 2013). This strain was first identified as
258 *B. cereus*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

268



269

270 **Figure 4.** An electrophotogram of the amplified 16S rRNA gene of isolate T10 with a size of
271 1500 bp. Marker (M): 1 kb DNA ladder



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Figure 5. A phylogenetic tree showing the relationship between strain T10 isolated from rhizospheres of Canna (*C. indica*) 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

280

3.3. Analysis of hydrolyzed products by the selected bacterial amylases using a TLC method

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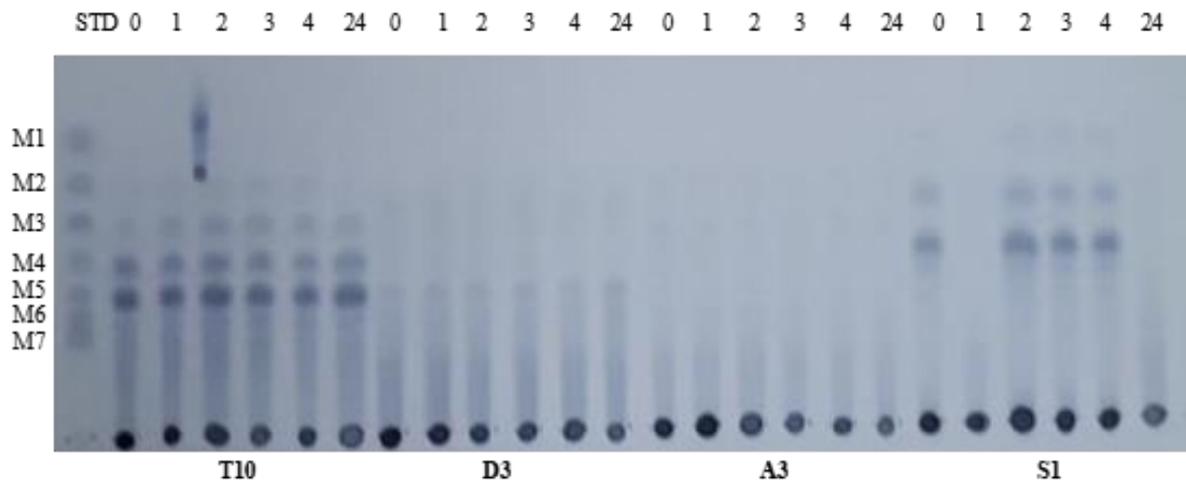
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The products of starch hydrolysis were assayed by oligosaccharide profile analysis on the amylase-hydrolyzed samples from the fourth isolates (T10, D3, A3, and S1) qualitatively. The results of TLC analysis showed that isolate T10 produced 3 bands, namely maltotriose (M3), maltotetraose (M4), and maltopentaose (M5), isolate S1 produced 2 bands, namely maltotriose (M3) and maltotetraose (M4), while two amylases of the isolates D3 and A3 were unable to hydrolyze starch (Figure 6).



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Figure 6. The product profile of starch hydrolyzed by amylase of the amyolytic bacteria (T10, D3, A3, and S1) using a TLC method with reaction times (hours): 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)

Based on the TLC chromatogram, the starch degraded by T10 amylase resulted in maltooigosachharides of maltotriose, maltotetraose, and maltopentaose. Amylases are able to break down starch polymer bonds into shorter, oligosaccharides or simple sugar molecules (Putri et al. 2012). The results showed that amyolytic bacteria with high amyolytic indexes (AI) did not correlated with their ability in degrading amyllum. The isolate T10 with its total diameter lower than isolate D3 showed higher ability in the breakdown of starch polymer bonds into shorter or oligosaccharides. The results of this study proved that a high AI value is not always accompanied by the ability of the amylase to break down starch polymer bonds. The ability of the T10 amylase to produce the malto-oligosaccharides 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 maltotriosa produced by amylase of *Brevibacterium* sp. using black potato

305 starch as substrate, while amylase of *Bacillus subtilis* strain SDP1 isolated from rhizosphere
306 of Acacia produces maltotriose and maltotetraose (Ozturk et al. 2014). Furthermore, Abdul
307 Manas et al. (2014) reported amylase of an alkaliphilic *Bacillus lehensis* G1 was capable
308 degrading oligosaccharides by producing malto-oligosaccharides with a higher degree of
309 polymerization than maltoheptaose observed on thin layer chromatography and high-
310 performance liquid chromatography analyses.

311

312 3.4. Crude enzyme production of a selected isolate and measurement of its amylase activity at
313 different culture incubations

314 Based on the ability of the fourth selected amylolytic bacteria in producing different
315 types of hydrolyzed product, isolate T10 was further assayed for its optimal amylase activity
316 at different incubation times. The results showed that incubation times affected amylase
317 activity of isolate T10 carried out in a 0.5% starch solution at 30°C in 50 mM acetate buffer
318 of pH 6. The amylase activity of T10 was optimal during incubation 1-3 days ranging from
319 0.546-0.717 U/mL, and the highest amylase activity was found at 24 hr incubation of 0.717
320 U/mL (Figure 7). The results also showed that amylase activity decreased after 72 h of
321 incubation. The amylase activity value at day 0 is quite high. This might be due to the
322 measurement of the enzyme activity using the DNS method, in which reducing sugar formed
323 from a carbon source (starch) is used by bacteria for the initial stages of growth; then the
324 bacteria will use the carbon source for the production of enzymes.

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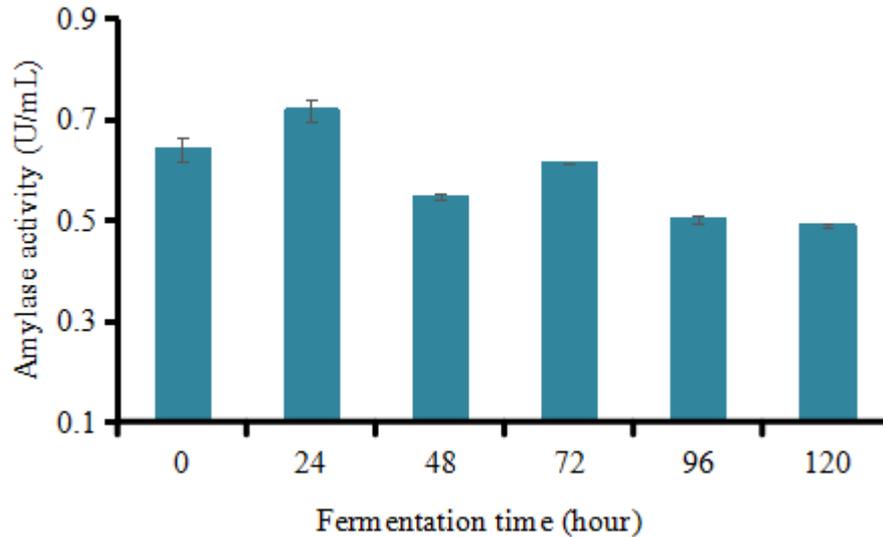


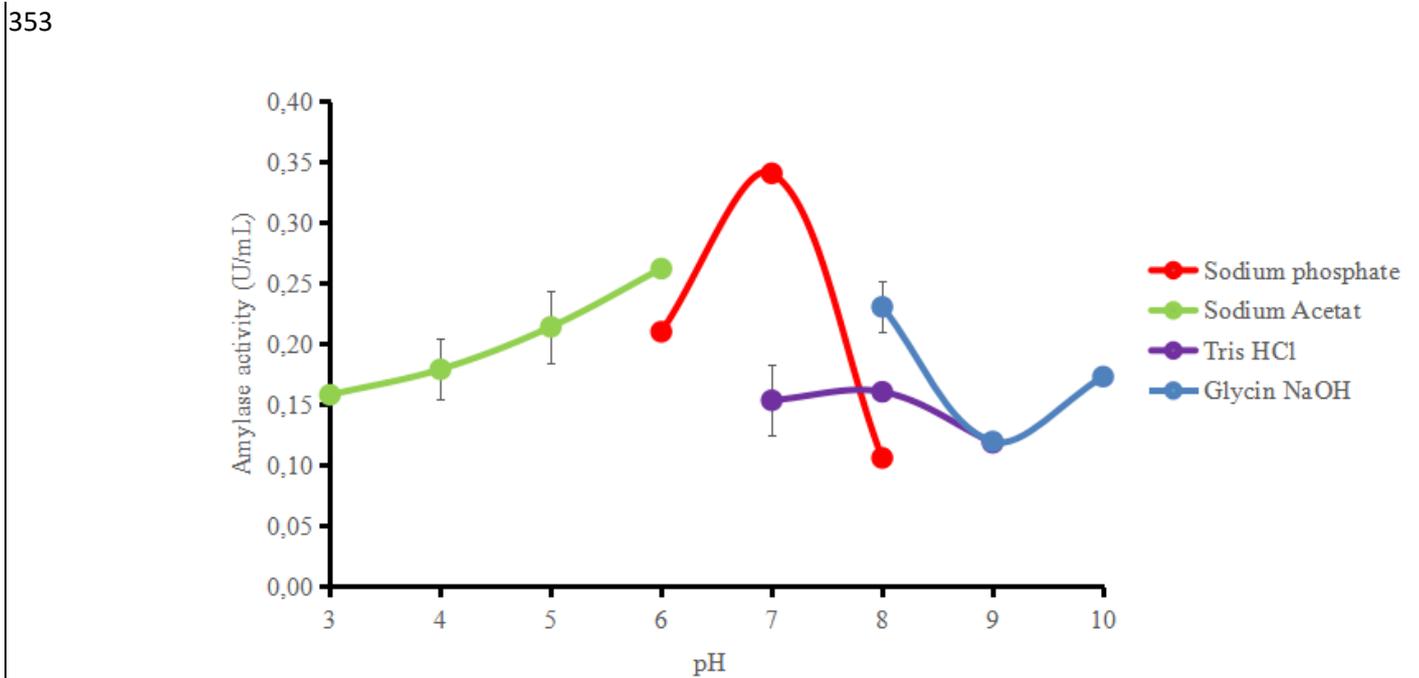
Figure 7. Amylase activity of T10 at different incubation times

The amylase activity of *Bacillus cereus* KN isolated from Ranu Ngebel and incubated for 3 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 3 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 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.

3.5. 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

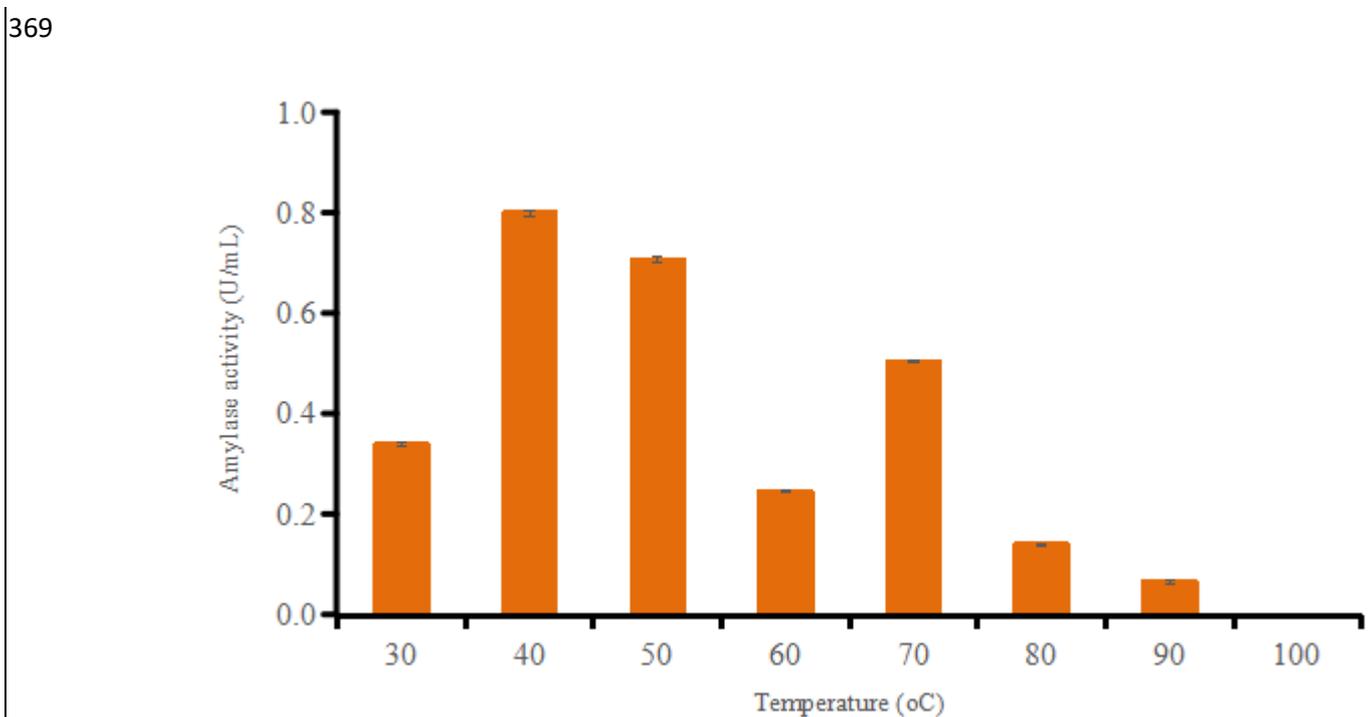
344 optimal pH of isolate T10 was in accordance with Naidu et al. (2019) for *Paenibacillus* sp.
345 D9 that its optimal pH for amylase activity is in the neutral range (pH 6-8). The increase in
346 pH beyond these values resulted in a decline in enzyme activity. Any change in pH caused a
347 change in the enzyme active site (Lim & Oslan 2021). Bajpai et al. (2015) reported that the
348 optimal pH for amylase activity of *Haloferax* sp. HA10 was at pH 7.0. According to Asgher
349 et al. (2007), each enzyme has an optimal pH to work most actively and the optimal pH of
350 amylase is varied from pH 3.8 to 9.5 depending on the type of enzyme and the source. Behal
351 et al. (2016) reported an amylase produced by *Bacillus* sp. AB04 had optimal activity at pH
352 8, and the enzyme is stable in neutral to alkaline (pH 7-10).



354
355 **Figure 8.** Amylase activity of T10 at different pH and buffers

356
357 The amylase activity of isolate T10 was observed at temperatures ranging from 30-
358 90°C at pH 7.0. Amylase activity of T10 isolate tended to be optimum at 40°C with an
359 activity value of 0.801 U/mL (Figure 9). A similar finding was also reported by
360 Sivaramakrishnan et al. (2006) for several species of *Bacillus* sp., *B. subtilis*, *B.*

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



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

372

373 The differences in the pH and temperature characteristics of enzyme activity indicated
374 that enzymes are specific, depending on the species that produces them. A decrease or
375 increase in temperature can affect the secretion of extracellular enzymes by changing the
376 physiology of the cell membrane (Rahmani et al. 2018). The optimum temperature is the
377 temperature that causes chemical reactions to occur at the greatest speed (Subagiyo et al.

378 2017). The results showed that after reaching the optimum condition, it was seen that the
379 activity of the T10 amylase decreased. High temperatures can cause enzymatic reactions to
380 decrease because enzyme proteins undergo conformational changes so that protein molecules
381 will experience denaturation (Yufinta et al. 2018).

382

384 The production of a specific malto-oligosaccharide in high yield through the
385 enzymatic hydrolysis of starch is of considerable commercial interest. This has been achieved
386 on an industrial scale after the discovery of a suitable malto-oligosaccharide-forming amylase
387 (MFA_{ses}), and since then several studies have tried to improve on existing methods by
388 increasing the yields of M3 and M5. These studies have included efforts to find new wild-
389 type strains producing MFA_{ses}, construct novel systems to achieve large-scale MFA_{ses}
390 expression, and immobilize MFA_{ses} to stability and productivity (Ben Ali et al. 2006). MFA_{ses}
391 from *Bacillus toyonensis*, a novel M5-amylase, seems promising for the manufacture of high
392 M5 syrups from starch and may be applicable to starch processing technologies due to its
393 highly specific activity, unique substrate specificity, and endo-type action pattern (Pan et al.
394 2017).

395

396 4. Conclusions

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

403

404 **Authors contribution**

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

407

408 **Acknowledgments**

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410 International Women University (IWU) for funding this research.

411

412 **Conflict of interest**

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

414

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1 Isolation and Characterization of Rhizospheric Bacteria Associated with Canna Plant 2 for Production of Maltooligosaccharide Amylase

3 4 5 **Abstract**

6 The objectives of the study were to isolate amylolytic bacteria from the rhizosphere and
7 plant tissue of *Canna edulis* Ker., as well as litter; to know oligosaccharide compounds
8 produced from starch hydrolysed by the bacterial enzymes, and to identify the amylolytic
9 bacteria based on phenetic and 16S rRNA gene sequences. From the rhizosphere, Canna plant
10 tissue, and litters obtained thirty-two amylolytic bacterial isolates. Eight isolates (TH6, TH7,
11 T5, T10, D2, D3, A3, S1) produced high clear zone diameters ranging from 18-30 mm;
12 especially an isolate T10, which was consistent in producing a total clear zone diameter of 20
13 mm. The hydrolysate of starch hydrolysed by the T10 amylase resulted in three
14 oligosaccharide compounds maltotriose, maltotetraose, and maltopentose. The amylase
15 activity of isolate T10 was optimal at a temperature of 40°C and pH 7 in an amount of 0.801
16 U/mL. The isolate T10 was identified as a species member of *Bacillus toyonensis* based on
17 phenotypic characterisation and 16S rDNA gene sequencing analysis with a similarity value
18 of 99.93%.

19
20 **Keywords:**

21 Amylolytic bacteria, Canna, Maltooligosaccharides, 16S rDNA gene.

22 23 **1. Introduction**

24 Oligosaccharides are members of an essential group of carbohydrates.
25 Macromolecules with short-chain polysaccharide sugars of 2 to 20 saccharide units.

26 Functional oligosaccharides such as galactooligosaccharide (GOS), fructooligosaccharide
27 (FOS), and maltooligosaccharide (MOS) are well-known prebiotics owing to their ability to
28 selectively stimulate beneficial bacteria in the intestines, particularly bifidobacterial species
29 (Zhao et al. 2017). Developing oligosaccharide products is one of the businesses with high
30 economic value. Plants such as Canna contain much starch, one of the crucial ingredients
31 (substrate) to produce oligosaccharides enzymatically. Canna plants (*Canna edulis* Ker.)
32 contain high levels of carbohydrates, mainly starch (93.3%), which consists of amylose
33 (33.48%) and amylopectin (59.82%) (El-Fallal et al. 2012). Starch is hydrolysed into smaller
34 oligosaccharides by α -amylase, one of the most important commercial enzymes (Jang et al.
35 2020).

36 The starch-processing industry has exploited amylase as a substitute for acid
37 hydrolysis in the production of starch hydrolysis. Amylase acts as a biocatalyst for the
38 hydrolysis of starch into simpler carbohydrates, such as glucose, maltose, and dextrin
39 (Divakaran et al. 2011; Abdalla et al. 2021). Amylolytic bacteria are producers of amylase
40 that can be used as biocatalysts in the starch hydrolysis process (Ding et al. 2021) to produce
41 various maltooligosaccharide products, such as maltotriose, maltotetraose, maltopentaose,
42 and maltohexaose (Pan et al. 2017).

43 Canna plants and its surrounding, including the rhizosphere and plant tissues, can be
44 sources of isolating amylolytic bacteria. The high starch content in canna tubers makes them
45 a suitable substrate for growing various bacteria, especially amylolytic bacteria. The bacteria
46 isolated from starch-rich sources generally have the potential to produce amylase with high
47 activity (Hellmuth & van den Brink 2013). In addition, the rhizosphere is known as the most
48 diverse microbial habitats concerning species richness and community size. The interaction
49 between plant roots and microorganisms is intensive around the rhizosphere, because the
50 plants secrete exudates containing carbohydrates, amino acids, and other nutrients utilised by

51 bacteria for growth. On the contrary, rhizospheric bacteria can produce protein and enzymes
52 that are important for the biological function of host plants (Afifah et al. 2018).

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

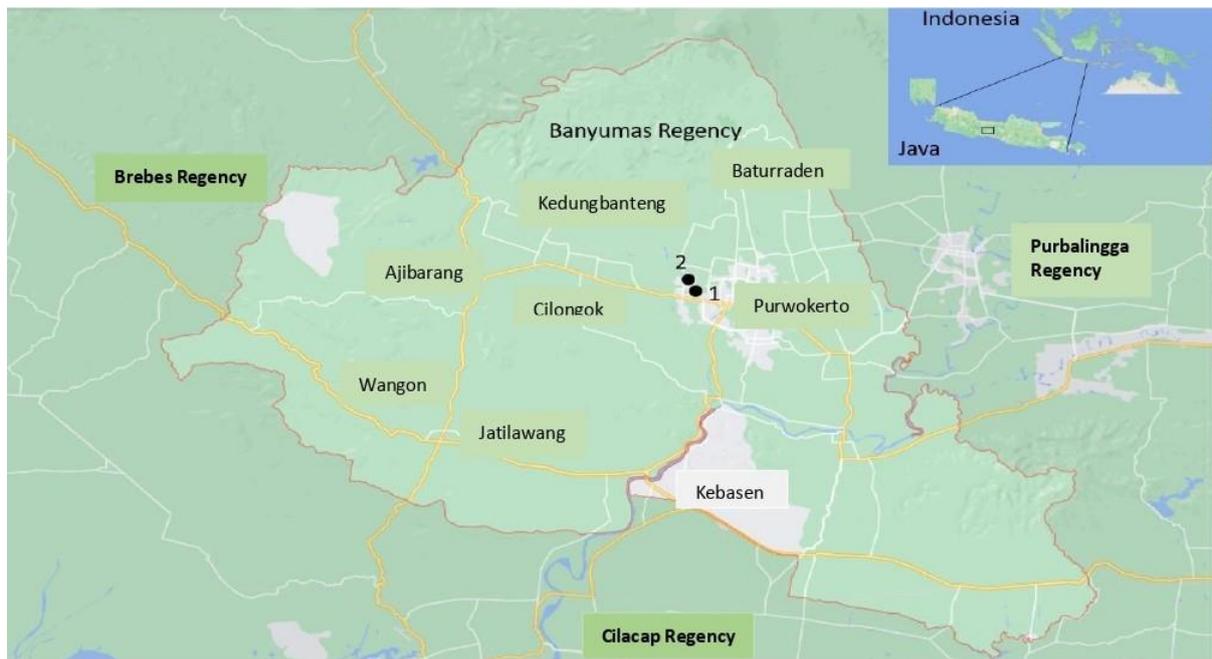
64 The objectives of the study were to isolate amylolytic bacteria from rhizosphere and
65 plant tissue of *Canna*, as well as litter; to know oligosaccharide compounds produced from
66 hydrolysate of starch hydrolysed by the bacterial enzymes, and to identify the selected
67 amylolytic bacteria based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

68

69 **2. Materials and methods**

70 **2.1. Sample collection and location of sampling**

71 Samples were taken from the rhizosphere and parts of *Canna* plant (*C. edulis* Ker.)
72 including tubers, stems, leaves, tissue, as well as litter growing in two places, namely in the
73 forest and the community gardens around the Perhutani Forest West Banyumas, Central Java,
74 Indonesia. The coordinates of the former are S 07°20.846 'E 109°06.410 and the latter is S 07
75 °20.812 'E 109°05.92 (Figure 1).



77

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

81

82 2.2. Isolation, screening, and morphological characterisation of amylolytic bacteria

83 Plant tissues and litter were cleaned with running water, then cut into 1 cm long
 84 pieces and separated according to the plant part. The sample pieces were immersed in 70%
 85 alcohol for 1 minute, then in 1% sodium hypochlorite solution for 3 minutes, after which they
 86 were soaked again using 70% alcohol for 1 minute, and rinsed with sterile distilled water
 87 three times (Duan et al. 2021, with modification). The sterile samples were placed on sterile
 88 tissue papers and then crushed using a mortar and one gram of each sample was diluted with
 89 9 mL of sterile distilled water, and then serial dilutions were made up to 10^{-7} .

90 One gram of *Canna* rhizospheric soil was put into a 20 mL of nutrient broth (NB)
 91 medium containing 1% soluble starch (Merck) in a 100 mL Erlenmeyer flask. The solution
 92 was then homogenised in an agitation speed shaker machine at 150 rpm and incubated for 24

93 hours at 30 °C. The amount of 1 mL of the solution was diluted with 9 mL of sterile distilled
94 water, and then serial dilutions were made up to 10⁻⁷.

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

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

107

108 2.3. Phenetic and phylogenetic characterisations of the selected bacterium

109 Phenetic characterisations of the selected bacterium (producing high diameter clear
110 zones and maltooligosaccharide enzyme) included colony morphology, cells morphology,
111 and biochemistry, were conducted by conventional methods (Smibert & Krieg 1981).
112 Biochemical tests were also conducted using the API 50CHB kit.

113 The 16S rDNA gene was amplified by polymerase chain reaction (PCR) technique
114 using a pair of primers (9F: 5'GAGTTTGATCCTCCTGGCTCAG-3') 1510R:
115 5'GGCTACCTTGTTACGA-3') (Yopi et al. 2017). The obtained bands were stained and
116 visualised by UV Transilluminator. The sequence was confirmed via 1st BASE Sequencing,
117 Malaysia. The 16S rDNA nucleotide sequences were analysed by nucleotide BLAST (Basic

118 Local Alignment Search Tool) search in the Gene Bank of National Center for Biotechnology
119 Information (NCBI) or BLAST for amino acid analysis (Zhuang et al. 2012). The
120 phylogenetic tree was constructed using a neighbour joining algorithm in MEGA 6.0
121 software (De Moraes Russo & Selvatti 2018).

122

123 2.4 Analysis of the hydrolysis products by a thin-layer chromatography method (Rahmani et
124 al. 2013)

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

130 The hydrolytic activity of amylase in a substrate solution was carried out at 30°C in
131 50 mM acetate buffer, pH 6, containing 0.5% of commercial starch. The enzyme-substrate
132 ratio (v/v) was 1:1 and the reaction times were in hours (0, 1, 2, 3, 4, 24). Reactions were
133 carried out in 2 mL Eppendorf containing 1 mL of reaction mixture in a Deep Well
134 Maximizer (Bioshaker M-BR-022UP, Taitec Japan).

135 A Thin Layer Chromatography (TLC) of maltooligosaccharide products was carried
136 out on silica gel 60F₂₅₄ plates (Merck Art 20-20 cm) and eluent using a solvent mixture of n-
137 butanol:acid:water (12:6:6, v/v/v). Spots formed were visualised by spraying the sugar
138 colours (0.5 g α -diphenylamine, 25 mL acetone, 2.5 mL phosphate acid, 0.5 mL aniline). All
139 samples were applied in equal quantities (4 μ L). Glucose (Sigma-Aldrich, U.S.A), maltose
140 (M2), maltotriose (M3), maltotetraose (M4), maltopentaose (M5), maltohexaose (M6), and
141 maltoheptaose (M7) (Megazyme) were used as standards.

142

143 2.5. Crude enzyme production and amylase activity at different fermentation time

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

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

$$152 \quad = \frac{c \times d \times 1000}{t \times mw} \text{ U/mL}$$

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

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

157

158 2.6. Effect of pH and temperature on enzyme activity of the selected isolate

159 The optimal pH of the enzyme activity was done at pH ranges of 3.0-10.0 under
160 standard assay conditions. Various buffers (0.05M) used were sodium acetate (pH 3.0-6.0),
161 sodium phosphate (6.0-8.0), Tris-HCL (pH 7.0-9.0), and Glycine-NaOH (pH 8.0-10.0). The
162 enzyme reactions were incubated at 40°C for 30 min in the presence of 0.5% (w/v) starch
163 solution (Merck)). The effect of temperatures on enzyme activity was conducted at
164 temperatures ranging from 30-90 °C in 50 mM acetate buffer at optimum pH for 30 min.
165 Amylase activity was assayed by DNS method (Miller 1959).

166

167 **3. Results and Discussion**

168 3.1 Isolation and an amyolytic assay of bacteria isolated from the rhizosphere and plant
169 tissue of Canna, and litter

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

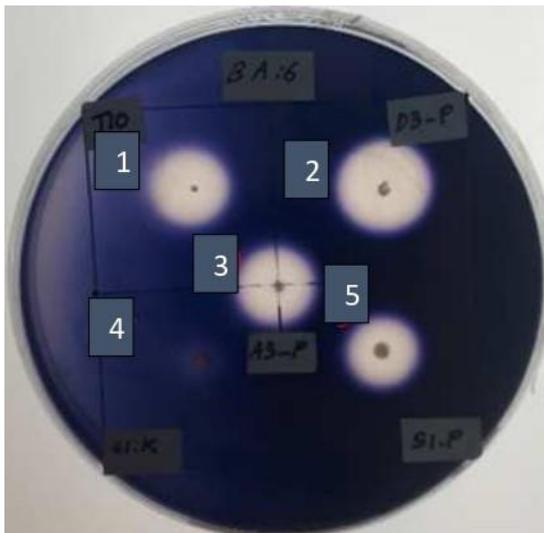
177 The ability of the bacteria to grow and to produce clear zones in the medium,
178 indicating that those bacteria were capable of producing amylase. The more amylase is
179 released, the wider clear zones are produced due to the degradation of amylum in the
180 medium, resulting in enhancing the amyolytic index (Ginting et al. 2021). The research
181 results showed that eight isolates of TH6, TH7, T5, T10, D2, D3, A3, and S1 showed high
182 total clear zone diameters (mm) of 18, 18, 18, 20, 18, 30, 18, and 18, respectively (Table 1).
183 The consistency of the bacterial isolates, resulting in the total clear zone diameter, was shown
184 by the isolates TH6, T10, D3, A3, and S1, while the other isolates tended to reduce or lose
185 their amyolytic activity (Figure 2). Based on the ability of isolates to produce a clear zone
186 diameter \geq 18 mm and consideration of source representatives, four isolates (TH6, T10, D3,
187 A3, and S1) were selected for further testing, namely their ability to hydrolyse starch.
188 Hasanah et al. (2020) reported that bacterial isolates having an amyolytic index of more than
189 9 mm were potentials for producing amylase. According to Ochoa-Solano & Olmos-Soto
190 (2006), bacterial isolates produce clear zones two or three times the diameter of the colony
191 are potential enzyme producers.

193 **Table 1.** Sources, number, and total clear zone diameter of amylolytic bacteria isolated from
 194 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

195



196

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

199

200 A high number of amyolytic bacteria isolated from the rhizosphere of Canna was by
 201 Vassekaran et al. (2010), who stated that bacteria isolated from starch-rich materials have
 202 better potential to produce amylase. Vijayalakshmi et al. (2012) found *Bacillus subtilis* KC3
 203 isolated from the rhizosphere of *Euphorbia hirta* produced a maximum halo zone of 23 mm

204 on a Starch Agar medium. Gebreyohannes (2015) reported that 16 bacterial isolates from
 205 soils could produce clear zones of 3-22 mm on starch agar plates. Ginting et al. (2021) found
 206 thermophilic bacteria of *Bacillus* sp. L3 and *B. caldotenax* L9 from a marine hydrothermal
 207 produces high amylolytic indexes of 3.04 and 3.52, respectively. The clear zone formed
 208 results from breaking starch compounds into simple compounds; the wider the clear zone
 209 formed, the higher the amylolytic activity (Zubaidah et al. 2019).

210 The characteristics of colonial morphology of the 34 isolates were rough, dry, bright,
 211 and pink; cells were Gram-positive, rod shape, motile, and had endospores. The endospore
 212 position of isolates of D1-D4 and S1-S2 was in terminal, while isolates of TH1-TH11, T1-
 213 T12, and D1-D4 had endospores in the centre. All isolates were able to hydrolyse starch and
 214 produce lecithinase (Table 2). Those characteristics indicated that the bacteria were members
 215 of the genus *Bacillus*. According to Logan & De Vos (2009), the main characteristics of the
 216 genus *Bacillus* are cells rod-shaped, straight or slightly curved, occurring singly and in pairs,
 217 some in chains, form endospores, Gram-positive or Gram-negative, motile, aerobes or
 218 facultative anaerobes, and mostly isolated from soil.

219

220 **Table 2.** Morphological and physiological properties of the bacteria isolated from Canna
 221 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	Rough, dry, bright and pink	Rough, dry, bright and	Rough, dry, bright, and pink

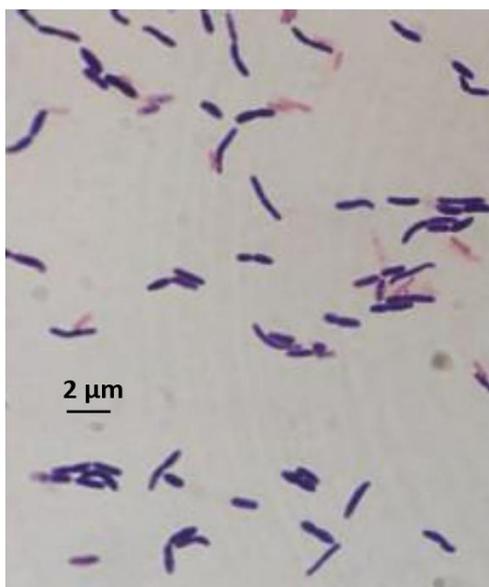
		pink		pink	
Gram reaction	+	+	+	+	+
Cell shape	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+
Presence of spore	+	+	+	+	+
Position of spore	Centre	Centre	Terminal	Centre	Terminal
Starch hydrolysis	+	+	+	+	+
Lecithinase production	+	+	+	+	+

222

223 3.2. Identification of the selected isolate of T10 based on phenetic and phylogenetic

224 characteristics

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



234

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

237

238 **Table 3.** Phenotypic characterisation 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	+

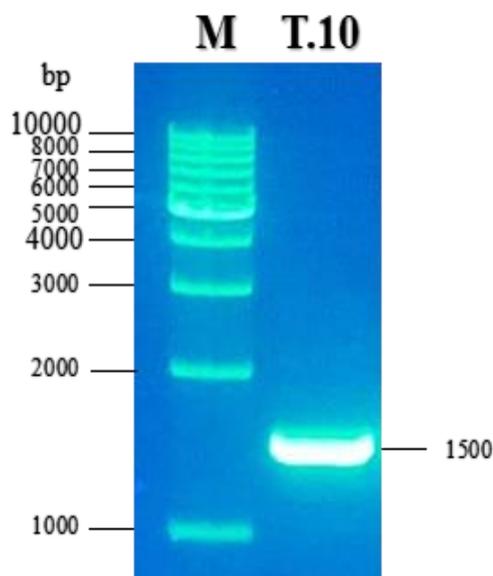
D-Mannose	+
Methyl- α D-glucopyranoside	+
Amygdalin	+w
Arbutin	+
Salicin	+
Cellobiose	-
D-saccharose	+
D-trehalose	+
Starch	+
Glycogen	+
D-turanose	+

239

240 The electrophoresis visualisation of the PCR product showed that the DNA of T10
241 produced a single band with a size of 1500 kb (Figure 4). The results of the comparison
242 between the 16S rRNA gene sequence of isolate T10 and nucleotide sequences in the
243 GeneBank (<http://blast.ncbi.nlm.nih.gov/>) showed that the bacterium is closely related to
244 species members of the genus *Bacillus*. The BLAST analysis results showed that isolating
245 T10 had a similarity of 99.3% with either *Bacillus toyonensis* SPa09NA, *B. toyonensis* PZ-
246 48, or *B. toyonensis* SMP1. The phylogenetic tree constructed using Neighbor-Joining, Model
247 Maximum Composite Likelihood, and 1000x Bootstrapping. A dendrogram resulted from
248 MEGA10 program showed that isolate T10 joined *B. toyonensis* SX04NA, *B. toyonensis*
249 Spa09NA, *B. toyonensis* SMP1, *B. toyonensis* PZ-48, *B. toyonensis* BCT-7112, and *B.*
250 *toyonensis* l3aM to form a separate cluster (Figure 5). Hence, isolate T10 was identified as
251 the species member of *B. toyonensis* based on the phenetic and phylogenetic characteristics.

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

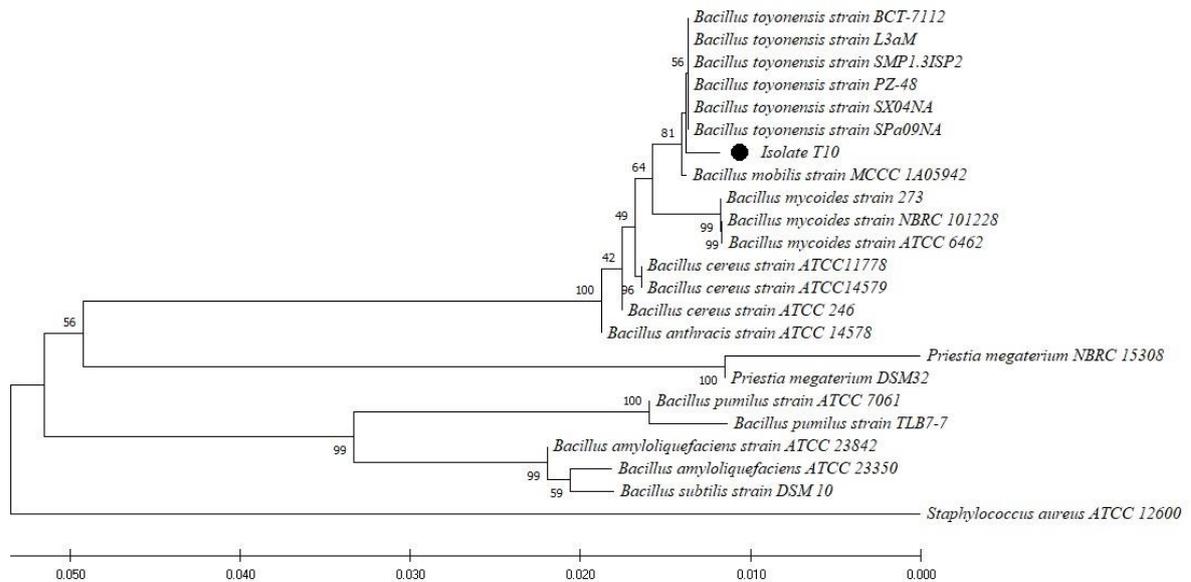
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265

266 **Figure 4.** An electropherogram of the amplified 16S rRNA gene of isolate T10 with a size of
267 1500 bp. Marker (M): 1 kb DNA ladder.

268



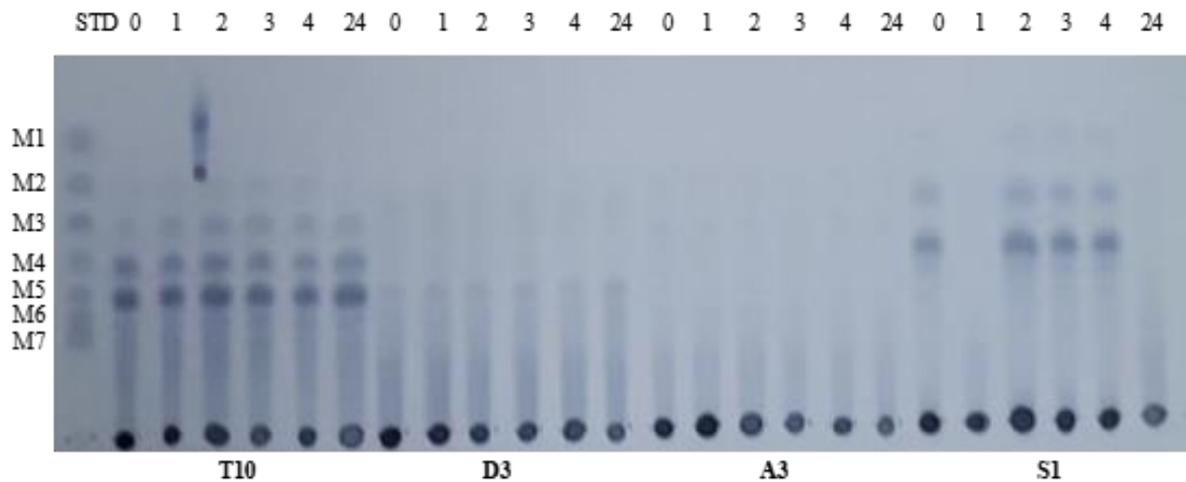
269

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

275

276 3.3. Analysis of hydrolysed products by the selected bacterial amylases using a TLC method

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



283

284 **Figure 6.** The product profile of starch hydrolysed by amylase of the amyolytic bacteria
 285 (T10, D3, A3, and S1) using a TLC method with reaction times (hours): of 0, 1, 2, 3, 4, and
 286 24 at 30°C. The Standards (STD): monosaccharide (M1), maltose (M2), maltotriose (M3),
 287 maltotetraose (M4), maltopentaose (M5), maltohexaose (M6) and maltohepta (M7).

288

289 Based on the TLC chromatogram, the starch degraded by T10 amylase resulted in
 290 malto-oligosachharides of maltotriose, maltotetraose, and maltopentaose. Amylases are able
 291 to break down starch polymer bonds into shorter oligosaccharides or simple sugar molecules
 292 (Putri et al. 2012). The results showed that amyolytic bacteria with high amyolytic indexes
 293 (AI) did not correlate with their ability to degrade amyllum. The isolate T10, with its total
 294 diameter lower than isolate D3, showed a higher ability to breakdown starch polymer bonds
 295 into shorter or oligosaccharides. The results of this study proved that a high AI value is only
 296 sometimes accompanied by the ability of the amylase to break down starch polymer bonds.
 297 The ability of the T10 amylase to produce the maltooligosaccharides was similar to the
 298 amylase of *Bacillus circulans* GRS 313 isolated from soil that also produced maltotriose,
 299 maltotetraose, and maltopentaose (Dey et al. 2002). On the contrary, Rahmani et al. (2013)
 300 found maltose and maltotriosa produced by amylase of *Brevibacterium* sp. using black potato

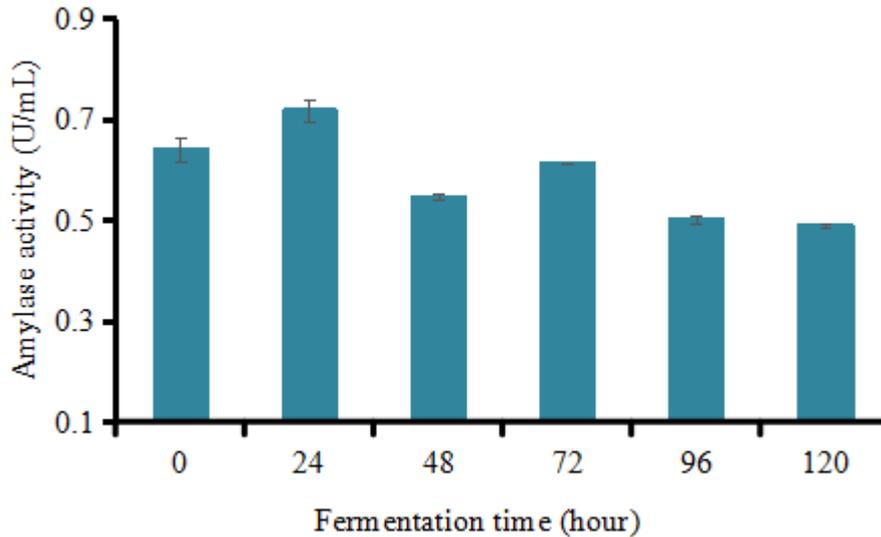
301 starch as substrate, while amylase of *Bacillus subtilis* strain SDP1 isolated from rhizosphere
302 of Acacia produces maltotriose and maltotetraose (Ozturk et al. 2014). Furthermore, Abdul
303 Manas et al. (2014) reported that amylase of an alkaliphilic *Bacillus lehensis* G1 could
304 degrade oligosaccharides by producing maltooligosaccharides with a higher degree of
305 polymerization than maltoheptaose observed on thin-layer chromatography and high-
306 performance liquid chromatography analyses.

307

308 3.4. Crude enzyme production of a selected isolate and measurement of its amylase activity at
309 different culture incubations

310 Based on the ability of the fourth selected amylolytic bacteria to produce different types
311 of hydrolysed product, isolate T10 was further assayed for its optimal amylase activity at
312 different incubation times. The results showed that incubation times affected the amylase
313 activity of isolate T10 carried out in a 0.5% starch solution at 30°C in 50 mM acetate buffer
314 of pH 6. The amylase activity of T10 was optimal during incubation 1-3 days ranging from
315 0.546-0.717 U/mL and the highest amylase activity was found at 24 hr incubation of 0.717
316 U/mL (Figure 7). The results also showed that amylase activity decreased after 72 h of
317 incubation. The amylase activity value at day 0 is quite high. This might be due to the
318 measurement of the enzyme activity using the DNS method, in which reducing sugar formed
319 from a carbon source (starch) is used by bacteria for the initial stages of growth; then, the
320 bacteria will use the carbon source for the production of enzymes.

321



322

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

324

325 The amylase activity of *Bacillus cereus* KN isolated from Ranu Ngebel and incubated
 326 for three days was 0.016 U/mL, while strain G20 isolated from Ranu Grati was lower at about
 327 0.0001 U/mL (Nisa et al. 2021). Luang et al. (2019) found *Bacillus* sp. 3.5AL2 isolated from
 328 soils of the unexplored Nasinuan Forest, Thailand and incubated for three days exhibiting
 329 amylase activity of 1.97 U/mg protein at the optimal conditions of 60°C and pH 7.0 after 30
 330 min incubation with 1% starch in 0.05 M phosphate buffer. Gebreyohannes (2015) reported
 331 that the amylase activity of *Bacillus* spp. it decreased after 48 h incubations due to the
 332 suppression and accumulation of other byproducts in the fermentation medium and also
 333 depletion of nutrients.

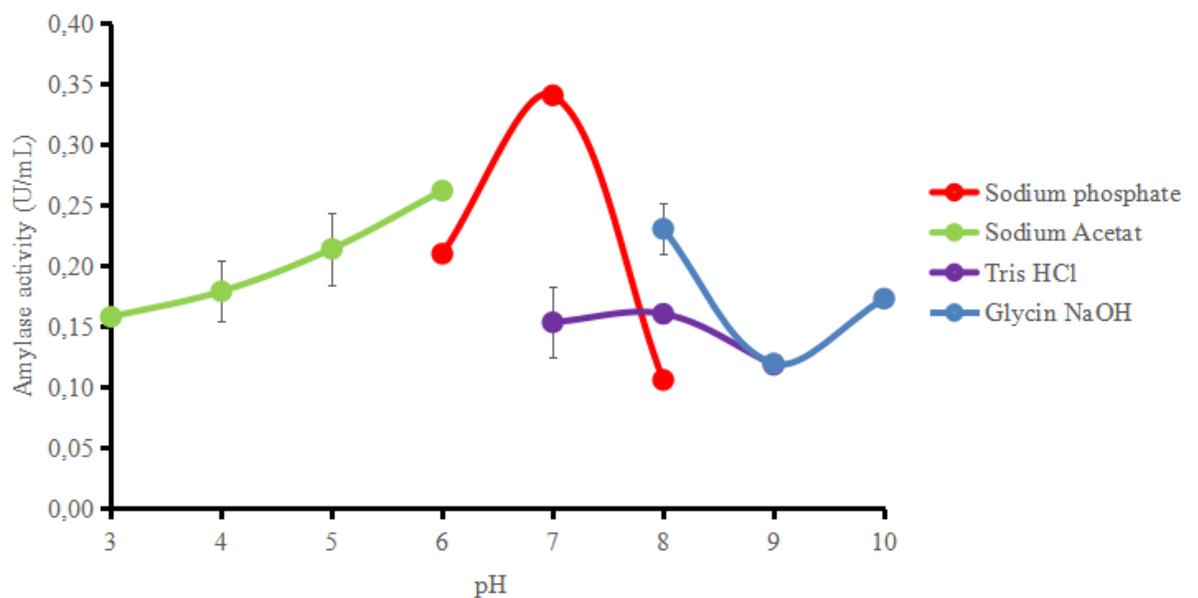
334

335 3.5. Enzyme characterisation: the effect of pH and temperature against enzyme activity of the
 336 selected isolate

337 The effects of pH's on the amylase activity of isolate T10 showed that optimum
 338 conditions were in sodium acetate buffer pH 6 with an amylase activity of 0.262 U/mL and in
 339 sodium phosphate buffer pH 7 with an amylase activity of 0.341 U/mL (Figure 8). The

340 optimal pH of isolate T10 was by Naidu et al. (2019) for *Paenibacillus* sp. D9 that its optimal
 341 pH for amylase activity is in the neutral range (pH 6-8). The increase in pH beyond these
 342 values resulted in a decline in enzyme activity. Any change in pH causes a change in the
 343 enzyme's active site (Lim & Oslan 2021). Bajpai et al. (2015) reported that the optimal pH
 344 for amylase activity of *Haloferax* sp. HA10 was at pH 7.0. According to Asgher et al.
 345 (2007), each enzyme has an optimal pH to work most actively, and the optimal pH of
 346 amylase is varied from pH 3.8 to 9.5 depending on the type of enzyme and the source. Behal
 347 et al. (2016) reported an amylase produced by *Bacillus* sp. AB04 had optimal activity at pH
 348 8. Moreover, the enzyme is stable in neutral to alkaline (pH 7-10).

349



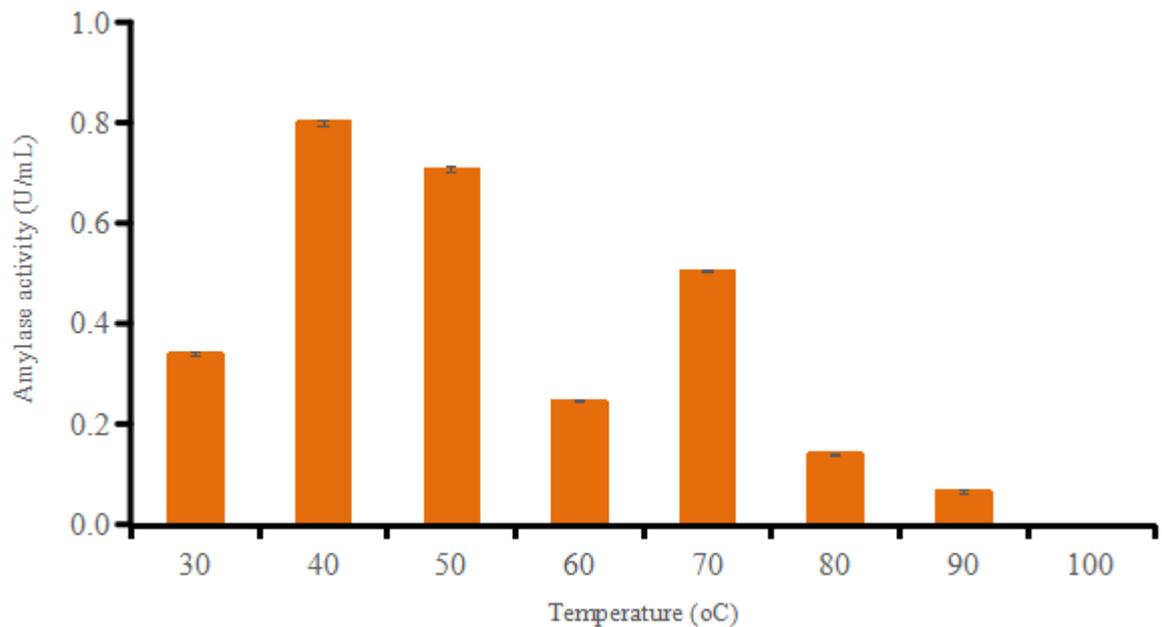
350

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

352

353 The amylase activity of isolate T10 was observed at temperatures ranging from 30-
 354 90°C at pH 7.0. Amylase activity of the T10 isolate tended to be optimum at 40°C with an
 355 activity value of 0.801 U/mL (Figure 9). A similar finding was also reported by
 356 Sivaramakrishnan et al. (2006) for several species of *Bacillus* sp., *B. subtilis*, *B.*

357 *stearothermophilus*, *B. licheniformis*, and *B. amiloliquefaciens* have optimum temperatures of
358 37-60°C. Gebreyohannes (2015) found that the maximum amylase activity of *Bacillus* spp.
359 was 40°C and *Streptomyces* spp. at 37°C, used 4% starch concentration at a neutral pH and
360 an incubated for 48 h. The crude enzyme of *Bacillus* sp. AB04 showed maximum activity at
361 pH 8 with an optimum temperature of 40° C with more than 75% activity in range of 50 - 80°
362 C (Behal et al. 2016). The results showed that either pH or temperature significantly affected
363 the enzyme activity of the T10 amylase which was optimum at pH 7.0 and a temperature of
364 40°C.
365



366
367 **Figure 9.** Amylase activity of isolate T10 at different temperatures.

368
369 The differences in the pH and temperature characteristics of enzyme activity indicated
370 that enzymes are specific, depending on the species that produces them. A decrease or
371 increase in temperature can affect the secretion of extracellular enzymes by changing the
372 physiology of the cell membrane (Rahmani et al. 2018). The optimum temperature is the
373 temperature that causes chemical reactions at the most incredible speed (Subagiyo et al.

374 2017). The results showed that after reaching the optimum condition, it was seen that the
375 activity of the T10 amylase decreased. High temperatures can cause enzymatic reactions to
376 decrease because enzyme proteins undergo conformational changes so that protein molecules
377 will experience denaturation (Yufinta et al. 2018).

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

388

389 **4. Conclusions**

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

396

397 **Authors contribution**

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

400

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404

405 **Conflict of interest**

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

407

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Research Article

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

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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%.

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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,

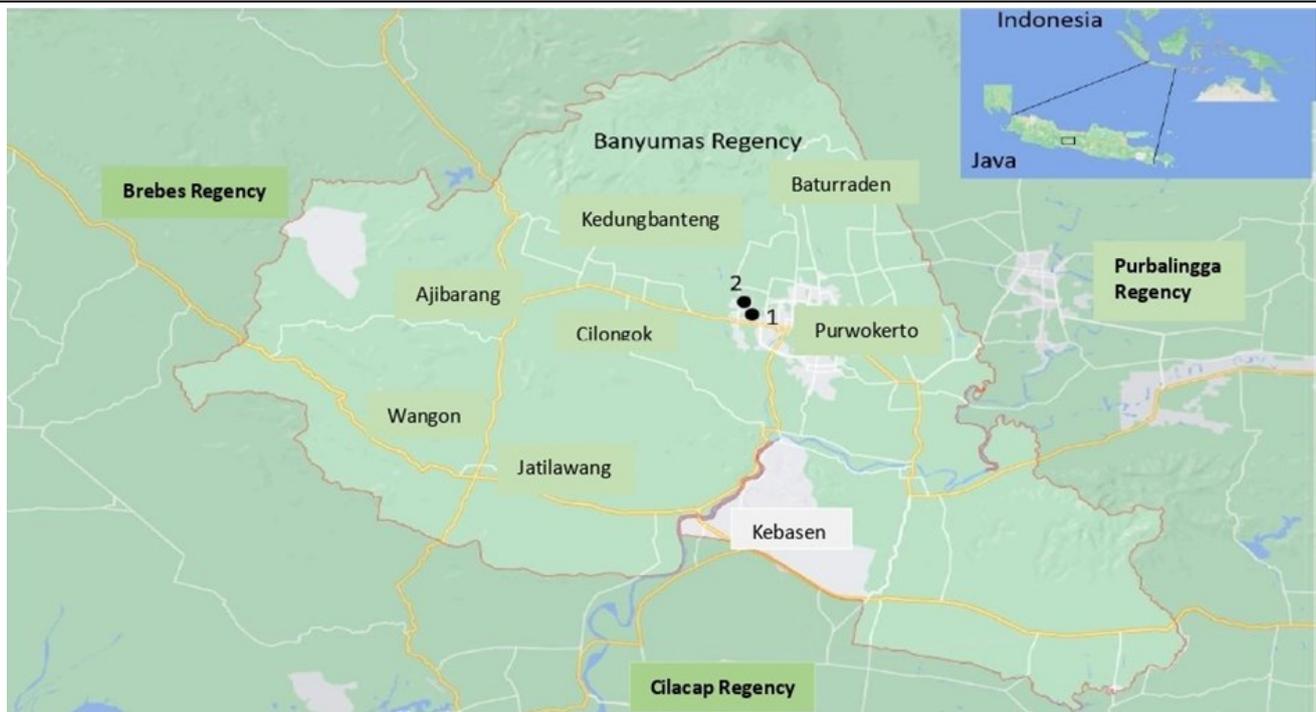


Figure 1. A map of Banyumas Regency and sampling sites: (1) the Perhutani Forest KPH West Banyumas, Central Java, (2) Community gardens around the Perhutani Forest KPH West Banyumas, Central Java.

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'GAGTTT-GATCCTCCTGGCTCAG-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 60F₂₅₄ 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 μ L). 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 μ mol 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 Amylytic 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 amyllum in the medium, resulting in enhancing the amylytic 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 amylytic activity (Figure 2). Based on the ability of isolates to produce a clear zone diameter ≥ 18 mm and consideration of source representatives, four 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 amylytic 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.

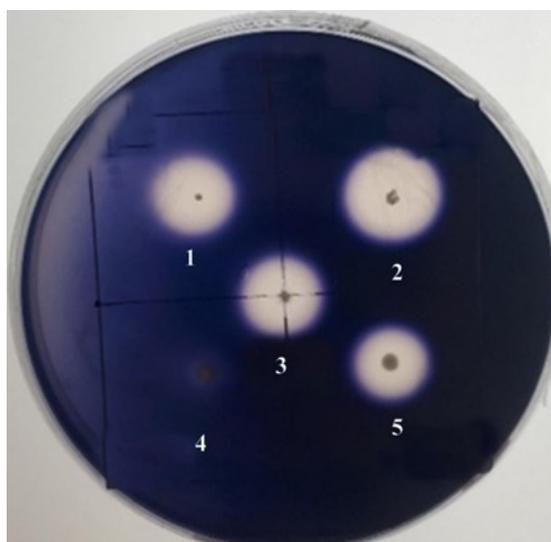


Figure 2. The amylytic zones produced by bacterial isolates of: (1) T10, (2) D3, (3) A3, (4) TH6, and (5) S1 on a NA medium + 1% soluble starch.

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 **enzyme** 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.nlm.nih.gov/>) showed that the bacterium is closely related to species members of the genus *Bacillus*. The BLAST analysis showed that isolating 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-71112, 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-71112^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.

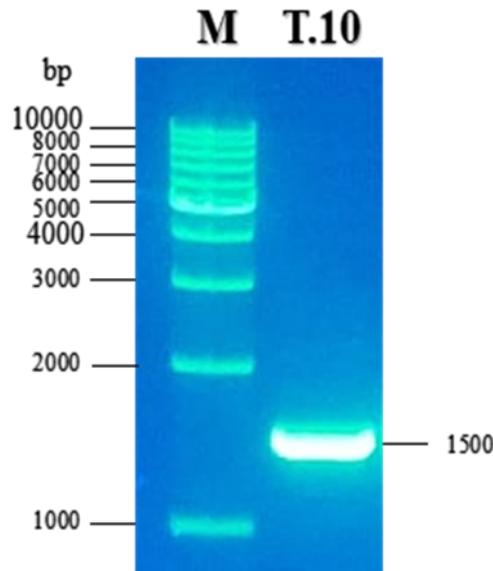


Figure 4. An electropherogram of the amplified 16S rRNA gene of isolate T10 with a size of 1500 bp. Marker (M): 1 kb DNA ladder.

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 amyolytic bacteria with high amyolytic indexes (AI) did not correlate with their ability to degrade amyllum. 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 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 amyolytic bacteria to produce different types of hydrolysed product, isolate T10 was further assayed for

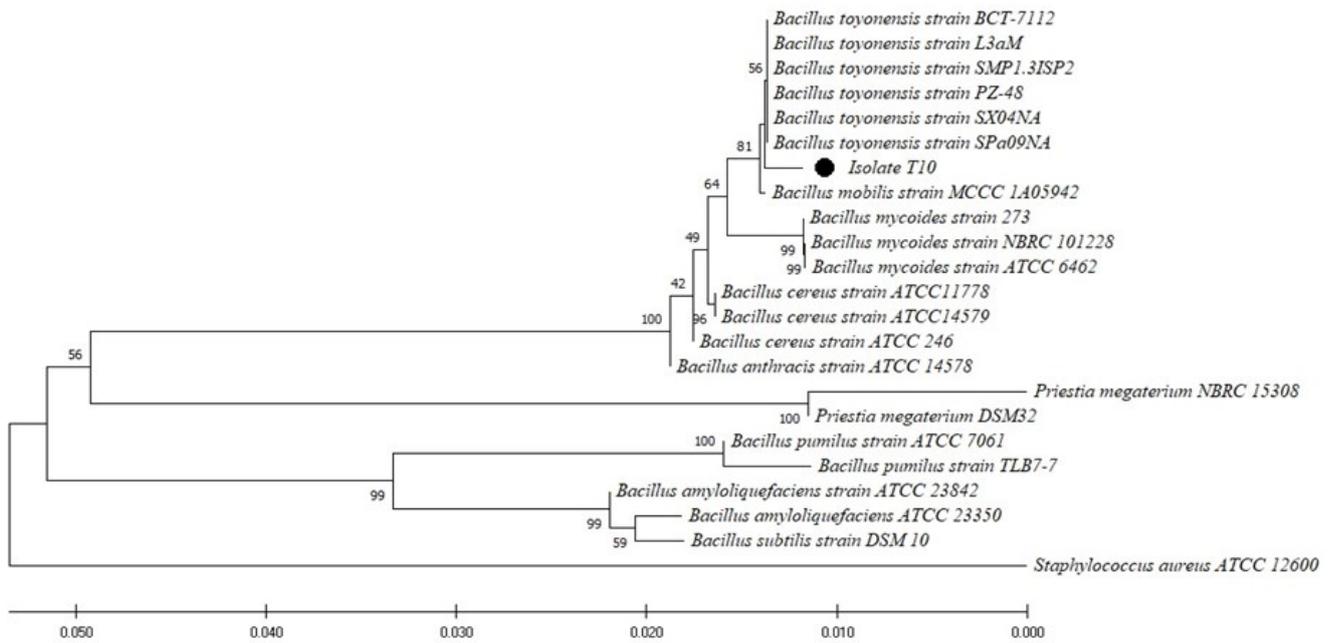


Figure 5. A phylogenetic tree showing the relationship between strain T10 isolated from rhizospheres of *Canna* (*C. edulis*) and several species members of the genus *Bacillus* on the basis of 16S rRNA gene sequence reconstructed based on Neighbor-Joining, Model Maximum Composite Likelihood, and 1000x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

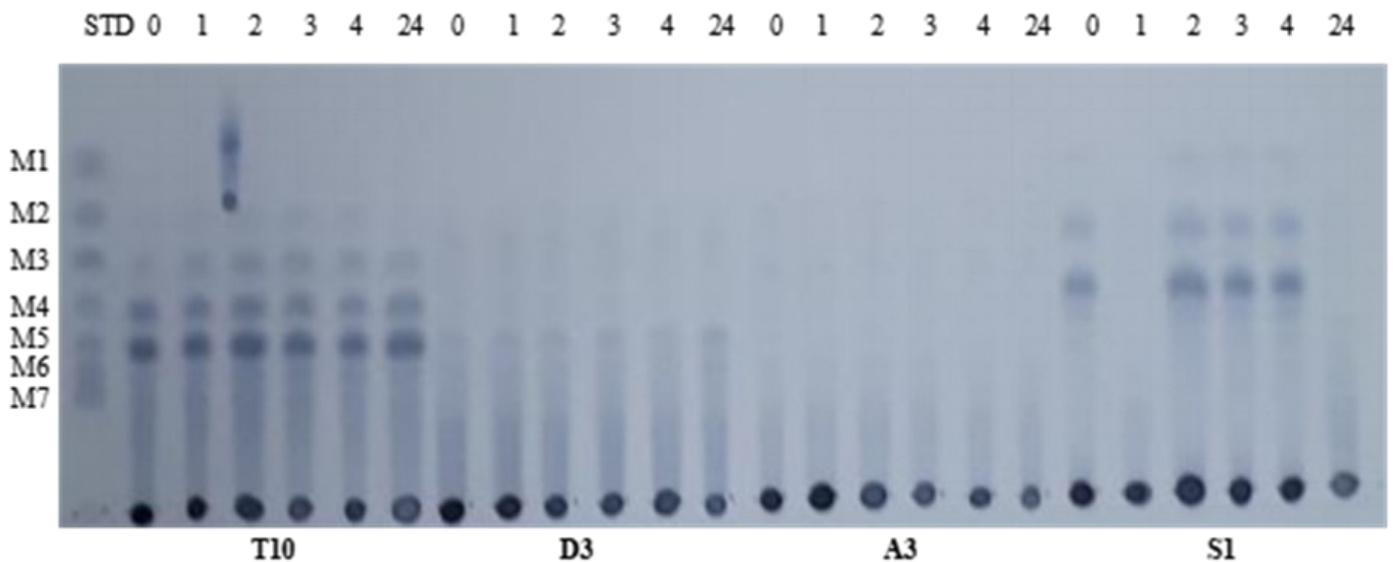


Figure 6. The product profile of starch hydrolyzed by amylase of the amylolytic bacteria (T10, D3, A3, and S1) using a TLC method with reaction times (hours): of 0, 1, 2, 3, 4, and 24 at 30°C. The Standards (STD): monosaccharide (M1), maltose (M2), maltotriose (M3), maltotetraose (M4), maltopentaose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

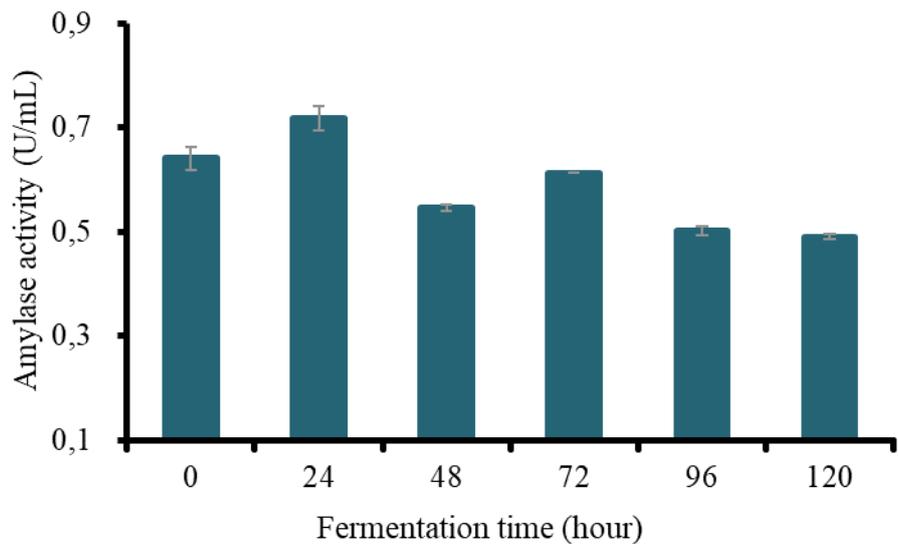


Figure 7. Amylase activity of T10 at different incubation times.

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.*

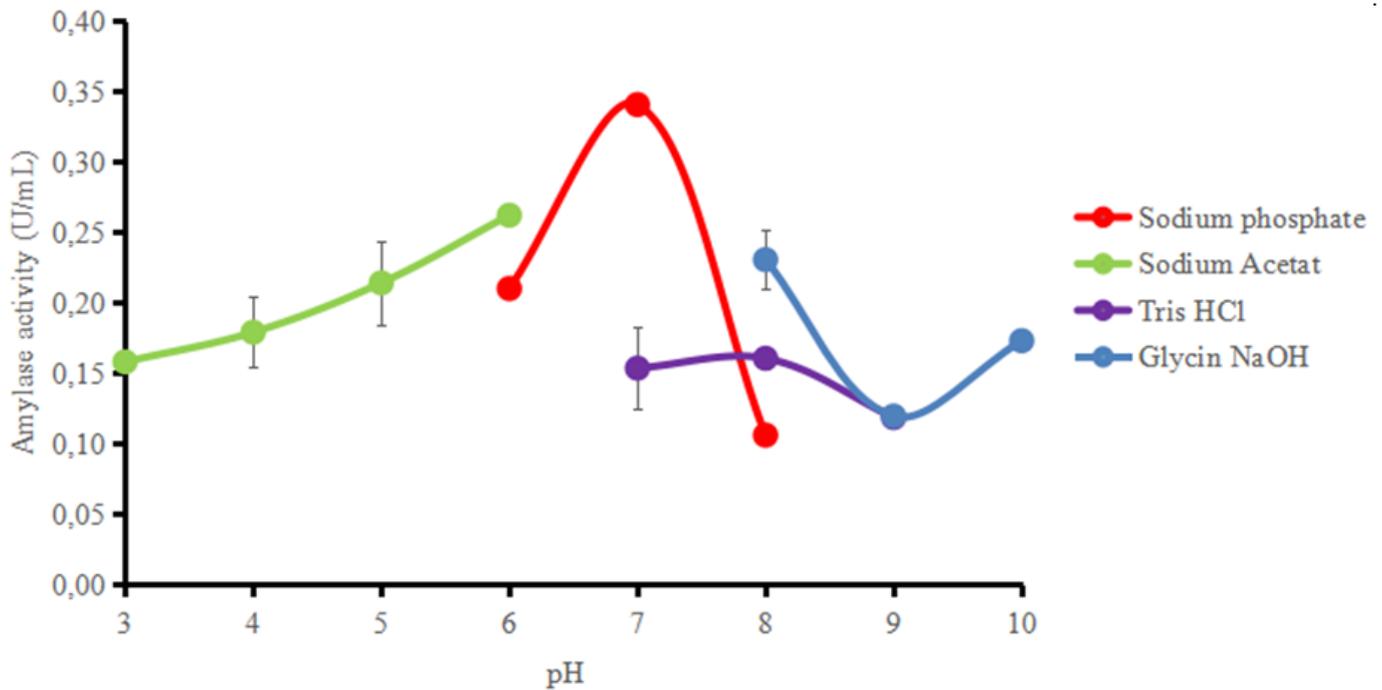


Figure 8. Amylase activity of T10 at different pH and buffers.

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.

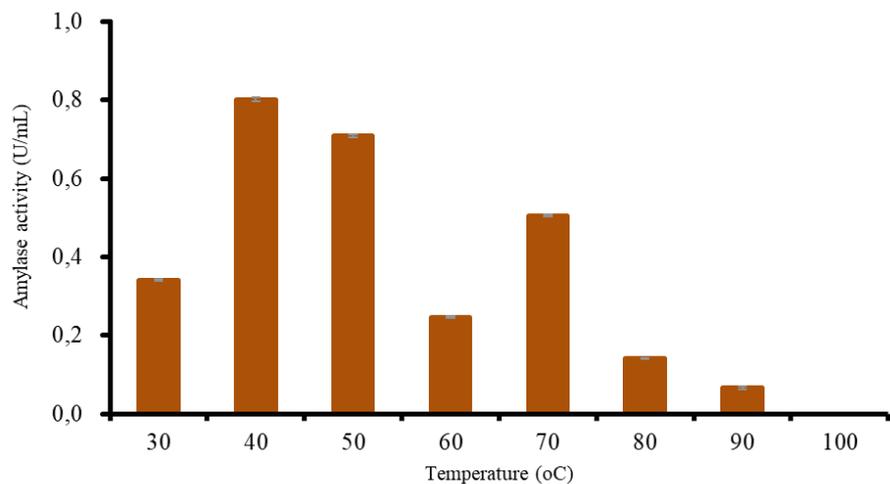


Figure 9. Amylase activity of isolate T10 at different temperatures.

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

3

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17

18

19 **Abstract**

20 The objectives of the study were to isolate amyolytic bacteria from the rhizosphere and
21 plant tissue of *Canna edulis* Ker., as well as litter; to know oligosaccharide compounds
22 produced from starch hydrolyzed by the bacterial enzymes, and to identify the amyolytic
23 bacteria based on phenetic and 16S rRNA gene sequences. From the rhizosphere, Canna plant
24 tissue, and litters obtained thirty-two amyolytic bacterial isolates. Eight isolates (TH6, TH7,
25 T5, T10, D2, D3, A3, S1) produced high clear zone diameters ranging from 18-30 mm;

26 especially an isolate T10, which was consistent in producing a total clear zone diameter of 20
27 mm. The hydrolysate of starch hydrolysed by the T10 amylase resulted in three oligosaccharide
28 compounds maltotriose, maltotetraose, and maltopentose. The amylase activity of isolate T10
29 was optimal at a temperature of 40°C and pH at 0.801 U/mL. The isolate T10 was identified as
30 a species member of *Bacillus toyonensis* based on phenotypic characterization and 16S rDNA
31 gene sequencing analysis with a similarity value of 99.93%.

32

33 Keywords:

34 Amylolytic bacteria, Canna, Maltooligosaccharides, 16S rDNA gene.

35

36 **1. Introduction**

37 Oligosaccharides are members of an essential group of carbohydrates. Macromolecules
38 with short-chain polysaccharide sugars of 2 to 20 saccharide units. Functional oligosaccharides
39 such as galactooligosaccharide (GOS), fructooligosaccharide (FOS), and maltooligosaccharide
40 (MOS) are well-known prebiotics owing to their ability to selectively stimulate beneficial
41 bacteria in the intestines, particularly bifidobacterial species (Zhao et al. 2017). Developing
42 oligosaccharide products is one of the businesses with high economic value. Plants such as
43 Canna contain much starch, one of the crucial ingredients (substrate) to produce
44 oligosaccharides enzymatically. Canna plants (*Canna edulis* Ker.) contain high levels of
45 carbohydrates, mainly starch (93.3%), which consists of amylose (33.48%) and amylopectin
46 (59.82%) (El-Fallal et al. 2012). Starch is hydrolysed into smaller oligosaccharides by α -
47 amylase, one of the most important commercial enzymes (Jang et al. 2020).

48 The starch-processing industry has exploited amylase as a substitute for acid hydrolysis
49 in producing starch hydrolysis. Amylase acts as a biocatalyst for the hydrolysis of starch into
50 simpler carbohydrates, such as glucose, maltose, and dextrin (Divakaran et al. 2011; Abdalla

51 et al. 2021). Amylolytic bacteria are producers of amylase that can be used as biocatalysts in
52 the starch hydrolysis process (Ding et al. 2021) to produce various maltooligosaccharide
53 products, such as maltotriose, maltotetraose, maltopentaose, and maltohexaose (Pan et al.
54 2017).

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

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

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

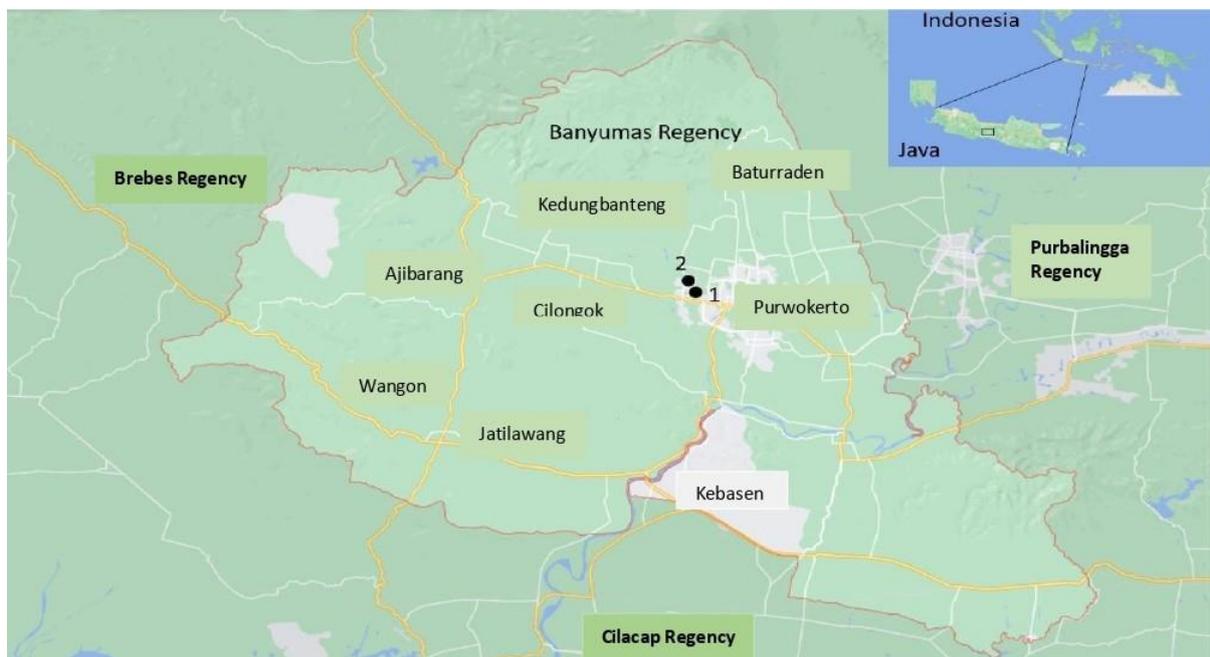
80

81 2. Materials and methods

82 2.1. Sample collection and location of sampling

83 Samples were taken from the rhizosphere and parts of *Canna* plant (*C. edulis* Ker.)
84 including tubers, stems, leaves, tissue, as well as litter growing in two places, namely in the
85 forest and the community gardens around the Perhutani Forest West Banyumas, Central Java,
86 Indonesia. The coordinates of the former are S 07°20.846 'E 109°06.410 and the latter is S 07
87 °20.812 'E 109°05.92 (Figure 1).

88



89

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

93

94 2.2. Isolation, screening, and morphological characterization of amylolytic bacteria

95 Plant tissues and litter were cleaned with running water, then cut into 1 cm long pieces
96 and separated according to the plant part. The sample pieces were immersed in 70% alcohol
97 for 1 minute, then in 1% sodium hypochlorite solution for 3 minutes, after which they were
98 soaked again using 70% alcohol for 1 minute, and rinsed with sterile distilled water three times
99 (Duan et al. 2021, with modification). The sterile samples were placed on sterile tissue papers
100 and then crushed using a mortar and one gram of each sample was diluted with 9 mL of sterile
101 distilled water, and then serial dilutions were made up to 10^{-7} .

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

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

111 The number of 0.5 μ L of bacterial cultures aged 24 hours at 30°C growing on NB
112 medium containing 1% soluble starch was spot inoculated onto NA medium containing 1%
113 commercial soluble starch, then incubated for 72 hours at 30°C. The growing colonies were
114 flooded with iodine solution, and the clear zones formed around the colonies were observed
115 and measured in their diameter (Vijayalakshmi et al. 2012). The isolates having high diameter
116 clear zones were selected and tested for their enzyme production. The colonial and cell

117 morphology of the isolated bacteria were characterized using conventional methods (Smibert
118 & Krieg 1981).

119

120 2.3. Phenetic and phylogenetic characterizations of the selected bacterium

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

125 The 16S rDNA gene was amplified by polymerase chain reaction (PCR) technique
126 using a pair of primers (9F: 5'GAGTTTGATCCTCCTGGCTCAG-3') 1510R:
127 5'GGCTACCTTGTTACGA-3') (Yopi et al. 2017). The obtained bands were stained and
128 visualized by UV Transilluminator. The sequence was confirmed via 1st BASE Sequencing,
129 Malaysia. The 16S rDNA nucleotide sequences were analyzed by nucleotide BLAST (Basic
130 Local Alignment Search Tool) search in the Gene Bank of National Center for Biotechnology
131 Information (NCBI) or BLAST for amino acid analysis (Zhuang et al. 2012). The phylogenetic
132 tree was constructed using a neighbour-joining algorithm in MEGA 6.0 software (De-Moraes-
133 Russo & Selvatti 2018).

134

135 2.4 Analysis of the hydrolysis products by a thin-layer chromatography method (Rahmani et 136 al. 2013)

137 An amount of 2 mL of each 24 h old bacterial culture (four selected isolates) was
138 inoculated into a 200 mL flask containing 20 mL of NB medium plus 1% (w/v) starch solution,
139 pH 6 (50 mM acetate buffer) and incubated at 30 °C for 24 h. The culture was sampling every
140 24 hour and then centrifugated, and the supernatant obtained was tested for its amyolytic
141 activity.

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

147 A Thin Layer Chromatography (TLC) of maltooligosaccharide products was carried
148 out on silica gel 60F₂₅₄ plates (Merck Art 20-20 cm) and eluent using a solvent mixture of n-
149 butanol:acid:water (12:6:6, v/v/v). Spots formed were visualized by spraying the sugar colours
150 (0.5 g α -diphenylamine, 25 mL acetone, 2.5 mL phosphate acid, 0.5 mL aniline). All samples
151 were applied in equal quantities (4 μ L). Glucose (Sigma-Aldrich, U.S.A), maltose (M2),
152 maltotriose (M3), maltotetraose (M4), maltopentaose (M5), maltohexaose (M6), and
153 maltoheptaose (M7) (Megazyme) were used as standards.

154

155 2.5. Crude enzyme production and amylase activity at different fermentation time

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

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

164
$$= \frac{c \times d \times 1000}{t \times mw} \text{ U/mL}$$

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

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

169

170 2.6. Effect of pH and temperature on enzyme activity of the selected isolate

171 The optimal pH of the enzyme activity was done at pH ranges of 3.0-10.0 under
172 standard assay conditions. Various buffers (0.05M) used were sodium acetate (pH 3.0-6.0),
173 sodium phosphate (6.0-8.0), Tris-HCL (pH 7.0-9.0), and Glycine-NaOH (pH 8.0-10.0). The
174 enzyme reactions were incubated at 40°C for 30 min in the presence of 0.5% (w/v) starch
175 solution (Merck)). The effect of temperatures on enzyme activity was conducted at
176 temperatures ranging from 30-90 °C in 50 mM acetate buffer at optimum pH for 30 min.
177 Amylase activity was assayed by DNS method (Miller 1959).

178

179 **3. Results and Discussion**

180 3.1 Isolation and an amyolytic assay of bacteria isolated from the rhizosphere and plant
181 tissue of Canna, and litter

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

189 The ability of the bacteria to grow and produce clear zones in the medium indicates that
190 those bacteria were capable of producing amylase. The more amylase is released, the wider

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

203

204 **Table 1.** Sources, number, and total clear zone diameter of amylolytic bacteria isolated from
 205 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	T1	15
in the people's gardens around the forest	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	D1	16
the people's gardens around the forest	D2	18
	D3	30
	D4	15
Roots of the Canna plant tissue growing in	A1	17
the people's gardens around the forest	A2	16
	A3	18
Litters of the Canna plant from the gardens of	S1	18
the residents around the forest	S2	16

206

207

208 **Figure 2.** The amyolytic zones produced by bacterial isolates of: (1) T10, (2) D3, (3) A3, (4)

209 TH6, and (5) S1 on a NA medium + 1% soluble starch.

210

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

221 The characteristics of colonial morphology of the 34 isolates were rough, dry, bright,
222 and pink; cells were Gram-positive, rod shape, motile, and had endospores. The endospore
223 position of isolates D1-D4 and S1-S2 was in terminal, while isolates of TH1-TH11, T1-T12,
224 and D1-D4 had endospores in the centre. All isolates were able to hydrolyse starch and produce
225 lecithinase (Table 2). Those characteristics indicated that the bacteria were members of the
226 genus *Bacillus*. According to Logan & De Vos (2009), the main characteristics of the genus
227 *Bacillus* are cells rod-shaped, straight or slightly curved, occurring singly and in pairs, some in
228 chains, form endospores, Gram-positive or Gram-negative, motile, aerobes or facultative
229 anaerobes, and mostly isolated from soil.

230

231 **Table 2.** Morphological and physiological properties of the bacteria isolated from Canna plants
232 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	+	+	+	+	+

233

234 3.2. Identification of the selected isolate of T10 based on phenetic and phylogenetic

235 characteristics

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



245

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

248

249 **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 ($^{\circ}\text{C}$)	10 - 45
Optimal growth temperature ($^{\circ}\text{C}$)	35
Salinity tolerance range (% NaCl)	≤ 4
API 50CHB	
Glycerol	-
D-Ribose	+

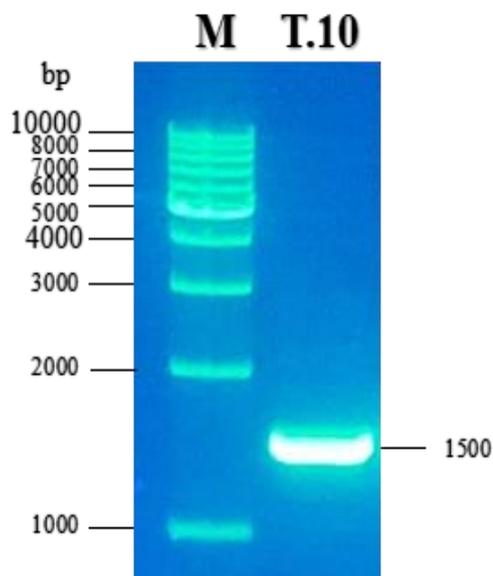
D-Mannose	+
Methyl- α D-glucopyranoside	+
Amygdalin	+w
Arbutin	+
Salicin	+
Cellobiose	-
D-saccharose	+
D-trehalose	+
Starch	+
Glycogen	+
D-turanose	+

250

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

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

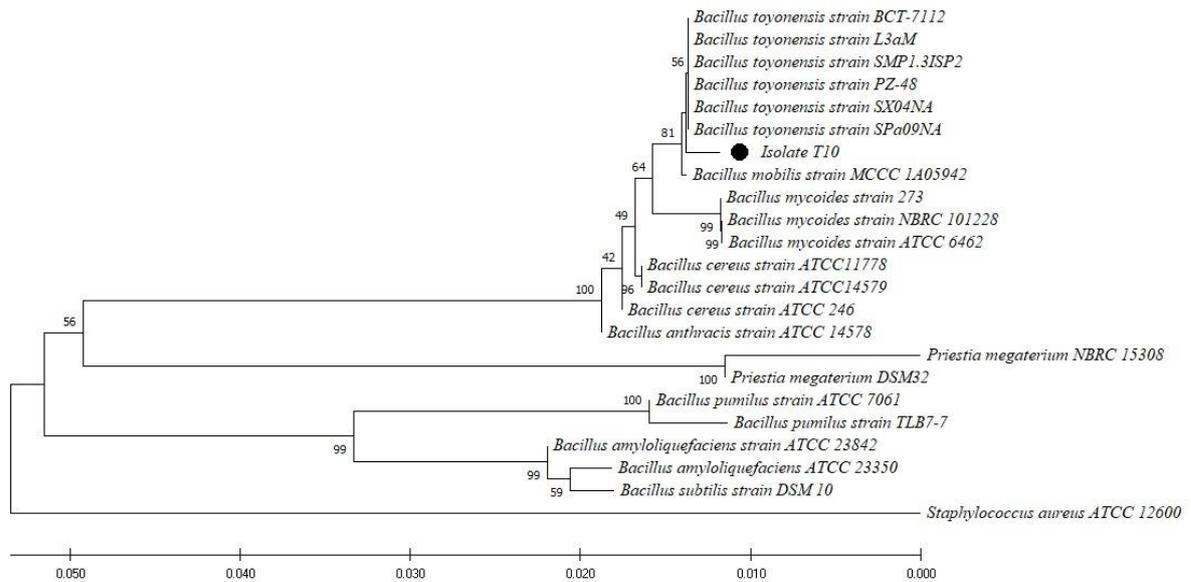
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276

277 **Figure 4.** An electropherogram of the amplified 16S rRNA gene of isolate T10 with a size of
278 1500 bp. Marker (M): 1 kb DNA ladder.

279



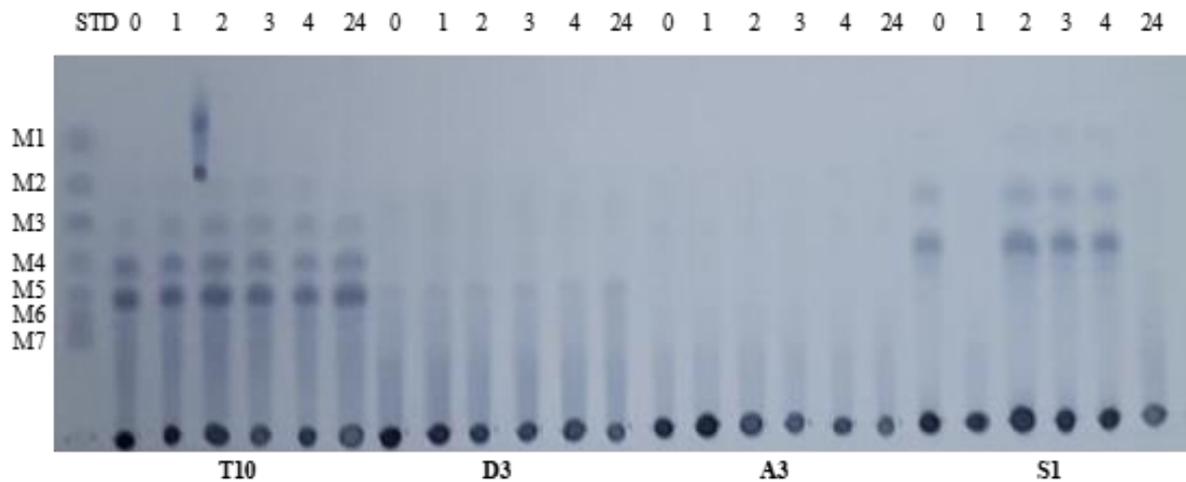
280

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

286

287 3.3. Analysis of hydrolysed products by the selected bacterial amylases using a TLC method

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



294

295 **Figure 6.** The product profile of starch hydrolyzed by amylase of the amyolytic bacteria
 296 (T10, D3, A3, and S1) using a TLC method with reaction times (hours): of 0, 1, 2, 3, 4, and
 297 24 at 30°C. The Standards (STD): monosaccharide (M1), maltose (M2), maltotriose (M3),
 298 maltotetraose (M4), maltopentaose (M5), maltohexaose (M6) and maltohepta (M7).

299

300 Based on the TLC chromatogram, the starch degraded by T10 amylase resulted in malto-
 301 oligosaccharides of maltotriose, maltotetraose, and maltopentaose. Amylases can break down
 302 starch polymer bonds into shorter oligosaccharides or simple sugar molecules (Putri et al.
 303 2012). The results showed that amyolytic bacteria with high amyolytic indexes (AI) did not
 304 correlate with their ability to degrade amyllum. The isolate T10, with its total diameter lower
 305 than isolate D3, showed a higher ability to break down starch polymer bonds into shorter or
 306 oligosaccharides. The results of this study proved that a high AI value is only sometimes
 307 accompanied by the ability of the amylase to break down starch polymer bonds. The ability of
 308 the T10 amylase to produce the maltooligosaccharides was similar to the amylase of *Bacillus*
 309 *circulans* GRS 313 isolated from soil that also produced maltotriose, maltotetraose, and
 310 maltopentaose (Dey et al. 2002). On the contrary, Rahmani et al. (2013) found maltose and
 311 maltotriose produced by amylase of *Brevibacterium* sp. using black potato starch as substrate,

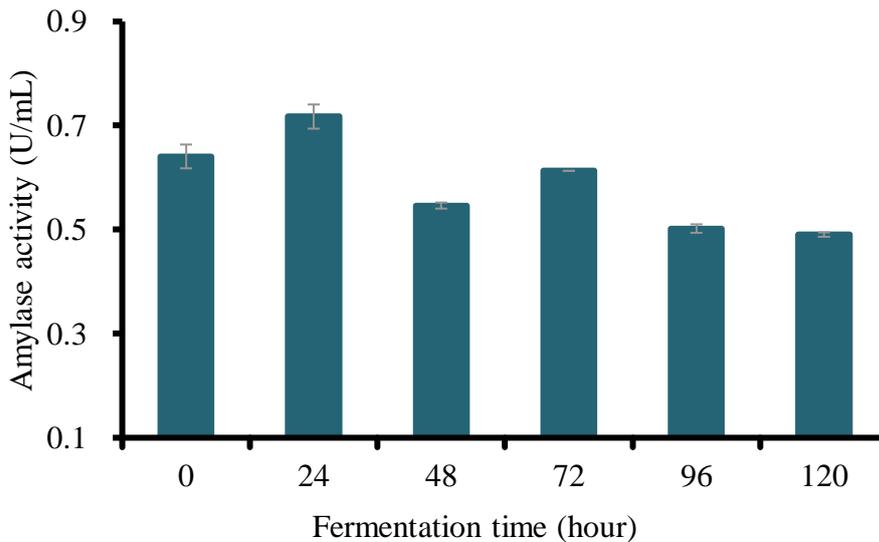
312 while amylase of *Bacillus subtilis* strain SDP1 isolated from rhizosphere of Acacia produces
313 maltotriose and maltotetraose (Ozturk et al. 2014). Furthermore, Abdul-Manas et al. (2014)
314 reported that amylase of an alkaliphilic *Bacillus lehensis* G1 could degrade oligosaccharides
315 by producing maltooligosaccharides with a higher degree of polymerization than
316 maltoheptaose observed on thin-layer chromatography and high-performance liquid
317 chromatography analyses.

318

319 3.4. Crude enzyme production of a selected isolate and measurement of its amylase activity at
320 different culture incubations

321 Based on the ability of the fourth selected amylolytic bacteria to produce different types
322 of hydrolysed product, isolate T10 was further assayed for its optimal amylase activity at
323 different incubation times. The results showed that incubation times affected the amylase
324 activity of isolate T10 carried out in a 0.5% starch solution at 30°C in 50 mM acetate buffer of
325 pH 6. The amylase activity of T10 was optimal during incubation 1-3 days ranging from 0.546-
326 0.717 U/mL and the highest amylase activity was found at 24 hr incubation of 0.717 U/mL
327 (Figure 7). The results also showed that amylase activity decreased after 72 h of incubation.
328 The amylase activity value at day 0 is quite high, this might be due to the measurement of the
329 enzyme activity using the DNS method, in which reducing sugar formed from a carbon source
330 (starch) is used by bacteria for the initial stages of growth; then, the bacteria will use the carbon
331 source for the production of enzymes.

332



333

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

335

336 The amylase activity of *Bacillus cereus* KN isolated from Ranu Ngebel and incubated
 337 for three days was 0.016 U/mL, while strain G20 isolated from Ranu Grati was lower at about
 338 0.0001 U/mL (Nisa et al. 2021). Luang et al. (2019) found *Bacillus* sp. 3.5AL2 isolated from
 339 soils of the unexplored Nasinuan Forest, Thailand and incubated for three days exhibiting
 340 amylase activity of 1.97 U/mg protein at the optimal conditions of 60°C and pH 7.0 after 30
 341 min incubation with 1% starch in 0.05 M phosphate buffer. Gebreyohannes (2015) reported
 342 that the amylase activity of *Bacillus* spp. decreased after 48 h incubations due to the suppression
 343 and accumulation of other byproducts in the fermentation medium and also depletion of
 344 nutrients.

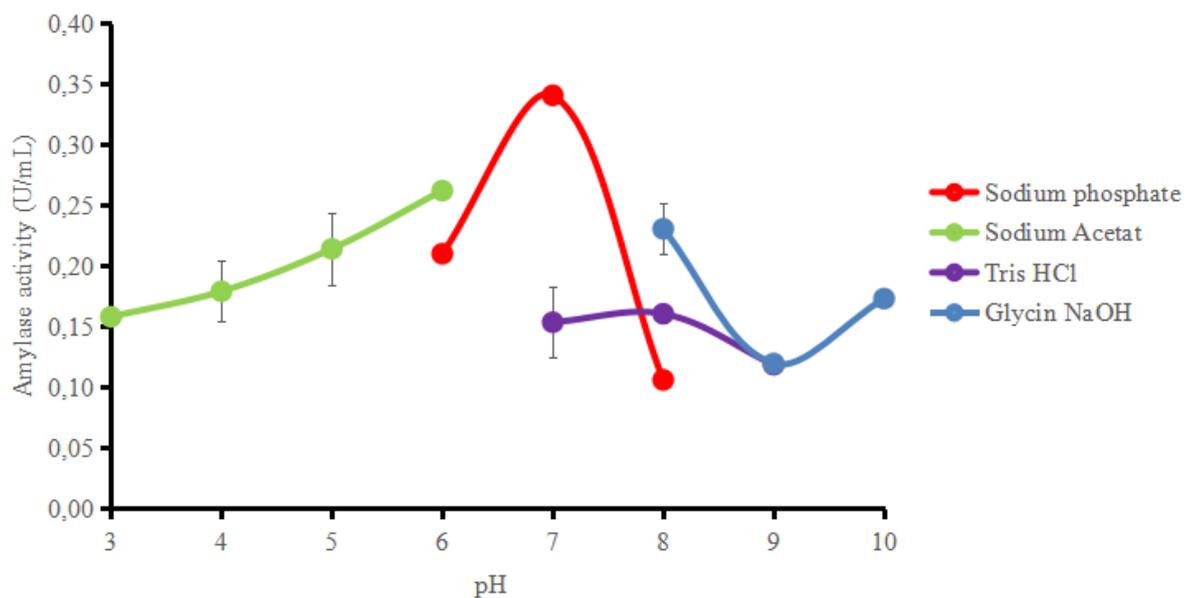
345

346 3.5. Enzyme characterization: the effect of pH and temperature against enzyme activity of the
 347 selected isolate

348 The effects of pH's on the amylase activity of isolate T10 showed that optimum
 349 conditions were in sodium acetate buffer pH 6 with an amylase activity of 0.262 U/mL and in
 350 sodium phosphate buffer pH 7 with an amylase activity of 0.341 U/mL (Figure 8). The optimal

351 pH of isolate T10 was by Naidu et al. (2019) for *Paenibacillus* sp. D9 that its optimal pH for
352 amylase activity is in the neutral range (pH 6-8). The increase in pH beyond these values
353 resulted in a decline in enzyme activity. Any change in pH causes a change in the enzyme's
354 active site (Lim & Oslan 2021). Bajpai et al. (2015) reported that the optimal pH for amylase
355 activity of *Haloferax* sp. HA10 was at pH 7.0. According to Asgher et al. (2007), each enzyme
356 has an optimal pH to work most actively, and the optimal pH of amylase is varied from pH 3.8
357 to 9.5 depending on the type of enzyme and the source. Behal et al. (2016) reported an amylase
358 produced by *Bacillus* sp. AB04 had optimal activity at pH 8. Moreover, the enzyme is stable
359 in neutral to alkaline (pH 7-10).

360



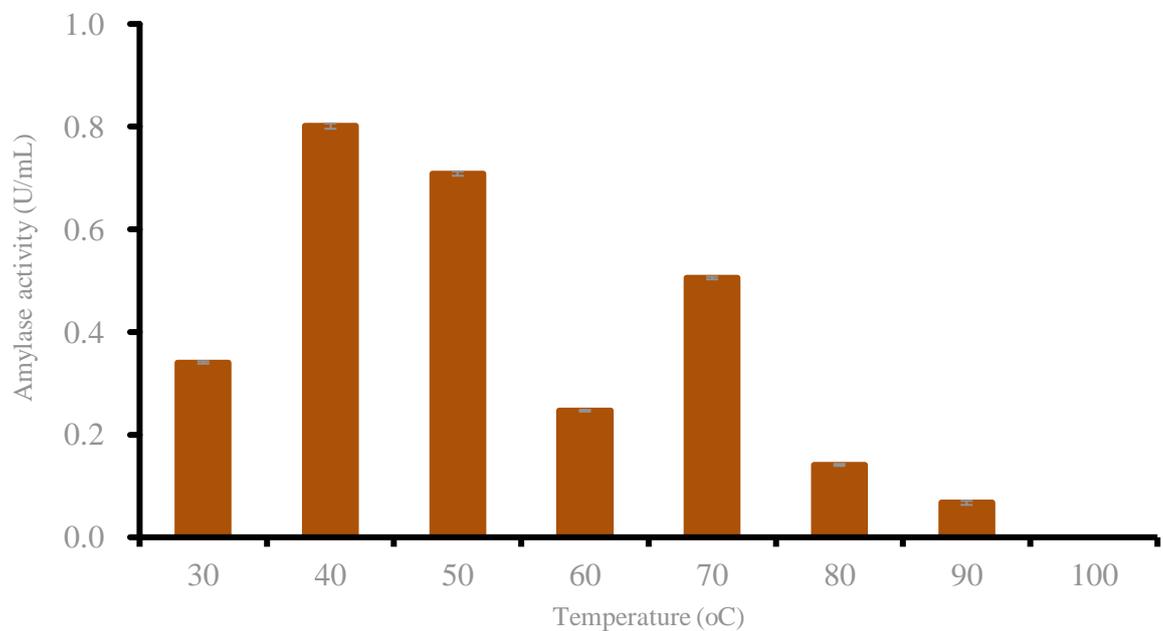
361

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

363

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

368 *licheniformis*, and *B. amiloliquefaciens* have optimum temperatures of 37-60°C.
369 Gebreyohannes (2015) found that the maximum amylase activity of *Bacillus* spp. was 40°C
370 and *Streptomyces* spp. at 37°C, used 4% starch concentration at a neutral pH and an incubated
371 for 48 h. The crude enzyme of *Bacillus* sp. AB04 showed maximum activity at pH 8 with an
372 optimum temperature of 40° C with more than 75% activity in range of 50 - 80° C (Behal et al.
373 2016). The results showed that either pH or temperature significantly affected the enzyme
374 activity of the T10 amylase which was optimum at pH 7.0 and a temperature of 40°C.
375



376
377 **Figure 9.** Amylase activity of isolate T10 at different temperatures.

378
379 The differences in the pH and temperature characteristics of enzyme activity indicated
380 that enzymes are specific, depending on the species that produces them. A decrease or increase
381 in temperature can affect the secretion of extracellular enzymes by changing the physiology of
382 the cell membrane (Rahmani et al. 2018). The optimum temperature is the temperature that
383 causes chemical reactions at the most incredible speed (Subagiyo et al. 2017). The results
384 showed that after reaching the optimum condition, it was seen that the activity of the T10

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

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

398

399 **4. Conclusions**

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

405

406 **Authors contribution**

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

409

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413

414 **Conflict of interest**

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

416

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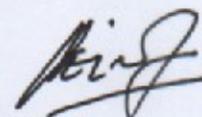
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1 **Isolation and Characterization of Rhizospheric Bacteria Associated with Canna Plant**
2 **for Production of Maltooligosaccharide Amylase**

3

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17

18

19 **Abstract**

20 The objectives of the study were to isolate amyolytic bacteria from the rhizosphere and
21 plant tissue of *Canna edulis* Ker., as well as litter; to know oligosaccharide compounds
22 produced from starch hydrolyzed by the bacterial enzymes, and to identify the amyolytic
23 bacteria based on phenetic and 16S rRNA gene sequences. From the rhizosphere, Canna plant
24 tissue, and litters obtained thirty-two amyolytic bacterial isolates. Eight isolates (TH6, TH7,
25 T5, T10, D2, D3, A3, S1) produced high clear zone diameters ranging from 18-30 mm;

26 especially an isolate T10, which was consistent in producing a total clear zone diameter of 20
27 mm. The hydrolysate of starch hydrolysed by the T10 amylase resulted in three oligosaccharide
28 compounds maltotriose, maltotetraose, and maltopentose. The amylase activity of isolate T10
29 was optimal at a temperature of 40°C and pH 7 in an amount of 0.801 U/mL. The isolate T10
30 was identified as a species member of *Bacillus toyonensis* based on phenotypic
31 characterization and 16S rDNA gene sequencing analysis with a similarity value of 99.93%.

32

33 Keywords:

34 Amylolytic bacteria, Canna, Maltooligosaccharides, 16S rDNA gene.

35

36 **1. Introduction**

37 Oligosaccharides are members of an essential group of carbohydrates. Macromolecules
38 with short-chain polysaccharide sugars of 2 to 20 saccharide units. Functional oligosaccharides
39 such as galactooligosaccharide (GOS), fructooligosaccharide (FOS), and maltooligosaccharide
40 (MOS) are well-known prebiotics owing to their ability to selectively stimulate beneficial
41 bacteria in the intestines, particularly bifidobacterial species (Zhao et al. 2017). Developing
42 oligosaccharide products is one of the businesses with high economic value. Plants such as
43 Canna contain much starch, one of the crucial ingredients (substrate) to produce
44 oligosaccharides enzymatically. Canna plants (*Canna edulis* Ker.) contain high levels of
45 carbohydrates, mainly starch (93.3%), which consists of amylose (33.48%) and amylopectin
46 (59.82%) (El-Fallal et al. 2012). Starch is hydrolysed into smaller oligosaccharides by α -
47 amylase, one of the most important commercial enzymes (Jang et al. 2020).

48 The starch-processing industry has exploited amylase as a substitute for acid hydrolysis
49 in the production of starch hydrolysis. Amylase acts as a biocatalyst for the hydrolysis of starch
50 into simpler carbohydrates, such as glucose, maltose, and dextrin (Divakaran et al. 2011;

51 Abdalla et al. 2021). Amylolytic bacteria are producers of amylase that can be used as
52 biocatalysts in the starch hydrolysis process (Ding et al. 2021) to produce various
53 maltooligosaccharide products, such as maltotriose, maltotetraose, maltopentaose, and
54 maltohexaose (Pan et al. 2017).

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

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

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

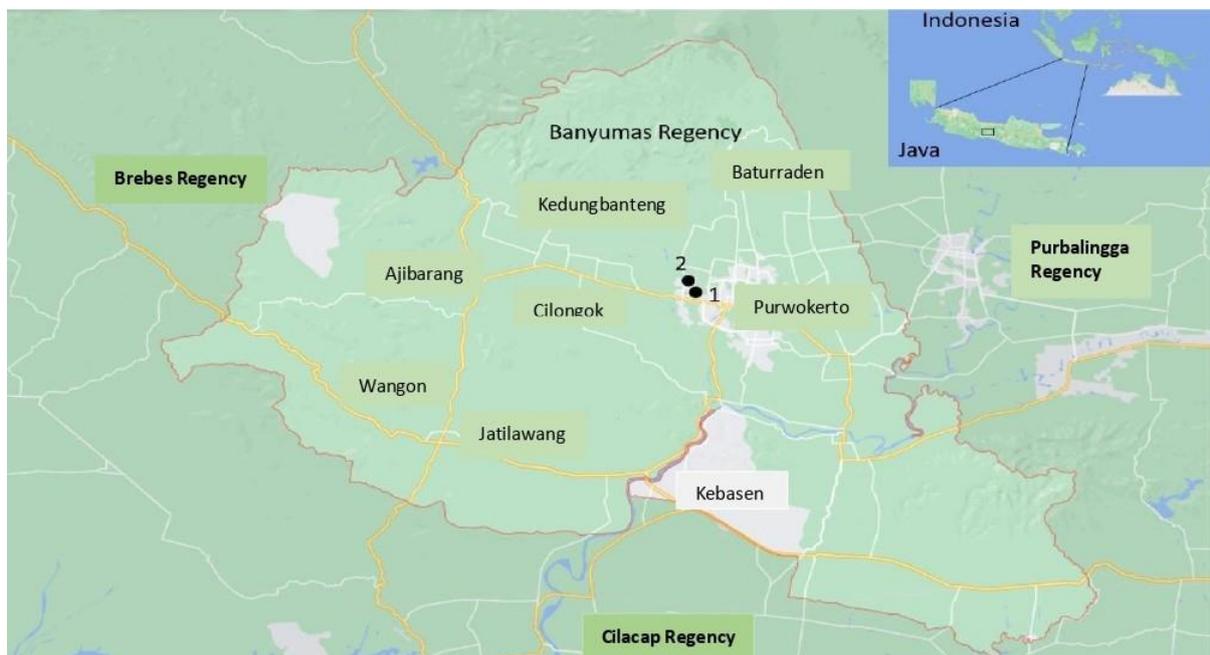
80

81 2. Materials and methods

82 2.1. Sample collection and location of sampling

83 Samples were taken from the rhizosphere and parts of *Canna* plant (*C. edulis* Ker.)
84 including tubers, stems, leaves, tissue, as well as litter growing in two places, namely in the
85 forest and the community gardens around the Perhutani Forest West Banyumas, Central Java,
86 Indonesia. The coordinates of the former are S 07°20.846 'E 109°06.410 and the latter is S 07
87 °20.812 'E 109°05.92 (Figure 1).

88



89

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

2.2. 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, 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 colonies was then inoculated onto an NA medium containing 1% soluble starch and purified using a streak quadrant method.

The number of 0.5 μ L 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

117 morphology of the isolated bacteria were characterized using conventional methods (Smibert
118 & Krieg 1981).

119

120 2.3. Phenetic and phylogenetic characterizations of the selected bacterium

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

125 The 16S rDNA gene was amplified by polymerase chain reaction (PCR) technique
126 using a pair of primers (9F: 5'GAGTTTGATCCTCCTGGCTCAG-3') 1510R:
127 5'GGCTACCTTGTTACGA-3') (Yopi et al. 2017). The obtained bands were stained and
128 visualized by UV Transilluminator. The sequence was confirmed via 1st BASE Sequencing,
129 Malaysia. The 16S rDNA nucleotide sequences were analyzed by nucleotide BLAST (Basic
130 Local Alignment Search Tool) search in the Gene Bank of National Center for Biotechnology
131 Information (NCBI) or BLAST for amino acid analysis (Zhuang et al. 2012). The phylogenetic
132 tree was constructed using a neighbour-joining algorithm in MEGA 6.0 software (De-Moraes-
133 Russo & Selvatti 2018).

134

135 2.4 Analysis of the hydrolysis products by a thin-layer chromatography method (Rahmani et 136 al. 2013)

137 An amount of 2 mL of each 24 h old bacterial cultures (four selected isolates) was
138 inoculated into a 200 mL flask containing 20 mL of NB medium plus 1% (w/v) starch solution,
139 pH 6 (50 mM acetate buffer) and incubated at 30 °C for 24 h. The culture was sampling every
140 24 hour and then centrifugated, and the supernatant obtained was tested for its amyolytic
141 activity.

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

147 A Thin Layer Chromatography (TLC) of maltooligosaccharide products was carried
148 out on silica gel 60F₂₅₄ plates (Merck Art 20-20 cm) and eluent using a solvent mixture of n-
149 butanol:acid:water (12:6:6, v/v/v). Spots formed were visualized by spraying the sugar colours
150 (0.5 g α -diphenylamine, 25 mL acetone, 2.5 mL phosphate acid, 0.5 mL aniline). All samples
151 were applied in equal quantities (4 μ L). Glucose (Sigma-Aldrich, U.S.A), maltose (M2),
152 maltotriose (M3), maltotetraose (M4), maltopentaose (M5), maltohexaose (M6), and
153 maltoheptaose (M7) (Megazyme) were used as standards.

154

155 2.5. Crude enzyme production and amylase activity at different fermentation time

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

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

164
$$= \frac{c \times d \times 1000}{t \times mw} \text{ U/mL}$$

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

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

169

170 2.6. Effect of pH and temperature on enzyme activity of the selected isolate

171 The optimal pH of the enzyme activity was done at pH ranges of 3.0-10.0 under
172 standard assay conditions. Various buffers (0.05M) used were sodium acetate (pH 3.0-6.0),
173 sodium phosphate (6.0-8.0), Tris-HCL (pH 7.0-9.0), and Glycine-NaOH (pH 8.0-10.0). The
174 enzyme reactions were incubated at 40°C for 30 min in the presence of 0.5% (w/v) starch
175 solution (Merck)). The effect of temperatures on enzyme activity was conducted at
176 temperatures ranging from 30-90 °C in 50 mM acetate buffer at optimum pH for 30 min.
177 Amylase activity was assayed by DNS method (Miller 1959).

178

179 **3. Results and Discussion**

180 3.1 Isolation and an amylolytic assay of bacteria isolated from the rhizosphere and plant
181 tissue of Canna, and litter

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

189 The ability of the bacteria to grow and to produce clear zones in the medium, indicating
190 that those bacteria were capable of producing amylase. The more amylase is released, the wider

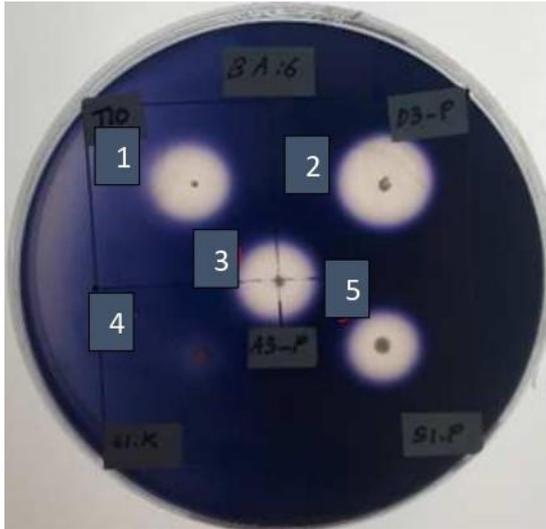
191 clear zones are produced due to the degradation of amylum in the medium, resulting in
 192 enhancing the amylolytic index (Ginting et al. 2021). The research results showed that eight
 193 isolates of TH6, TH7, T5, T10, D2, D3, A3, and S1 showed high total clear zone diameters
 194 (mm) of 18, 18,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

203

204 **Table 1.** Sources, number, and total clear zone diameter of amylolytic bacteria isolated from
 205 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	T1	15
in the people's gardens around the forest	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	D1	16
the people's gardens around the forest	D2	18
	D3	30
	D4	15
Roots of the Canna plant tissue growing in	A1	17
the people's gardens around the forest	A2	16
	A3	18
Litters of the Canna plant from the gardens of	S1	18
the residents around the forest	S2	16



207

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

210

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

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

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

230

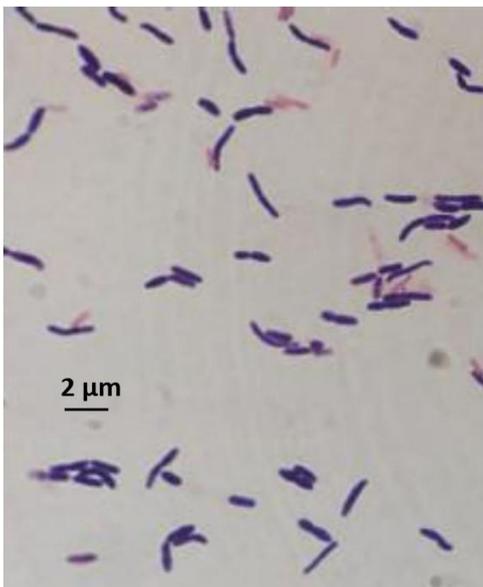
231 **Table 2.** Morphological and physiological properties of the bacteria isolated from Canna plants
 232 and their surrounding

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	+	+	+	+	+

233

234 3.2. Identification of the selected isolate of T10 based on phenetic and phylogenetic
 235 characteristics

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



245
 246 **Figure 3.** The appearance of bacterial cells isolate T10 under a microscope with a
 247 magnification of 1000x. The cells appear single or in chains.

248
 249 **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- α D-glucopyranoside	+
Amygdalin	+w
Arbutin	+
Salicin	+
Cellobiose	-
D-saccharose	+
D-trehalose	+
Starch	+
Glycogen	+
D-turanose	+

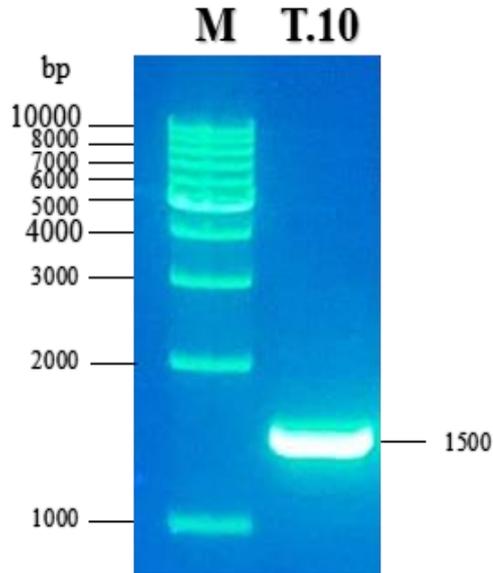
250

251 The electrophoresis visualization of the PCR product showed that the DNA of T10
252 produced a single band with a size of 1500 kb (Figure 4). The results of the comparison between
253 the 16S rRNA gene sequence of isolate T10 and nucleotide sequences in the GeneBank
254 (<http://blast.ncbi.nlm.nih.gov/>) showed that the bacterium is closely related to species

255 members of the genus *Bacillus*. The BLAST analysis results showed that isolating T10 had a
256 similarity of 99.3% with either *Bacillus toyonensis* SPa09NA, *B. toyonensis* PZ-48, or *B.*
257 *toyonensis* SMP1. The phylogenetic tree constructed using Neighbor-Joining, Model
258 Maximum Composite Likelihood, and 1000x Bootstrapping. A dendrogram resulted from
259 MEGA10 program showed that isolate T10 joined *B. toyonensis* SX04NA, *B. toyonensis*
260 Spa09NA, *B. toyonensis* SMP1, *B. toyonensis* PZ-48, *B. toyonensis* BCT-7112, and *B.*
261 *toyonensis* l3aM to form a separate cluster (Figure 5). Hence, isolate T10 was identified as the
262 species member of *B. toyonensis* based on the phenetic and phylogenetic characteristics.

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

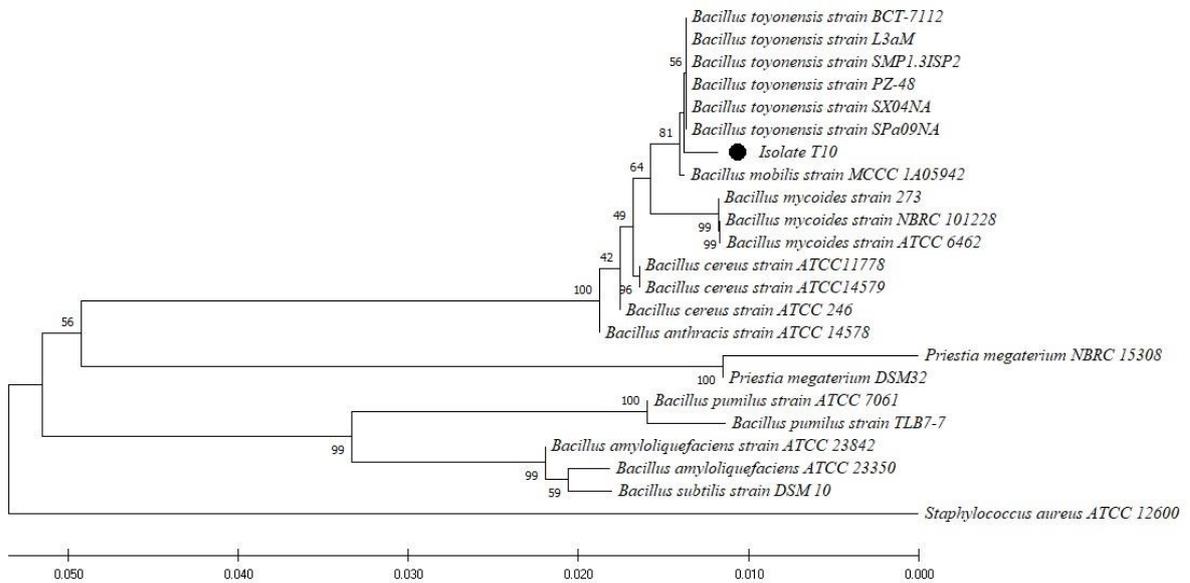
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276

277 **Figure 4.** An electropherogram of the amplified 16S rRNA gene of isolate T10 with a size of
 278 1500 bp. Marker (M): 1 kb DNA ladder.

279



280

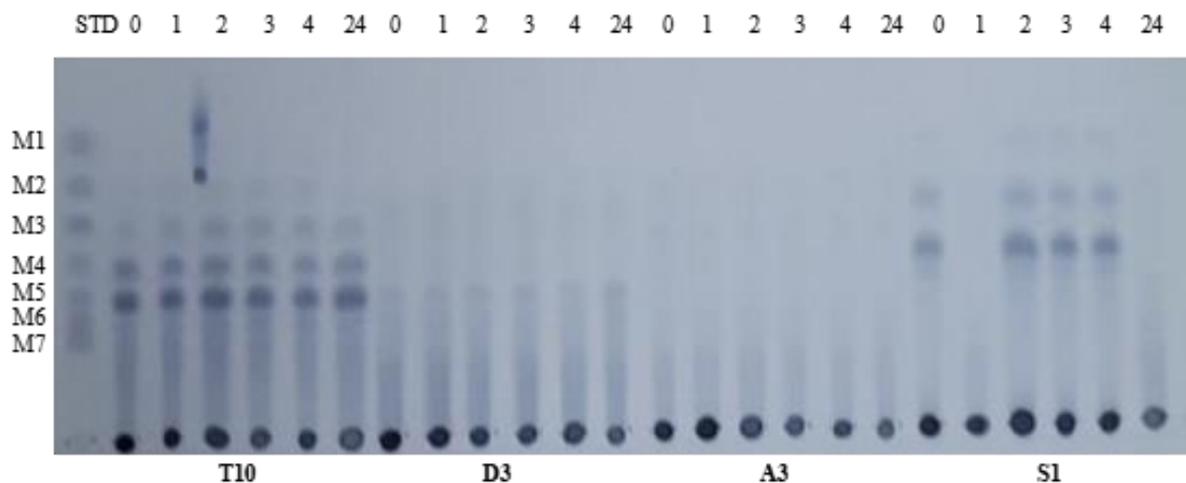
281 **Figure 5.** A phylogenetic tree showing the relationship between strain T10 isolated from
 282 rhizospheres of *Canna* (*C. edulis*) and several species members of the genus *Bacillus* on the
 283 basis of 16S rRNA gene sequence reconstructed based on Neighbor-Joining, Model Maximum

284 Composite Likelihood, and 1000x Bootstrapping. The analysis used a MEGA10 program and
285 *Staphylococcus aureus* ATCC 12600 as an outgroup.

286

287 3.3. Analysis of hydrolysed products by the selected bacterial amylases using a TLC method

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



294

295 **Figure 6.** The product profile of starch hydrolyzed by amylase of the amylolytic bacteria
296 (T10, D3, A3, and S1) using a TLC method with reaction times (hours): of 0, 1, 2, 3, 4, and
297 24 at 30°C. The Standards (STD): monosaccharide (M1), maltose (M2), maltotriose (M3),
298 maltotetraose (M4), maltopentaose (M5), maltohexaose (M6) and maltohepta (M7).

299

300 Based on the TLC chromatogram, the starch degraded by T10 amylase resulted in malto-
301 oligosaccharides of maltotriose, maltotetraose, and maltopentaose. Amylases are able to break

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

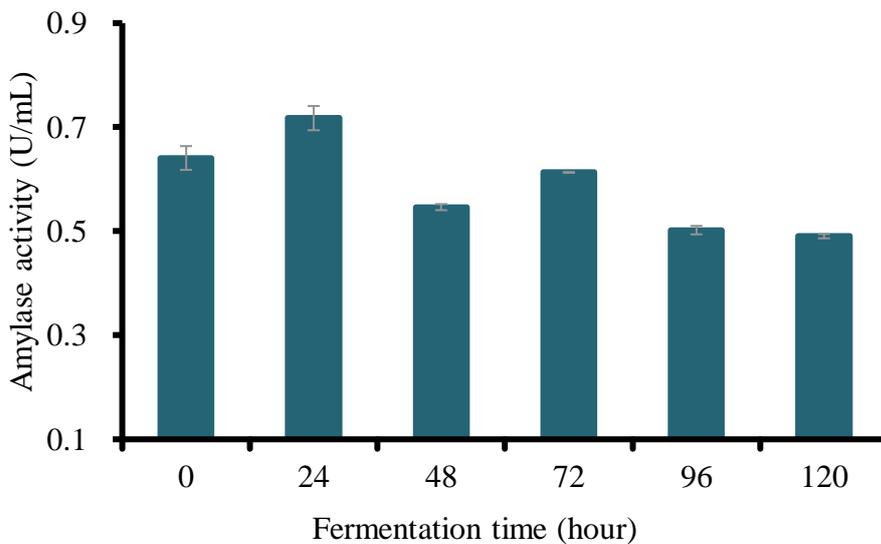
318

319 3.4. Crude enzyme production of a selected isolate and measurement of its amylase activity at
320 different culture incubations

321 Based on the ability of the fourth selected amylolytic bacteria to produce different types
322 of hydrolysed product, isolate T10 was further assayed for its optimal amylase activity at
323 different incubation times. The results showed that incubation times affected the amylase
324 activity of isolate T10 carried out in a 0.5% starch solution at 30°C in 50 mM acetate buffer of
325 pH 6. The amylase activity of T10 was optimal during incubation 1-3 days ranging from 0.546-
326 0.717 U/mL and the highest amylase activity was found at 24 hr incubation of 0.717 U/mL

327 (Figure 7). The results also showed that amylase activity decreased after 72 h of incubation.
328 The amylase activity value at day 0 is quite high. This might be due to the measurement of the
329 enzyme activity using the DNS method, in which reducing sugar formed from a carbon source
330 (starch) is used by bacteria for the initial stages of growth; then, the bacteria will use the carbon
331 source for the production of enzymes.

332



333

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

335

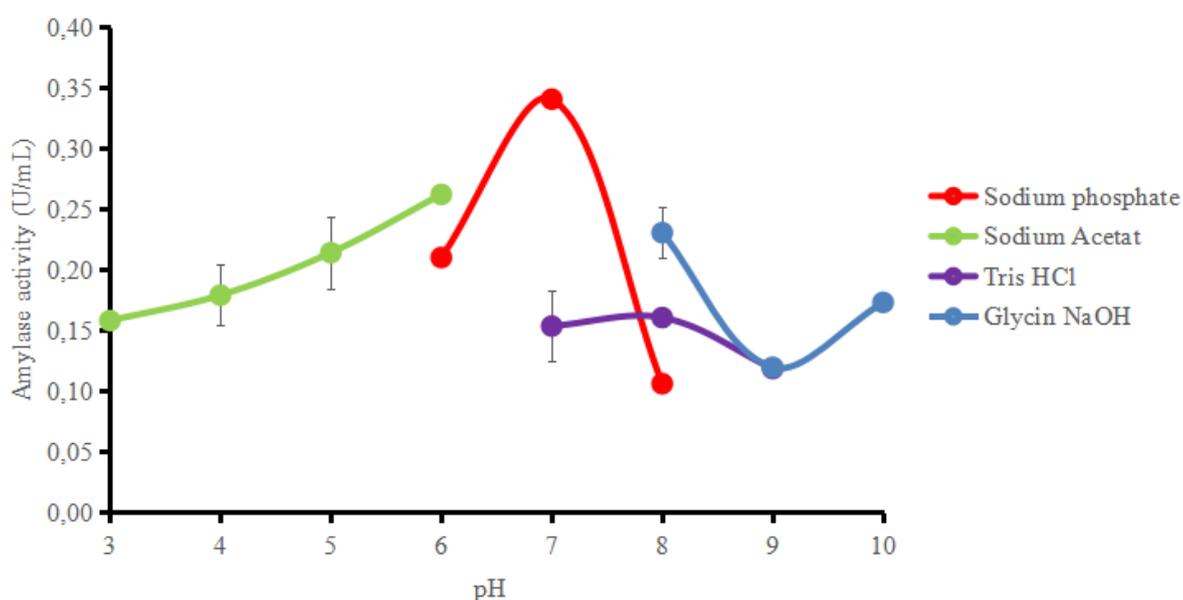
336 The amylase activity of *Bacillus cereus* KN isolated from Ranu Ngebel and incubated
337 for three days was 0.016 U/mL, while strain G20 isolated from Ranu Grati was lower at about
338 0.0001 U/mL (Nisa et al. 2021). Luang et al. (2019) found *Bacillus* sp. 3.5AL2 isolated from
339 soils of the unexplored Nasinuan Forest, Thailand and incubated for three days exhibiting
340 amylase activity of 1.97 U/mg protein at the optimal conditions of 60°C and pH 7.0 after 30
341 min incubation with 1% starch in 0.05 M phosphate buffer. Gebreyohannes (2015) reported
342 that the amylase activity of *Bacillus* spp. it decreased after 48 h incubations due to the
343 suppression and accumulation of other byproducts in the fermentation medium and also
344 depletion of nutrients.

345

346 3.5. Enzyme characterization: the effect of pH and temperature against enzyme activity of the
347 selected isolate

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

360



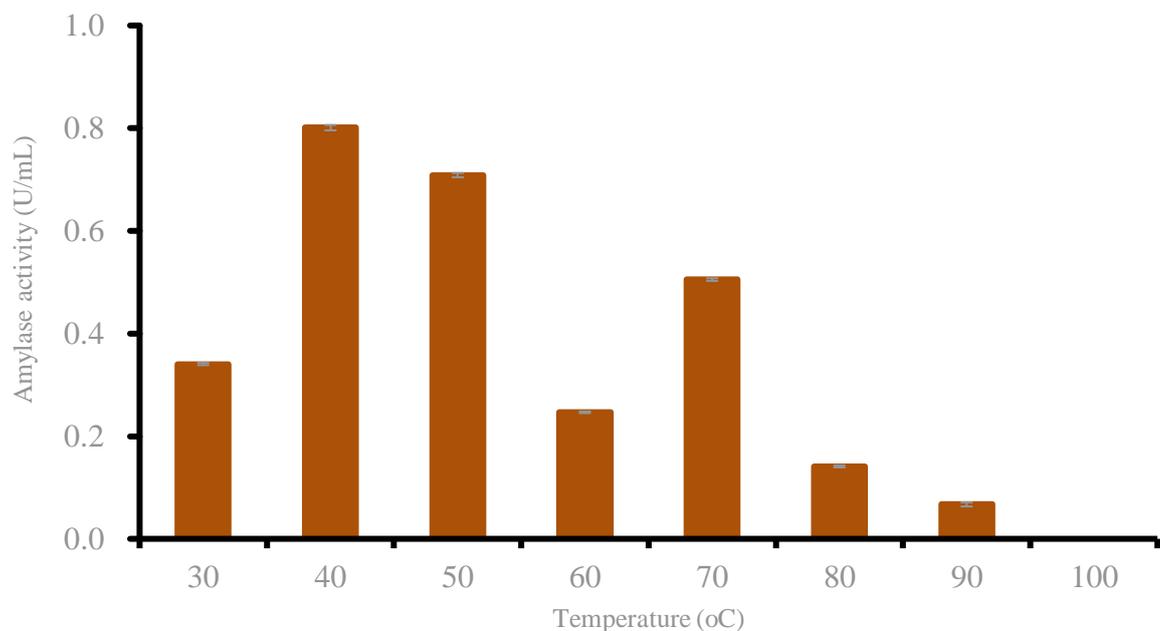
361

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

363

364 The amylase activity of isolate T10 was observed at temperatures ranging from 30-90°C
365 at pH 7.0. Amylase activity of the T10 isolate tended to be optimum at 40°C with an activity
366 value of 0.801 U/mL (Figure 9). A similar finding was also reported by Sivaramakrishnan et
367 al. (2006) for several species of *Bacillus* sp., *B. subtilis*, *B. stearotherophilus*, *B.*
368 *licheniformis*, and *B. amiloliquefaciens* have optimum temperatures of 37-60°C.
369 Gebreyohannes (2015) found that the maximum amylase activity of *Bacillus* spp. was 40°C
370 and *Streptomyces* spp. at 37°C, used 4% starch concentration at a neutral pH and an incubated
371 for 48 h. The crude enzyme of *Bacillus* sp. AB04 showed maximum activity at pH 8 with an
372 optimum temperature of 40° C with more than 75% activity in range of 50 - 80° C (Behal et al.
373 2016). The results showed that either pH or temperature significantly affected the enzyme
374 activity of the T10 amylase which was optimum at pH 7.0 and a temperature of 40°C.

375



376

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

378

379 The differences in the pH and temperature characteristics of enzyme activity indicated
380 that enzymes are specific, depending on the species that produces them. A decrease or increase
381 in temperature can affect the secretion of extracellular enzymes by changing the physiology of
382 the cell membrane (Rahmani et al. 2018). The optimum temperature is the temperature that
383 causes chemical reactions at the most incredible speed (Subagiyo et al. 2017). The results
384 showed that after reaching the optimum condition, it was seen that the activity of the T10
385 amylase decreased. High temperatures can cause enzymatic reactions to decrease because
386 enzyme proteins undergo conformational changes so that protein molecules will experience
387 denaturation (Yufinta et al. 2018).

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

398

399 **4. Conclusions**

400 It can be concluded that amounts of 32 amylolytic bacteria were isolated from
401 rhizosphere and plant tissue of *Canna edulis*, as well as litter; the selected amylolytic bacterial
402 isolate of T10 was capable of hydrolysing starch by producing maltotriose (M3), maltotetraose

403 (M4) and maltopentaose (M5); and the identity of the selected isolate T10 belonged to a species
404 member of *B. toyonensis* based on phenotypic and phylogenetic characterizations.

405

406 **Authors contribution**

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

409

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413

414 **Conflict of interest**

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

416

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Research Article

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

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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%.

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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,

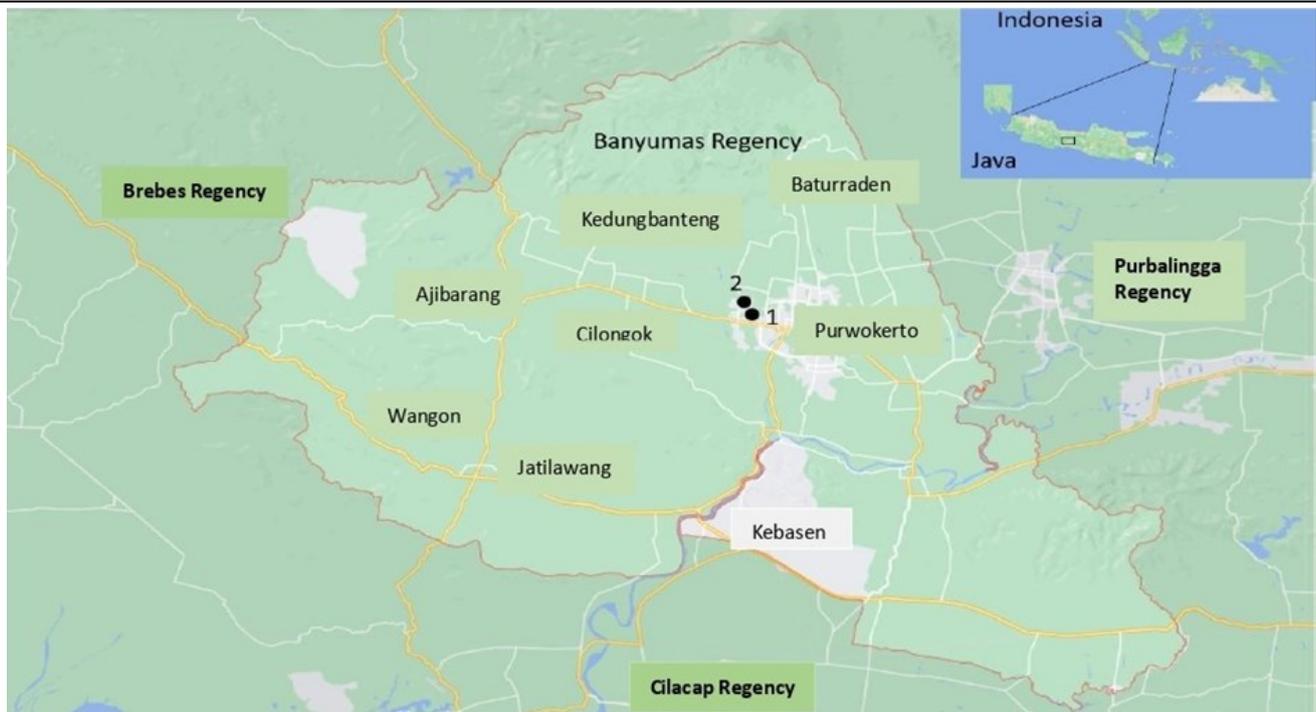


Figure 1. A map of Banyumas Regency and sampling sites: (1) the Perhutani Forest KPH West Banyumas, Central Java, (2) Community gardens around the Perhutani Forest KPH West Banyumas, Central Java.

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 maltoligosaccharide 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'GAGTTT-GATCCTCCTGGCTCAG-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 60F₂₅₄ 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 μ L). 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 μ mol 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 Amylytic 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 amyllum in the medium, resulting in enhancing the amylytic 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 amylytic activity (Figure 2). Based on the ability of isolates to produce a clear zone diameter ≥ 18 mm and consideration of source representatives, four 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 amylytic 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.

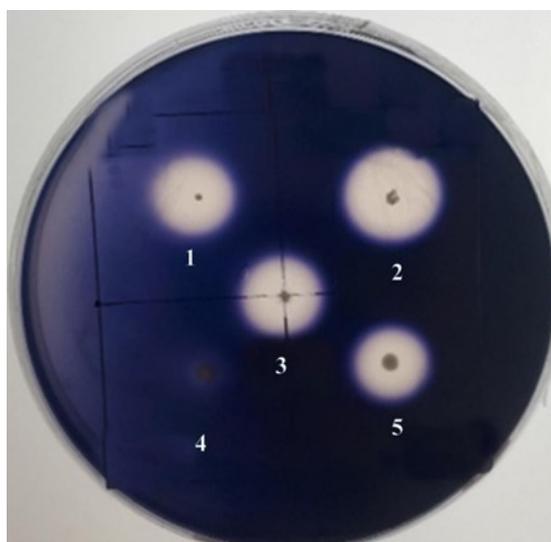


Figure 2. The amylytic zones produced by bacterial isolates of: (1) T10, (2) D3, (3) A3, (4) TH6, and (5) S1 on a NA medium + 1% soluble starch.

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 **enzyme** 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).

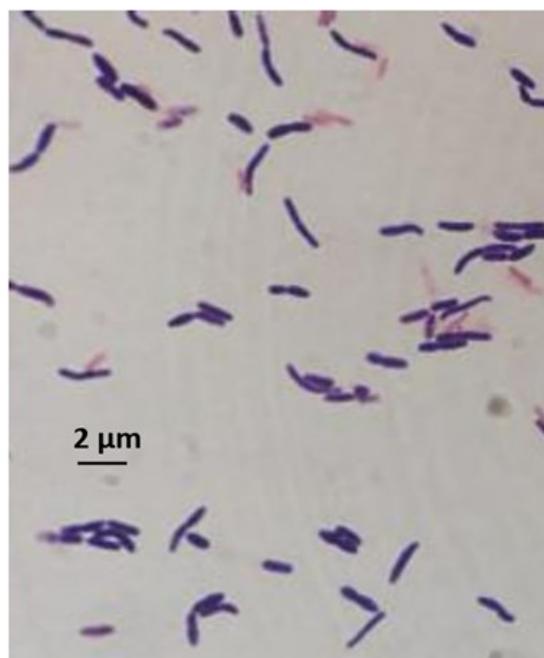


Figure 3. The appearance of bacterial cells isolate T10 under a microscope with a magnification of 1000x. The cells appear single or in chains.

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.nlm.nih.gov/>) showed that the bacterium is closely related to species members of the genus *Bacillus*. The BLAST analysis showed that isolating 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-71112, 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-71112^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.

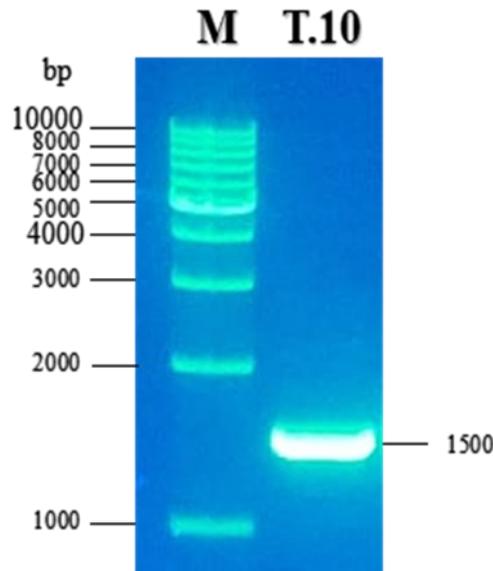


Figure 4. An electropherogram of the amplified 16S rRNA gene of isolate T10 with a size of 1500 bp. Marker (M): 1 kb DNA ladder.

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 amyolytic bacteria with high amyolytic indexes (AI) did not correlate with their ability to degrade amyllum. 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 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 amyolytic bacteria to produce different types of hydrolysed product, isolate T10 was further assayed for

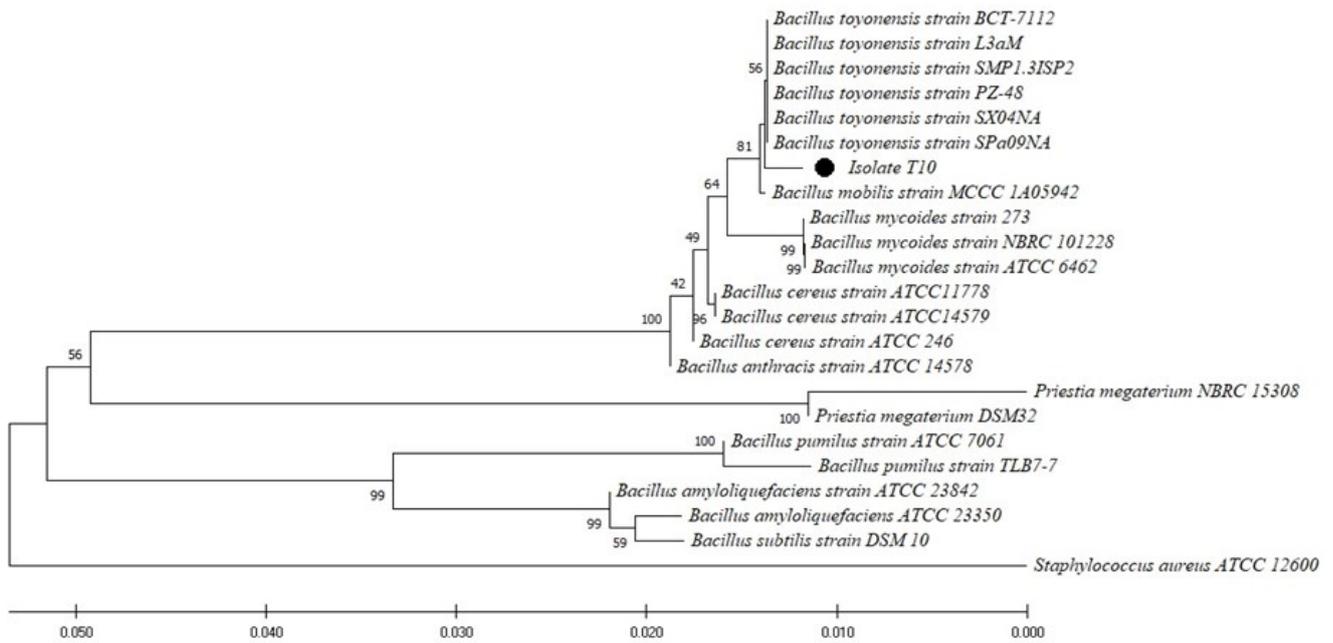


Figure 5. A phylogenetic tree showing the relationship between strain T10 isolated from rhizospheres of *Canna* (*C. edulis*) and several species members of the genus *Bacillus* on the basis of 16S rRNA gene sequence reconstructed based on Neighbor-Joining, Model Maximum Composite Likelihood, and 1000x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

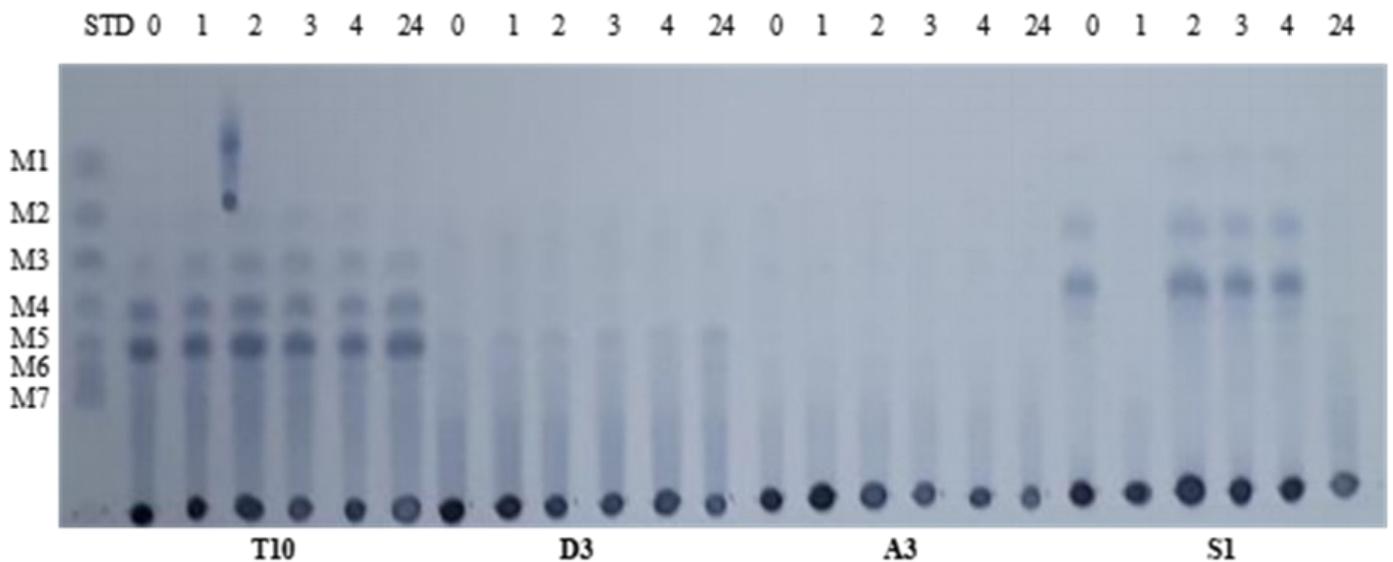


Figure 6. The product profile of starch hydrolyzed by amylase of the amylolytic bacteria (T10, D3, A3, and S1) using a TLC method with reaction times (hours): of 0, 1, 2, 3, 4, and 24 at 30°C. The Standards (STD): monosaccharide (M1), maltose (M2), maltotriose (M3), maltotetraose (M4), maltopentaose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

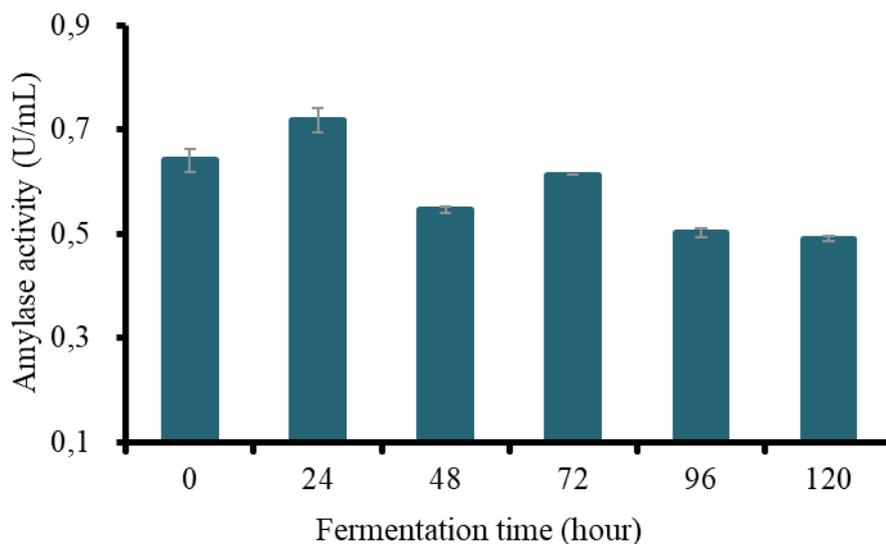


Figure 7. Amylase activity of T10 at different incubation times.

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.*

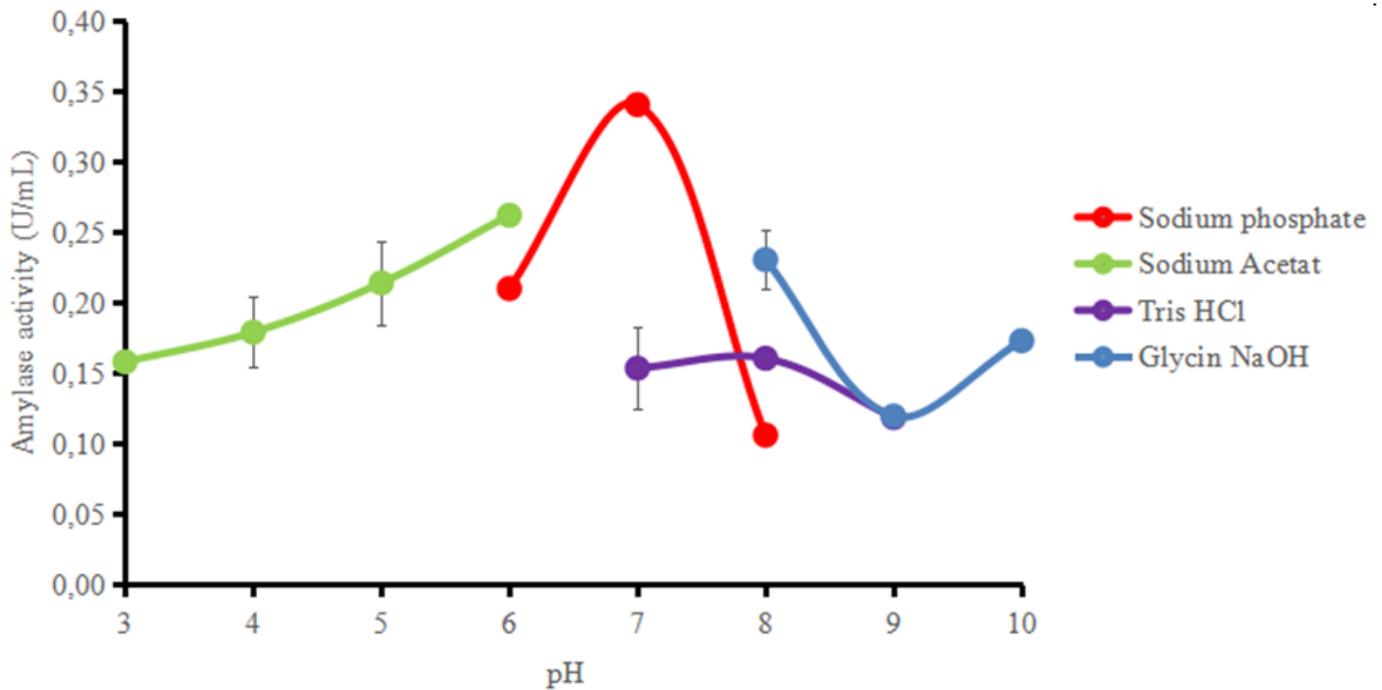


Figure 8. Amylase activity of T10 at different pH and buffers.

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.

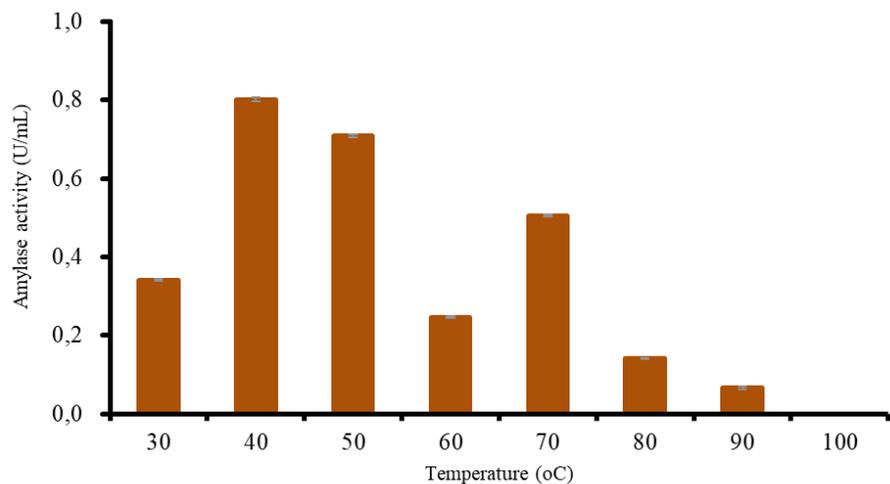


Figure 9. Amylase activity of isolate T10 at different temperatures.

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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