[JTBB] Notice for Initial Review Re

gle.com/mail/u/0/?tab=rm&ogbl#search/jtbb+agustiani/FMfcgzGqRGbvTHtVCbrtdbzKWcXPWvrL

jtbb agustiani

Active

5,860

25

[JTBB] Notice for Initial Review Result

External Inbox ×

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

Tue, Oct 25, 2022, 10:5

to Rina, me, Nanik, Nuraeni

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>

Tue, Oct 25, 2022, 3:2

to Rina

Reply Forward

nnect to chat

Search

[JTBB] Editor Decision - oedjijono

gle.com/mail/u/0/?tab=rm&ogbl#search/jtbb+agustiani/FMfcgzGrbcCBKdFQFVvrwLXMkqBQFcpr

jtbb agustiani

Active

5,860

25

[JTBB] Editor Decision

External Inbox ×

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

to me, Rina, Nanik, Nuraeni

Mon, Dec 19, 2022, 4:

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

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

One attachment • Scanned by Gmail

jtbb-Author's Res...

Search

[JTBB] Editor Decision - oedjijono

gle.com/mail/u/0/?tab=rm&ogbl#search/jtbb+agustiani/FMfcgzGrbbrvjWXnZfXvPSwwFjKQmhQc

jtbb agustiani

Active

5,860

25

[JTBB] Editor Decision

External Inbox ×

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

Mon, Dec 12, 2022, 10:

to me, Rina, Nanik, Nuraeni

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.

Search

The manuscript also needs linguistic editing and paraphrasing.

5,860

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.

25

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 bacteria! 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.

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 Nanik

Dec 26, 2022, 12:

Author's Response - oedjijono@

gle.com/mail/u/0/?tab=rm&ogbl#search/jtbb+agustiani/KtbxLrjNcfXLTXnxWSsVvKLcmWKdZjQJtg

jtbb agustiani

Active

5,860

Author's Response

External Inbox ×

Oedjijono 1 <oedjijono@unsoed.ac.id> Mon, Dec 26, 2022, 9:

to Liya

Dear the Editor of JTBB

25

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


78346-272681-AR...


78346-280500-1-...

REVIEWER A & B_...

GRAMMARLY CH...

Liya Audinah <liyaaudinah15@gmail.com> Mon, Dec 26, 2022, 9:

to me

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

Search

[JTBB] Editor Decision

External Inbox ×

Dr Miftahul Ilmi <m.ilmi@ugm.ac.id> Mon, Jan 30, 10:

to me, Rina, Nanik, Nuraeni

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 Ilmi
Faculty of Biology, Universitas Gadjah Mada
m.ilmi@ugm.ac.id

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

Oedjijono 1 <oedjijono@unsoed.ac.id> Mon, Jan 30, 11:

to Dr

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

Sincerely yours,

Oedjijono

[JTBB] Editor Decision_similaritycheck

External Inbox ×

5,860

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

to me, Rina, Nanik, Nuraeni

Thu, Feb 2, 10:

Dear Dr. Oedjijono, M.Sc.,

25

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

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 Liya, Rina, Nanik, Nuraeni

Fri, Feb 3, 9:

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

5,860

25

[JTBB] Copyediting Completed

External Inbox ×

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

Mon, Mar 13, 5:

to me

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 Comp

gle.com/mail/u/0/?tab=rm&ogbl#search/jtbb+agustiani/FMfcgzGsIbHVxHqFZGCSsISwZbjxMDPB

jtbb agustiani

Active

5,860

[JTBB] Copyediting Review Completed

External Inbox ×

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

25

Oedjijono 1
Thanks a lot.

Salwa Wafi <wafisalwa@gmail.com>
to me

Mon, Mar 20, 12:0

Dear Mr. Oejijono,

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 attache
Thank you very much for your cooperation.

Best regards,
Salwa Shabria Wafi
Admin of JTBB.

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

•••

One attachment • Scanned by Gmail

Article 6 JTBB-Ag...

Oedjijono 1

Search

[JTBB] Proofread Layout - oedjijo

gle.com/mail/u/0/?tab=rm&ogbl#search/jtbb+agustiani/FMfcgzGslklsvgvrsKhLMdHQGlFDxXvZ

jtbb agustiani

Active

5,860

25

[JTBB] Proofread Layout

External Inbox ×

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.

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

One attachment • Scanned by Gmail

PDF

Article 6 JTBB-Ag...

Search

Journal of
Tropical Biodiversity and Biotechnology

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			
	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.		

	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."		
	The background of why the research is specifically focused on malto-oligosaccharides amylase instead of other oligosaccharides needs to be explained in the manuscript.		
	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!) 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 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.		
	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.		
	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 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.		

Journal of
Tropical Biodiversity and Biotechnology

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			
	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.	The organization of the section Materials and methods, we rearranged as the following: 2.1. Sample collection and location of sampling 2.2. Isolation, screening, and morphological characterization of amylolytic 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)	Line 71-165

	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."	We already change the title to become: "Isolation and characterization of rhizospheric bacteria associated with Canna sp. for production of malto-oligosaccharide amylase."	Line 1-2
	The background of why the research is specifically focused on malto-oligosaccharides amylase instead of other oligosaccharides needs to be explained in the manuscript.	The type of oligosaccharide produced by enzymatic hydrolysis depend on the type of carbohydrate polymer and enzymes type which we used in study We used starch and amylase for this research so hydrolysis product of starch by amylase is maltooligosaccharide.	Line 23-67
	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!)	According to a plant systematic expert, the Canna plant used in this research is *Canna indica*	Line 7, 72, 274, 397
	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	In the Introduction We already delete the word of *Aspergillus oryzae*	Line 58

	mentioned *Aspergillus oryzae* 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!		
	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.	In the methodology We already change the literature of Niu, 2017 to become Duan et al. 2021	Line 88
	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.	a. Yes, we missed one step…. Actually after washing, the samples then put on sterile tissue papers 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.	Line 88-89 Line128-129
	In the results and discussion: a. To facilitate interpretation, results are better presented in graphs rather than tables.	a. We already presented the results in graphs rather than tables, such as: Table 3 to become Figure 7, Table 4 to become Figure 8, and Table 5 to become Figure 9.	Line 326 Line 354 Line 370

		We also change the image caption in Figure 6.	Line 287
	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.	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.	
	c. Results for the electrophotogram (Figure 5): Please give details for the size of each band of the marker	b. We already add the size of each band of the marker in the electrophotogram Figure 4.	Line 269
	Some formatting inconsistencies have been observed in some references that need correction.	We already revision some references following authors guidelines from the journal.	

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.	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. We also improve the Figure 6 to make more beautiful figure	Line 326 Line 354 Line 370 Line 287

Abstract

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

Introduction

Line	Suggestion	Author's Response (Blue color)
Line 55	Bacillus 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 figure1	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 ≥ 18mm 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 *Bacillus* 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 amylolytic 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 *Bacillus* 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 *B.toyonensis*?	We already added about the potency of *B. toyonensis* 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

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

Abstract

The objectives of the study were to isolate amylolytic bacteria from rhizosphere and plant tissue of *Canna indica*, 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. Thirty-two isolates of amylolytic bacteria were obtained from the rhizosphere, Canna plant tissue, and litters. 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 hydrolyzed by the T10 amylase resulted in three oligosaccharide compounds of maltotriose, maltotetraose, and maltopentose. The amylase activity of isolate T10 was optimal at a temperature 40°C and pH 7 in an amount of 0.801 U/mL. The isolate T10 was identified as a species member of *Bacillus toyonensis* based on phenotyphic characterization and 16S rDNA gene sequencing analysis with a similarity value of 99.93%.

Keywords:

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

1. Introduction

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

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

Amylase has been exploited by the starch processing industry as a substitute for acid hydrolysis in the production of 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 malto-oligosaccharide 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 good substrate for the growth of various bacteria, especially the 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 habitats with respect to 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 the growth. On the contrary, rhizospheric bacteria can produce protein and enzymes that are important for the biological function of the host plants (Afifah et al. 2018).

Bacteria, fungi, plants, and animals play an important role in the utilization of polysaccharides. Members of the genus *Bacillus* were known to be able 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 that is, 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 hydrolized by the bacterial enzymes, and to identify the selected amylolytic bacteria based on 16S rRNA gene sequences.

2. Materials and methods

2.1. Sample collection and location of sampling

Samples were taken from the rhizosphere and parts of Canna plant (C. *indica*) 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 later is S 07 °20.812 'E 109°05.92 (Figure 1).

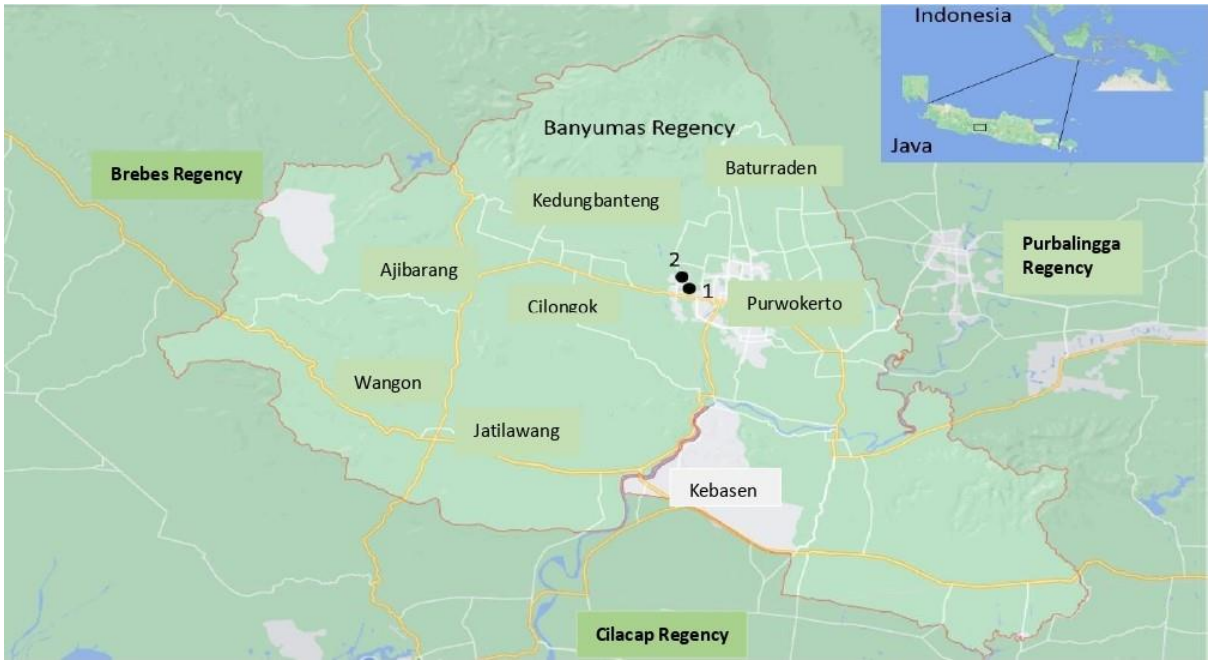


Figure 1. A map of Banyumas Regency and sampling sites: (1) the Perhutani Forest KPH West Banyumas, Central Java, (2) Community gardens around the Perhutani Forest KPH West Banyumas, Central Java.

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 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. 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 of the growing bacterial colonies was then inoculated onto NA medium containing 1% soluble starch, and purified using a streak quadrant method.

Amount of 0.5µL 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. Characterization of colonial and cell morphology of the isolated bacteria were carried out using conventional methods (Smibert & Krieg 1981).

2.3. Phenetic and phylogenetic characterizations of the selected bacterium

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

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

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

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

Amount of 2 mL of each 24 h old bacterial cultures (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 malto-oligosaccharide products was carried out on silica gel $60F_{254}$ plates (Merck Art 20-20 cm) and eluent using a solvent mixture of n-butanol:acid:water (12:6:6, v/v/v). Spots formed were visualized by spraying the sugar colors (0.5 g α-diphenylamine, 25 mL acetone, 2.5 mL phosphate acid, 0.5 mL aniline). All samples were applied in equal quantities (4 µL). Glucose (Sigma-Aldrich, U.S.A), maltose (M2),

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

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

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 sampling every 24 hour and then centrifuge and the supernatant obtained was tested for its amylase activity.

Measurement of amylase activity used a DNS method (Miller 1959) and the enzyme reaction was conducted as above. 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:

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

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 1 µmol of D-Glucose per minute under the experimental condition given.

2.6. Effect of pH and temperature toward 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 sodium 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).

3. Results and Discussion

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

The results of the study found 32 bacterial isolates growing on NA medium supplemented with 1% soluble starch, with details: 11 isolates were from the rhizosphere of the Canna growing in the forest, 12 isolates from rhizosphere of the Canna growing in the people's gardens around the forest, 4 isolates from the leave tissue of the Canna growing in the people's gardens around the forest, 3 isolates from the roots of the Canna growing in the people's gardens around the forest, and 2 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 to produce clear zones in the medium, indicating that those bacteria were capable of producing amylase. The more amylase is released, the wider clear zones are produced due to the degradation of amylum in the medium, resulting in the enhancement of the amylolytic index (Ginting et al. 2021). The research results showed that eight isolates of TH6, TH7, T5, T10, D2, D3, A3, and S1 showing 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 in 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 loss their amylolytic activity (Figure 2). Based on the ability of isolates to produce a clear zone diameter $\geq$ 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 amylolytic

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

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

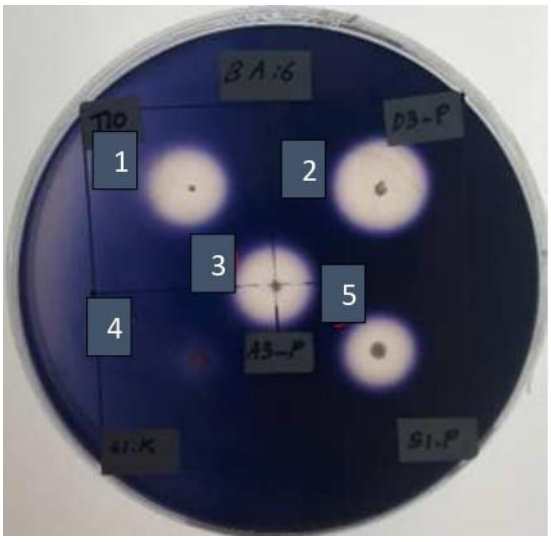


Figure 2. The amylolytic zones produced by bacterial isolates of: (1) T10, (2) D3, (3) A3, (4) TH6, and (5) S1 on a NA medium + 1% soluble starch.

A high number of amylolytic bacteria isolated from the rhizosphere of Canna was in accordance with Vassekaran et al. (2010) who stated that bacteria isolated from starch rich materials may have better potential to produce amylase. Vijayalakshmi et al. (2012) found *Bacillus subtilis* KC3 isolated from the rhizosphere of *Euphorbia hirta* producing maximum halo zone of 23 mm on a Starch Agar medium. Gebreyohannes (2015) reported that 16 bacterial isolates from soils were capable of producing 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 producing high amylolytic indexes of 3.04 and 3.52, respectively. The clear zone formed is the result of a reaction from the breakdown of 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 of D1-D4 and S1-S2 was in terminal, while isolates of TH1-TH11, T1-T12, and D1-D4 had endospores in the center. All of the isolates were able to hydrolyze starch and to produce lecithinase (Table 2). Those characteristics indicated that the bacteria were members of the genus *Bacillus*. According to Logan & De 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.

Table 2. Morphological and physiological properties of the bacteria isolated from Canna 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 maltoooligosachharides
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).

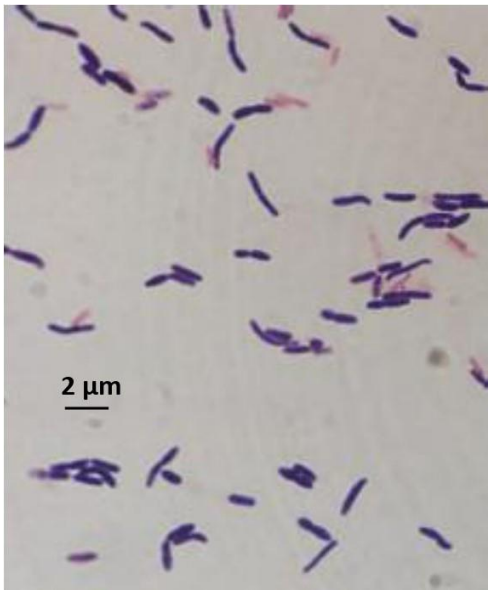


Figure 3. The appearance of bacterial cells isolates T10 under a microscope with a magnification of 1000x. The cells appear single or in chains

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

B. toyonensis strain BCT-7112^{T} was firstly 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 use in 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 are involved in the production of siderophores, bacteriocins, and nonribosomal peptide synthetases. Wang et al. (2021) reported that *Bacillus toyonensis* XIN-YC13 produced a novel antibiotic namely toyoncin, with antimicrobial activity against *B. cereus* and *Listeria monocytogenes*. This antibiotic exerts bactericidal activity and induces cell membrane damage.

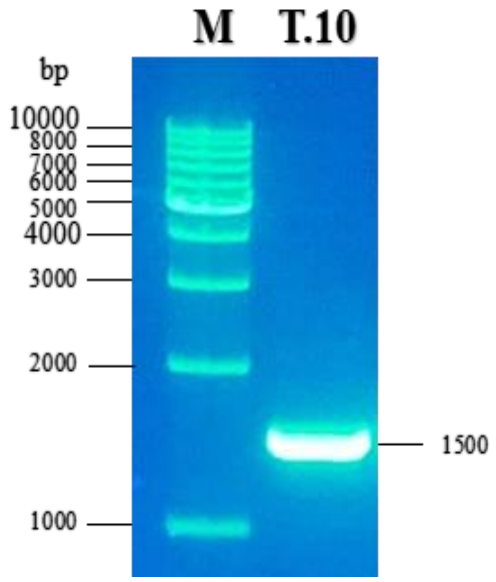


Figure 4. An electrophotogram of the amplified 16S rRNA gene of isolate T10 with a size of 1500 bp. Marker (M): 1 kb DNA ladder

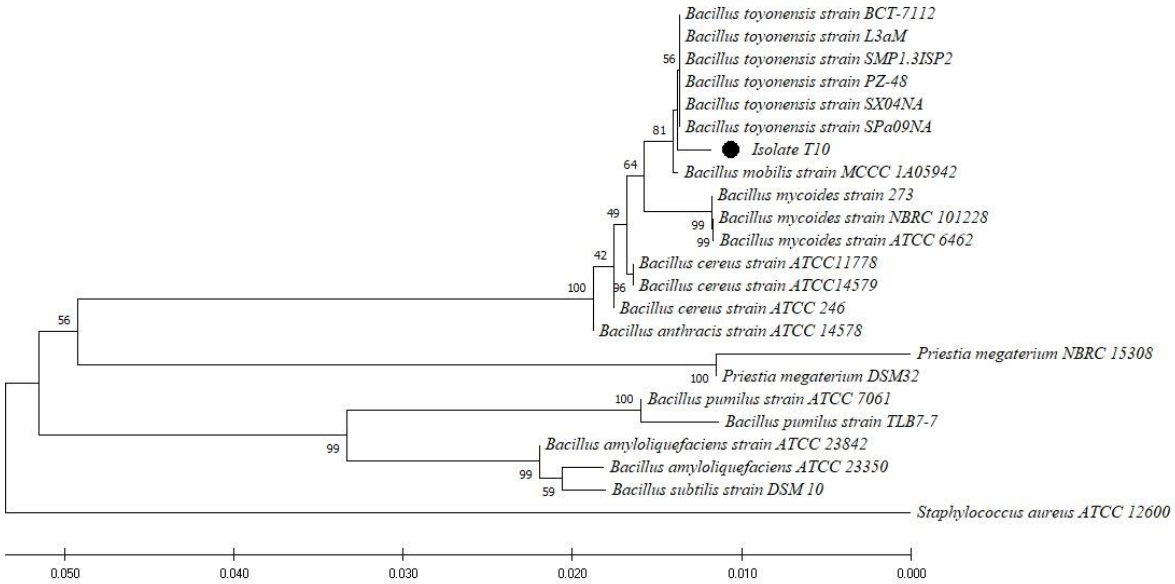


Figure 5. A phylogenetic tree showing the relationship between strain T10 isolated from rhizospheres of Canna (*C. indica*) and several species members of the genus *Bacillus* on the basis of 16S rRNA gene sequence reconstructed based on Neighbor-Joining, Model Maximum Composite Likelihood, and 1000x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

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

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).

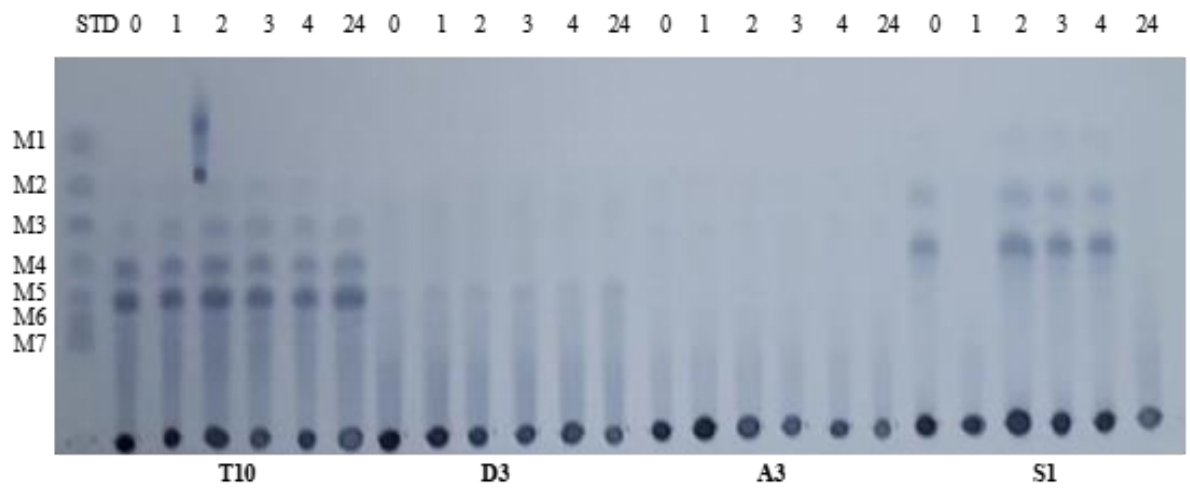


Figure 6. The product profile of starch hydrolyzed by amylase of the amylolytic bacteria (T10, D3, A3, and S1) using a TLC method with reaction times (hours): 0, 1, 2, 3, 4, and 24 at 30°C. The Standards (STD): monosaccharide (M1), maltose (M2), maltotriose (M3), maltotetraose (M4), maltopentaose (M5),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

Based on the TLC chromatogram, the starch degraded by T10 amylase resulted in maltoooligosachharides 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 amylolytic bacteria with high amylolytic indexes (AI) did not correlated with their ability in degrading amylum. 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

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 amylase of an alkaliphilic *Bacillus lehensis* G1 was capable degrading oligosaccharides by producing malto-oligosaccharides with a higher degree of polymerization than maltoheptaose observed on thin layer chromatography and high-performance liquid chromatography analyses.

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

Based on the ability of the fourth selected amylolytic bacteria in producing different types of hydrolyzed product, isolate T10 was further assayed for its optimal amylase activity at different incubation times. The results showed that incubation times affected 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 reducing 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.

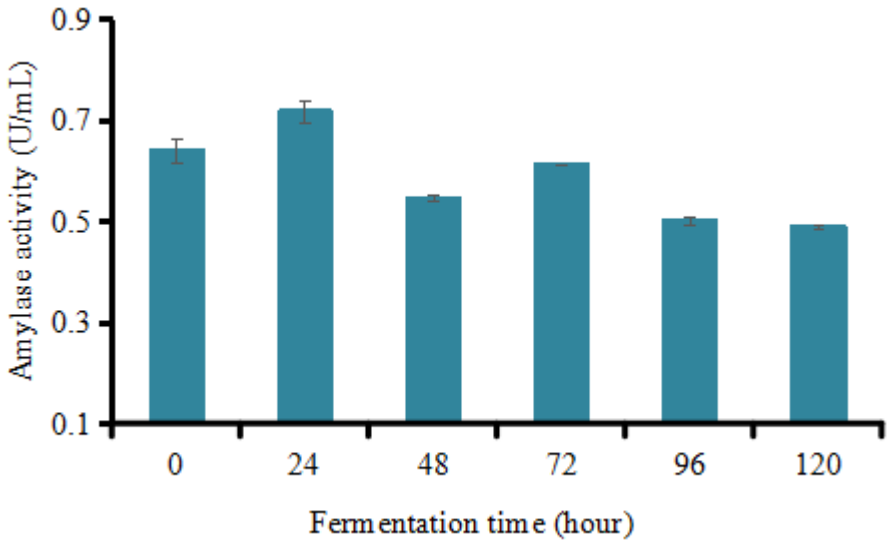


Figure 7. Amylase activity of T10 at different incubation times

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

optimal pH of isolate T10 was in accordance with 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 caused a change in the enzyme 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, and the enzyme is stable in neutral to alkaline (pH 7-10).

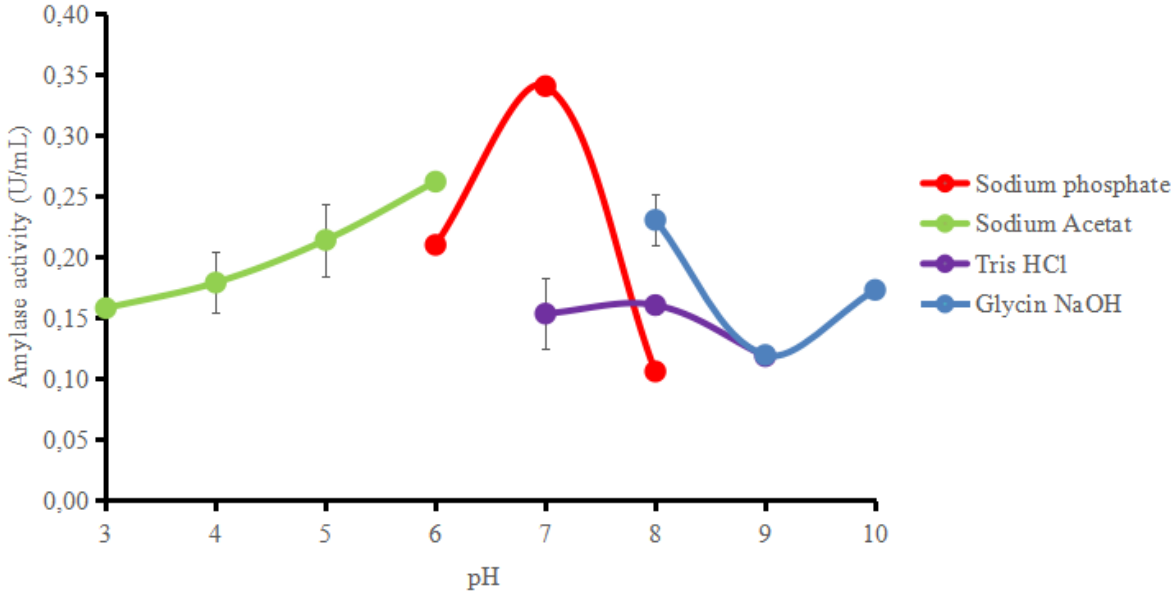


Figure 8. Amylase activity of T10 at different pH and buffers

The amylase activity of isolate T10 was observed at temperatures ranging from 30-90ºC at pH 7.0. Amylase activity of 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. licheniformis, and *B. amiloliquefaciens,* which have optimum temperatures of 37-60°C. Gebreyohannes (2015) found that maximum amylase activity of *Bacillus* spp. was 40°C and *Streptomyces* spp. at 37°C using 4% starch concentration at a neutral pH and an incubation of 48 h. The crude enzyme of *Bacillus* sp. AB04 showed maximum activity at pH 8 with 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.

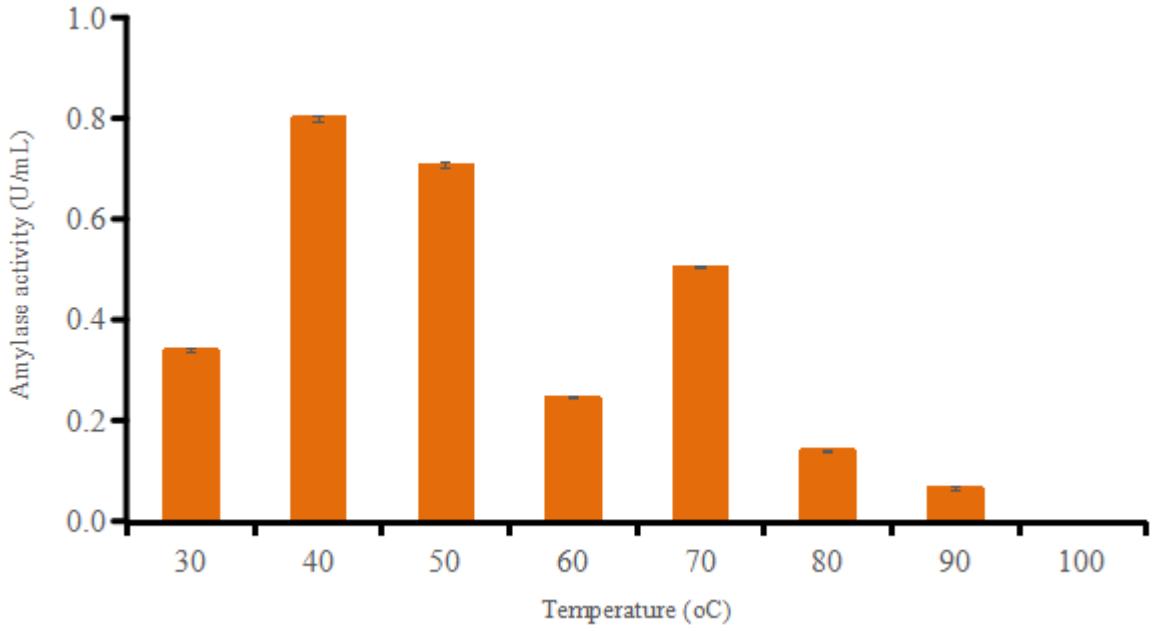


Figure 9. Amylase activity of isolate T10 at different temperatures

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 to occur at the greatest speed (Subagiyo et al.

2017). The results showed that after reaching the optimum condition, it was seen that the activity of the T10 amylase decreased. 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 malto-oligosaccharide in high yield through the enzymatic hydrolysis of starch is of considerable commercial interest. This has been achieved on an industrial scale after the discovery of a suitable malto-oligosaccharide-forming amylase (MFA_{ses}), and since then several studies have tried to improve on 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} to 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 be applicable to starch processing technologies due to its highly specific activity, unique substrate specificity, and endo-type action pattern (Pan et al. 2017).

4. Conclusions

It can be concluded that amounts of 32 amylolytic bacteria were isolated from rhizosphere and plant tissue of *Canna indica*, as well as litter; the selected amylolytic bacterial isolate of T10 was capable of hydrolyzing 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 analyzed the research data, O, NR and NE supervised all the process, and re-wrote the manuscript.

Acknowledgments

We would like to thank the Research Center for Biotechnology (BRIN) and International Women University (IWU) for funding this research.

Conflict of interest

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

References

Abdalla, M. et al., 2021. One-pot production of maltoheptaose (DP7) from starch by sequential addition of cyclodextrin glucotransferase and cyclomaltodextrinase. *Enzyme and Microbial Technology*, 149 (6), 109847. doi: 10.1016/j.enzmictec.2021.109847

Abdul-Manas, N.H. et al., 2014. The characterisation of an alkali-stable maltogenic amylase from *Bacillus lehensis* G1 and improved malto-oligosaccharide production by hydrolysis suppression. *PLoS ONE* 9(9). doi: 10.1371/journal.pone.0106481

Afifah, N., Putri, D.H. & Irdawati, 2018. Isolation and identification of rhizosfer bacteria from the Andalas plant stem (*Morus macroura* Miq.). *Bioscience*, 2(1), pp.72-75.

Agamennone, V. et al., 2019. Genome annotation and antimicrobial properties of *B. toyonensis* VU-DES13, isolated from the *Folsomia candida* gut. *Entomologia Experimentalis et Aplicata* 167, pp:269-285. doi: 10.1111/eea.12763.

Ashger, M. et al., 2007. A thermostable α-amylase from moderately thremophilic *Bacillus subtilis* strain for starch processing. *J Food Eng*., 79, pp.950-955.

Bajpai, B., Chaudhary, M. & Saxena, J., 2015. Production and characterization of α-Amylase from an extremely halophilic archaeon, *Haloferax* sp. HA10. *Food Technol Biotechnol.*, 53(1), pp.11-17, doi: 10.17113/ftb.53.01.15.3824

Ben-Ali, M. et al., 2006. Thermostability enhancement and change in starch hydrolysis profile of the maltohexaose-forming amylase of *Bacillus stearothermophilus* US100 strain. *Biochemical Journal*, 394(1), pp.51–56. doi: 10.1042/BJ20050726

Behal, A. et al., 2016. Characterization of alkaline α- amylase from *Bacillus* sp. AB 04. *IJAB*, 8(1), pp.80-83.

De-Moraes-Russo, C.A. & Selvatti, A.P., 2018. Bootstrap and rogue identification tests for phylogenetic analyses. *Molecular Biology and Evolution*, 35(9), pp.2327–2333. doi: 10.1093/molbev/msy118.

Dey, G. et al., 2002. Purification and characterization of maltooligosaccharide-forming amylase from *Bacillus circulans* GRS 313. *Journal of Industrial Microbiology and Biotechnology*, 28(4), pp.193-200. DOI: 10.1038/sj/jim/7000220.

Ding, N. et al., 2021. Carbohydrate-binding module and linker allow cold adaptation and salt tolerance of maltopentaose-forming amylase from marine bacterium *Saccharophagus degradans* 2-40T. *Frontiers in Microbiology*, 12(7), pp.1–14. doi: 10.3389/fmicb.2021.708480.

Divakaran, D., Chandran, A. & Pratap-Chandran, R., 2011. Comparative study on production of α-amylase from *Bacillus licheniformis* strains. *Brazilian Journal of Microbiology*, 42(4), pp.1397–1404. doi: 10.1590/S1517-83822011000400022.

Duan, Y. et al., 2021. Isolation, identification, and antibacterial mechanisms of *Bacillus amyloliquefaciens* QSB-6 and its effect on plant roots. *Frontiers in Microbiology*, 12:746799. doi:10.3389/fmicb.2021.746799.

El-Fallal, A. et al., 2012. Starch and microbial α-amylases: from concepts to biotechnological applications. In *Carbohydrates-Comprehensive Studies on Glycobiology and Glycotechnology*, Intech Open Science, pp.459-488. doi: 10.5772/51571

Gebreyohannes, G., 2015. Isolation and optimization of amylase producing bacteria and actinomycetes from soil samples of Maraki and Tewedros campus, University of Gondar, North West Ethiopia. *African Journal of Microbiology Research*, 9(31), pp.1877-1882.

Ginting, E.L. et al., 2021. Isolation and identification of thermophilic amylolytic bacteria from Likupang Marine Hydrothermal, North Sulawesi, Indonesia. Biodiversitas, 22(6), pp.3326-3332. doi: 10.13057/biodiv/d220638.

Gupta, R. et al., 2003. Microbial α-amylases: A biotechnological perspective. *Process Biochemistry*, 38(11), pp.1599–1616. doi: 10.1016/S0032-9592(03)00053-0.

Hasanah, U. et al., 2020. Amylolytic activity of bacterial strains isolated from sago pulp of the traditional sago industry in Palopo, South Sulawesi. *AIP Conference Proceedings* 2231, 040073 (2020), https://doi.org/10.1063/5.0002487.

Hellmuth, K. & van-den Brink, J.M., 2013. Microbial production of enzymes used in food applications. In *Microbial Production of Food Ingredients, Enzymes and Nutraceuticals*. Woodhead Publishing Limited. doi: 10.1533/9780857093547.2.262.

Jang, E.Y. et al., 2020. Amylase-producing maltooligosaccharide provides potential relief in rats with loperamide-induced constipation. *Evidence-Based Complementary and Alternative Medicine*. doi: 10.1155/2020/5470268.

Jiménez, G. et al., 2013. Description of *Bacillus toyonensis* sp. nov., a novel species of the *Bacillus cereus* group, and pairwise genome comparisons of the species of the group by means of ANI calculations. *Systematic and Applied Microbiology* 36(6), pp.383-391. doi: 10.1016/j.syapm.2013.04.008.

Lim, S.J. & Oslan, S.N., 2021. Native to designed: Microbial α-Amylases for industrial applications. *PeerJ*, 9, pp.1–30. doi: 10.7717/peerj.11315.

Logan, N.A. & De Vos, P., 2009. Genus I. Bacillus Cohn 1872. In *Bergey's Manual of Systematic Bacteriology Second Edition Volume Three The Firmicutes*. Springer, Springer Dordrecht Heidelberg London New York. pp.21-127. doi: 10.1007/b92997.

Luang-In, V. et al., 2019. Isolation and identification of amylase-producing bacteria from soil in Nasinuan community forest, Maha Sarakham, Thailand. *Biomedical & Pharmacology Journal*, 12(3), pp.1061-1068.

Luo, J-c. et al., 2021. Characterization of a deep sea *Bacillus toyonensis* isolate: genomic and pathogenic features. *Frontiers in Cellular and Infection Microbiology*, 11, 629116. doi: 10.3389/fcimb.2021.629116.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31(3), pp.426–428. doi: 10.1021/ac60147a030

Moradi, M. et al., 2014. Screening and isolation of powerful amylolytic bacterial strains. *International Journal of Current Microbiology and Applied Sciences*, 3(2), pp.758–768.

Naidu, K. et al., 2019. Purification and characterization of α-amylase from *Paenibacillus* sp. D9 and *Escherichia coli* recombinants. *Biocatalysis and Biotransformation*, 38(1), pp.24-34. doi: 10.1080/10242422.2019.1628738.

Nisa, I.K. et al., 2021. The potential of amylase enzyme activity against bacteria isolated from several lakes in East Java, Indonesia. *Biodiversitas*, 22(1), pp.42-49.

500 Niu, D. et al., 2017. Highly efficient enzymatic preparation of isomalto-oligosaccharides
501 from starch using an enzyme cocktail. *Electronic Journal of Biotechnology*, 26,
502 pp.46–51. doi: 10.1016/j.ejbt.2016.12.002.
503 Ochoa-Solano, J.L. & Olmos-Soto, J., 2006. The functional property of Bacillus for Shrimp
504 feeds. *Food Microbiology* 23(6), pp.519–525, doi: 10.1016/j.fm.2005.10.004
505 Ozturk, H.U. et al., 2014. A *maltooligosaccharides* producing α-amylase from *Bacillus*
506 *subtilis* SDP1 isolated from rhizosphere of *Acacia cyanophylla* Lindley. *Food*
507 *Biotechnology*, 28(4), pp.309-332. doi: 10.1080/08905436.2014.963600.
508 Pan, S. et al., 2017. Maltooligosaccharide-forming amylase: Characteristics, preparation, and
509 application. *Biotechnology Advances*, 35(5), pp.619–632. doi:
510 10.1016/j.biotechadv.2017.04.004.
511 Putri, W.D.R. et al., 2012. Isolation and characterization of amylolytic lactic acid bacteria
512 during growol fermentation, an Indonesian traditional food. *Jurnal Teknologi*
513 *Pertanian*, 13(1), pp.52-60.
514 Rahmani, N. et al., 2013. Production of maltooligosaccharides from black potato (*Coleus*
515 *tuberosusi)* starch by α-amylase from a marine bacterium (*Brevibacterium* sp.).
516 *Microbiology Indonesia*, 7(3), pp.129-136. doi: 10.5454/mi.7.3.6.
517 Rahmani, N. et al., 2018. Xylanase and feruloyl esterase from actinomycetes cultures could
518 enhance sugarcane bagasse hydrolysis in the production of fermentable sugars.
519 *Bioscience Biotechnology and Biochemistry*, 82(5), pp.904–915. doi:
520 10.1080/09168451.2018.1438169.
521 Santos, F.D.S. et al., 2018. *Bacillus toyonensis* improves immune response in the mice
522 vaccinated with recombinant antigen of bovine herpesvirus type 5. *Benef. Microbes*,
523 9(1), pp.133–142. doi: 10.3920/BM2017.0021.

524 Sivaramakrishnan, S. et al., 2006. α-Amylase from microbial sources: An overview on recent
525 developments. *Food Technol. Biotechnol.*, 44 (2), pp.173–184.
526 Smibert, R.M. & Krieg, N.R., 1981. General Characterization. In *Manual Methods for*
527 *General Bacteriology*. American Society for Microbiology, Washington.
528 Subagiyo, Djarod, M.S.R. & Setyati, W.A., 2017. Potensi ekosistem mangrove sebagai
529 sumber bakteri untuk produksi protease, amilase, dan selulase. *Jurnal Kelautan*
530 *Tropis*, 20(2), pp.106–111.
531 Vaseekaran, S., Balakumar, S. & Arasaratnam, V., 2010. Isolation and identification of a
532 bacterial strain producing thermostable α- amylase. *Tropical Agricultural Research*,
533 22 (1), pp.1-11. doi: 10.4038/tar.v22i1.2603.
534 Vijayalakshmi, et al., 2012. Isolation and characterization of *Bacillus subtilis* KC3 for
535 amylolytic activity. *International Journal of Bioscience, Biochemistry and*
536 *Bioinformatics*, 2(5), pp.336-341. doi: 10.7763/IJBBB.2012.V2.128.
537 Wang, J. et al., 2021. Toyoncin, a novel leaderless bacteriocin that is produced by *Bacillus*
538 *toyonensis* XIN-YC13 and specifically targets *B. cereus* and *Listeria*
539 *monocytogenes. Applied and Environmental Microbiology*, 87(12), e00185-21. doi:
540 10.1128/AEM.00185-21.
541 Yopi, et al., 2017. Isolation and characterization of mannanase, xylanase, and cellulase from
542 marine bacteria *Bacillus* sp. *Biofarmasi Journal of Natural Product Biochemistry*,
543 15(1), pp.15–20. doi: 10.13057/biofar/f150103.
544 Yufinta, C.P., Julyantoro, P.G.S. & Pratiwi, M.A., 2018. Pengaruh penambahan *Bacillus* sp.
545 terhadap kelulushidupan pasca larva udang Vannamei (*Litopenaeus vannamei*) yang
546 terinfeksi vibriosis. *Current Trends in Aquatic Science*, 1(1), pp.89-95.

Zhao, C. et al., 2017. Functional properties, structural studies and chemo-enzymatic synthesis of oligosaccharides. *Trends in Food Science and Technology,* 66, pp.135–145. doi: 10.1016/j.tifs.2017.06.008.

Zhuang, Y., Zhou, X. & Wang, S., 2012. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Plant Systematics and Evolution,* 298(7), pp.3389–3402. doi: 10.2503/jjshs.58.977.

Zubaidah, A. et al., 2019. Screening bakteri selulolitik dan amilolitik pada rumen sapi sebagai kandidat probiotik pada budidaya ikan secara *in vitro. Jurnal Riset Akuakultur,* 14(4), pp.261-271.

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

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 hydrolysed 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 7 in an amount of 0.801 U/mL. The isolate T10 was identified as a species member of *Bacillus toyonensis* based on phenotyphic characterisation and 16S rDNA gene sequencing analysis with a similarity value of 99.93%.

Keywords:

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

1. 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 hydrolised 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 the production of 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 habitats 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 utilised by

bacteria for growth. On the contrary, rhizospheric bacteria can produce protein 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 utilising polysaccharides. Members of the genus *Bacillus* were known to be able 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 hydrolised by the bacterial enzymes, and to identify the selected amylolytic bacteria based on 16S rRNA gene sequences.

2. Materials and methods

2.1. 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).

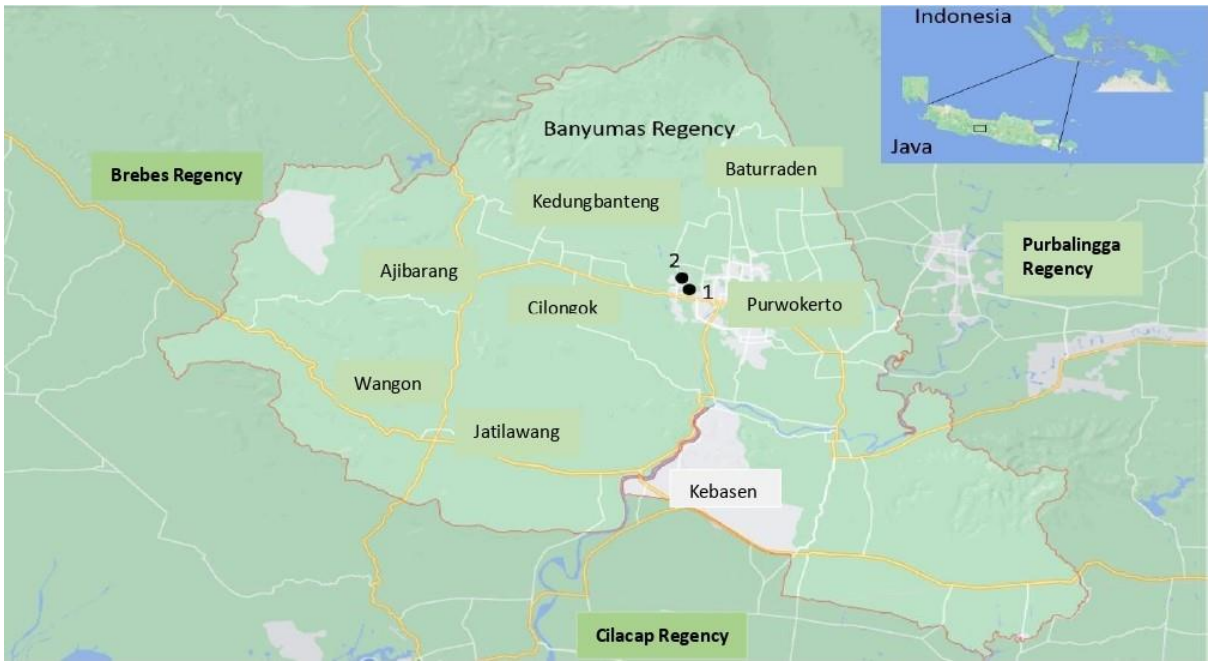


Figure 1. A map of Banyumas Regency and sampling sites: (1) the Perhutani Forest KPH West Banyumas, Central Java, (2) Community gardens around the Perhutani Forest KPH West Banyumas, Central Java.

2.2. Isolation, screening, and morphological characterisation 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 homogenised 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 µL 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 characterised using conventional methods (Smibert & Krieg 1981).

2.3. Phenetic and phylogenetic characterisations of the selected bacterium

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

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

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

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

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

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

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

3. Results and Discussion

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

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

The ability of the bacteria to grow and to produce clear zones in the medium, indicating that those bacteria were capable of producing amylase. The more amylase is released, the wider clear zones are produced due to the degradation of amylum in the medium, resulting in enhancing the amylolytic index (Ginting et al. 2021). The research results showed that eight isolates of TH6, TH7, T5, T10, D2, D3, A3, and S1 showed high total clear zone diameters (mm) of 18, 18, 18, 20, 18, 30, 18, and 18, respectively (Table 1). The consistency of the bacterial isolates, resulting in the total clear zone diameter, was shown by the isolates TH6, T10, D3, A3, and S1, while the other isolates tended to reduce or lose their amylolytic activity (Figure 2). Based on the ability of isolates to produce a clear zone diameter $\geq$ 18 mm and consideration of source representatives, four isolates (TH6, T10, D3, A3, and S1) were selected for further testing, namely their ability to hydrolyse starch. Hasanah et al. (2020) reported that bacterial isolates having an amylolytic index of more than 9 mm were potentials for producing 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.

192

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

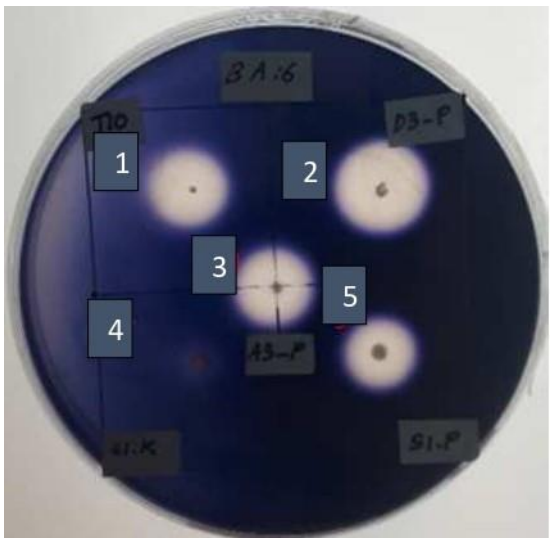


Figure 2. The amylolytic zones produced by bacterial isolates of: (1) T10, (2) D3, (3) A3, (4) TH6, and (5) S1 on a NA medium + 1% soluble starch.

A high number of amylolytic bacteria isolated from the rhizosphere of Canna was by Vassekaran 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 of 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 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.

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

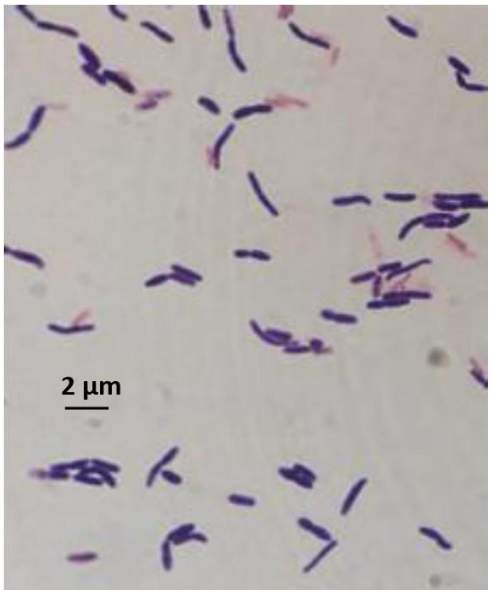
Characteristics	Isolate code				
	TH1-TH11	T1-T12	D1-D4	A1-A3	S1-S2
Colonial morphology on NA Agar	Rough, dry, bright, and Pink	Rough, dry, bright and	Rough, dry, bright and pink	Rough, dry, bright and	Rough, dry, bright, and pink

		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-αD-glucopyranoside	+
Amygdalin	+w
Arbutin	+
Salicin	+
Cellobiose	-
D-saccharose	+
D-trehalose	+
Starch	+
Glycogen	+
D-turanose	+

The electrophoresis visualisation 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 the comparison between the 16S rRNA gene sequence of isolate T10 and nucleotide sequences in the GeneBank (http://blast.ncbi.mlm.nih.gov/) showed that the bacterium is closely related to species members of the genus *Bacillus.* The BLAST analysis results 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 constructed using Neighbor-Joining, Model Maximum Composite Likelihood, and 1000x Bootstrapping. A dendrogram resulted from MEGA10 program showed that isolate T10 joined *B. toyonensis* SX04NA, *B. toyonensis* Spa09NA, *B. toyonensis* SMP1, *B. toyonensis* PZ-48, *B. toyonensis* BCT-7112, and *B. toyonensis* l3aM to form a separate cluster (Figure 5). Hence, isolate T10 was identified as the species member of *B. toyonensis* based on the phenetic and phylogenetic characteristics.

B. toyonensis strain BCT-7112^{T} was first isolated in 1966 in Japan from a survey designed to obtain naturally occurring microorganisms as pure cultures in the laboratory for use as probiotics in animal nutrition (Jiménez et al. 2013). This strain was first identified as *B. cereus* var. *toyoi*, and it has been used as the active ingredient of the preparation TOYOCERIN, is 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.

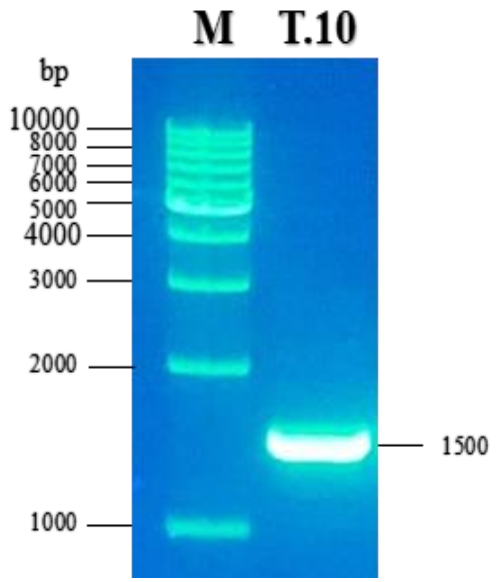


Figure 4. An electropherogram of the amplified 16S rRNA gene of isolate T10 with a size of 1500 bp. Marker (M): 1 kb DNA ladder.

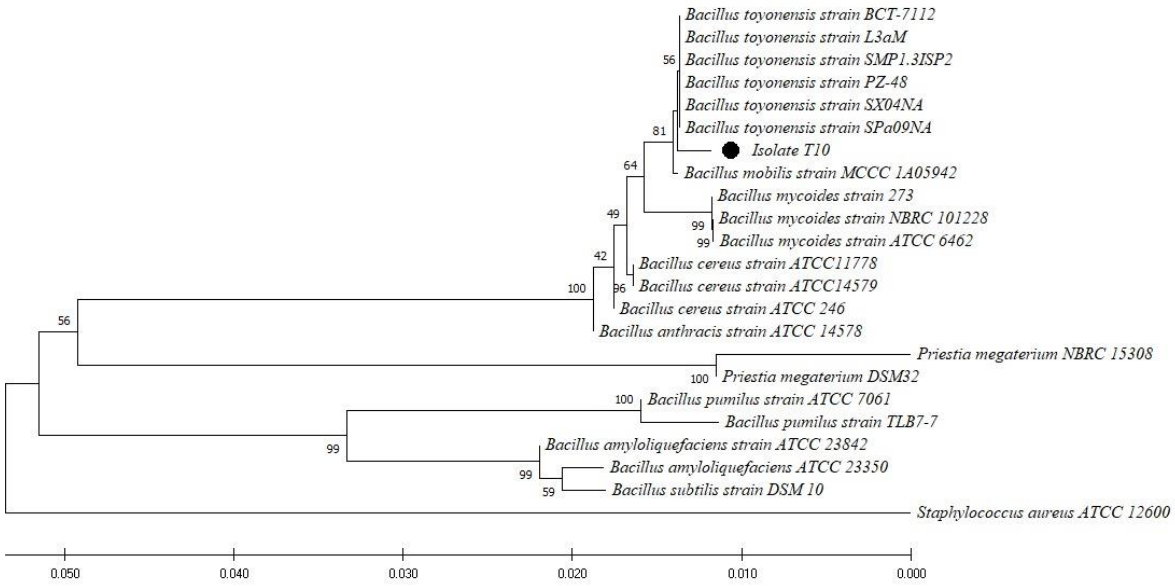


Figure 5. A phylogenetic tree showing the relationship between strain T10 isolated from rhizospheres of Canna (*C. edulis*) and several species members of the genus ***Bacillus*** on the basis of 16S rRNA gene sequence reconstructed based on Neighbor-Joining, Model Maximum Composite Likelihood, and 1000x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

3.3. 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 hydrolyse starch (Figure 6).

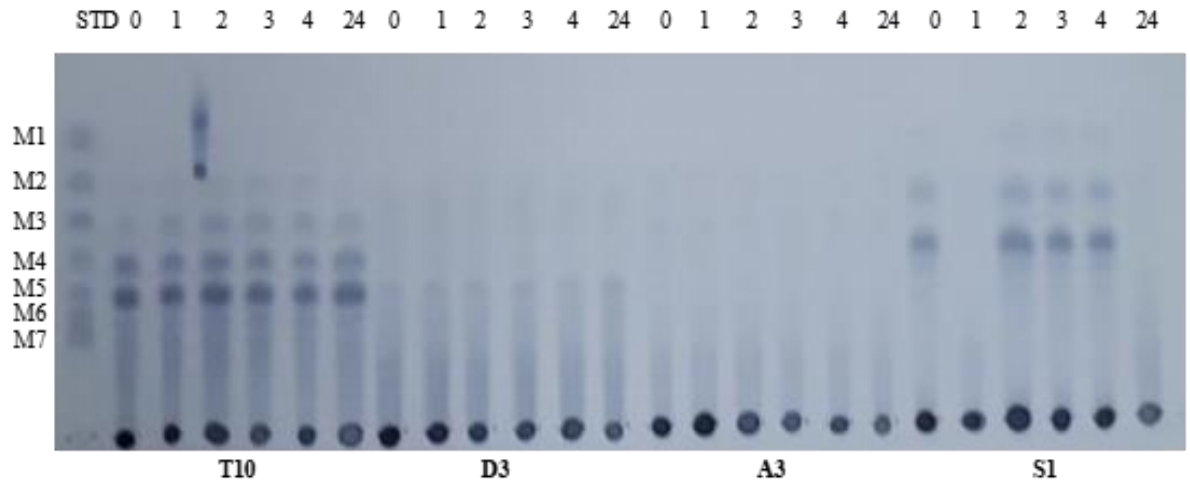


Figure 6. The product profile of starch hydrolysed by amylase of the amylolytic bacteria (T10, D3, A3, and S1) using a TLC method with reaction times (hours): of 0, 1, 2, 3, 4, and 24 at 30°C. The Standards (STD): monosaccharide (M1), maltose (M2), maltotriose (M3), maltotetraose (M4), maltopentaose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

Based on the TLC chromatogram, the starch degraded by T10 amylase resulted in malto-oligosachharides 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 amylolytic bacteria with high amylolytic indexes (AI) did not correlate with their ability to degrade amylum. The isolate T10, with its total diameter lower than isolate D3, showed a higher ability to breakdown 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 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 hydrolised 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

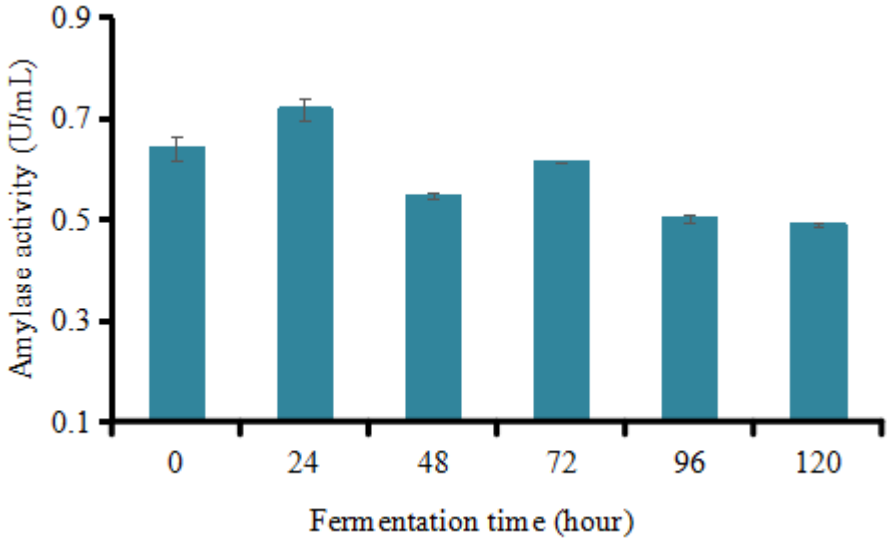


Figure 7. Amylase activity of T10 at different incubation times.

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. it 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 characterisation: 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).

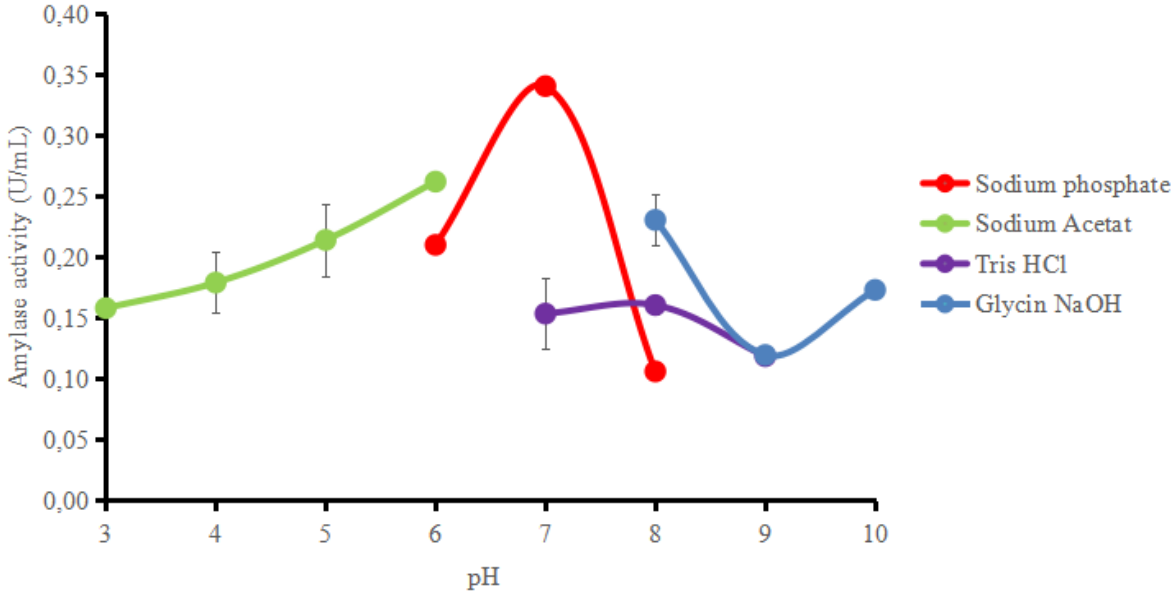
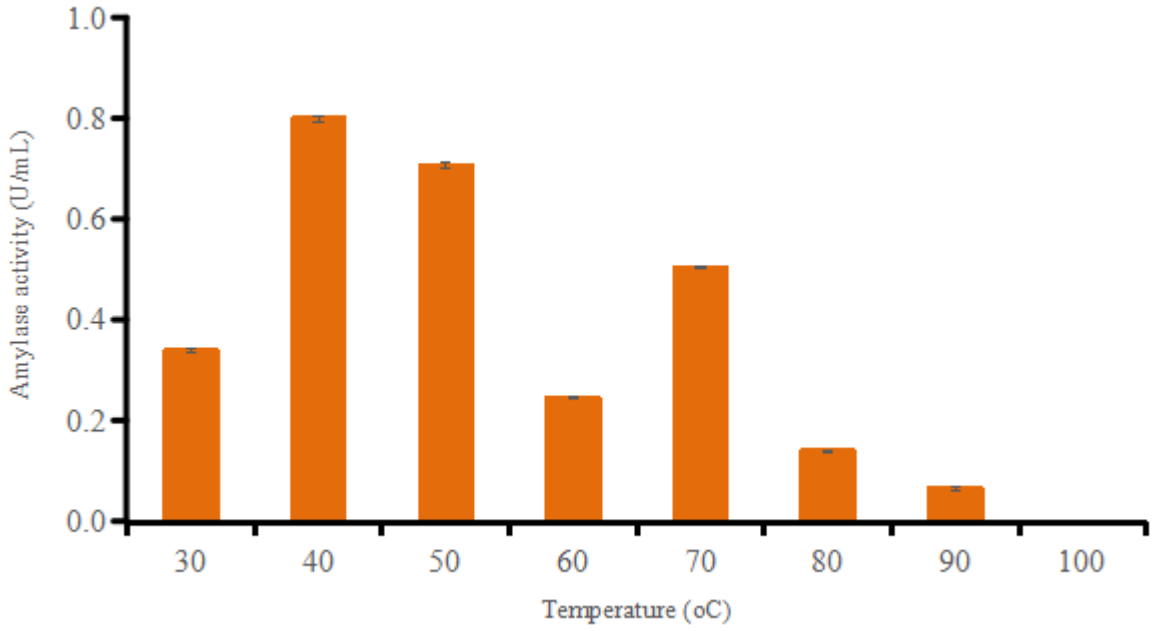


Figure 8. Amylase activity of T10 at different pH and buffers.

The amylase activity of isolate T10 was observed at temperatures ranging from 30-90^{o}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.*

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.

2017). The results showed that after reaching the optimum condition, it was seen that the activity of the T10 amylase decreased. 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 immobilise 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).

4. 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 characterisations.

Authors contribution

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

Acknowledgments

We would like to thank the Research Center for Biotechnology (BRIN) and International Women University (IWU) for funding this research.

Conflict of interest

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

References

Abdalla, M. et al., 2021. One-pot production of maltoheptaose (DP7) from starch by sequential addition of cyclodextrin glucotransferase and cyclomaltodextrinase. *Enzyme and Microbial Technology,* 149 (6), 109847. doi: 10.1016/j.enzmictec.2021.109847

Abdul-Manas, N.H. et al., 2014. The characterisation of an alkali-stable maltogenic amylase from *Bacillus lehensis* G1 and improved maltooligosaccharide production by hydrolysis suppression. *PLoS ONE* 9(9). doi: 10.1371/journal.pone.0106481

Afifah, N., Putri, D.H. & Irdawati, 2018. Isolation and identification of endophytic bacteria from the Andalas plant stem (*Morus macroura* Miq.). *Bioscience,* 2(1), pp.72-75.

Agamennone, V. et al., 2019. Genome annotation and antimicrobial properties of *B. toyonensis* VU-DES13, isolated from the *Folsomia candida* gut. *Entomologia Experimentalis et Aplicata* 167, pp:269-285. doi: 10.1111/eea.12763.

Ashger, M. et al., 2007. A thermostable α-amylase from moderately thermophilic *Bacillus subtilis* strain for starch processing. *J Food Eng*., 79, pp.950-955.

423 Bajpai, B., Chaudhary, M. & Saxena, J., 2015. Production and characterization of α-Amylase
424 from an extremely halophilic archaeon, *Haloferax* sp. HA10. *Food Technol*
425 *Biotechnol.,* 53(1), pp.11-17, doi: 10.17113/ftb.53.01.15.3824
426 Ben-Ali, M. et al., 2006. Thermostability enhancement and change in starch hydrolysis
427 profile of the maltohexaose-forming amylase of *Bacillus stearothermophilus* US100
428 strain. *Biochemical Journal,* 394(1), pp.51–56. doi: 10.1042/BJ20050726
429 Behal, A. et al., 2016. Characterization of alkaline α- amylase from *Bacillus* sp. AB 04.
430 *IJAB,* 8(1), pp.80-83.
431 De-Moraes-Russo, C.A. & Selvatti, A.P., 2018. Bootstrap and rogue identification tests for
432 phylogenetic analyses. *Molecular Biology and Evolution*, 35(9), pp.2327–2333. doi:
433 10.1093/molbev/msy118.
434 Dey, G. et al., 2002. Purification and characterization of maltooligosaccharide-forming
435 amylase from *Bacillus circulans* GRS 313. *Journal of Industrial Microbiology and*
436 *Biotechnology*, 28(4), pp.193-200. DOI: 10.1038/sj/jim/7000220.
437 Ding, N. et al., 2021. Carbohydrate-binding module and linker allow cold adaptation and salt
438 tolerance of maltopentaose-forming amylase from marine bacterium
439 *Saccharophagus degradans* 2-40T. *Frontiers in Microbiology*, 12(7), pp.1–14. doi:
440 10.3389/fmicb.2021.708480.
441 Divakaran, D., Chandran, A. & Pratap-Chandran, R., 2011. Comparative study on production
442 of α-amylase from *Bacillus licheniformis* strains. *Brazilian Journal of Microbiology*,
443 42(4), pp.1397–1404. doi: 10.1590/S1517-83822011000400022.
444 Duan, Y. et al., 2021. Isolation, identification, and antibacterial mechanisms of *Bacillus*
445 *amyloliquefaciens* QSB-6 and its effect on plant roots. *Frontiers in Microbiology,*
446 12:746799. doi:10.3389/fmicb.2021.746799.

El-Fallal, A. et al., 2012. Starch and microbial α-amylases: from concepts to biotechnological applications. In *Carbohydrates-Comprehensive Studies on Glycobiology and Glycotechnology*, Intech Open Science, pp.459-488. doi: 10.5772/51571

Gebreyohannes, G., 2015. Isolation and optimization of amylase producing bacteria and actinomycetes from soil samples of Maraki and Tewedros campus, University of Gondar, North West Ethiopia. *African Journal of Microbiology Research*, 9(31), pp.1877-1882.

Ginting, E.L. et al., 2021. Isolation and identification of thermophilic amylolytic bacteria from Likupang Marine Hydrothermal, North Sulawesi, Indonesia. Biodiversitas, 22(6), pp.3326-3332. doi: 10.13057/biodiv/d220638.

Gupta, R. et al., 2003. Microbial α-amylases: A biotechnological perspective. *Process Biochemistry*, 38(11), pp.1599–1616. doi: 10.1016/S0032-9592(03)00053-0.

Hasanah, U. et al., 2020. Amylolytic activity of bacterial strains isolated from sago pulp of the traditional sago industry in Palopo, South Sulawesi. *AIP Conference Proceedings* 2231, 040073 (2020), https://doi.org/10.1063/5.0002487.

Hellmuth, K. & van-den Brink, J.M., 2013. Microbial production of enzymes used in food applications. In *Microbial Production of Food Ingredients, Enzymes and Nutraceuticals*. Woodhead Publishing Limited. doi: 10.1533/9780857093547.2.262.

Jang, E.Y. et al., 2020. Amylase-producing maltooligosaccharide provides potential relief in rats with loperamide-induced constipation. *Evidence-Based Complementary and Alternative Medicine*. doi: 10.1155/2020/5470268.

Jiménez, G. et al., 2013. Description of *Bacillus toyonensis* sp. nov., a novel species of the *Bacillus cereus* group, and pairwise genome comparisons of the species of the group by means of ANI calculations. *Systematic and Applied Microbiology* 36(6), pp.383-391. doi: 10.1016/j.syapm.2013.04.008.

Lim, S.J. & Oslan, S.N., 2021. Native to designed: Microbial α-Amylases for industrial applications. *PeerJ,* 9, pp.1–30. doi: 10.7717/peerj.11315.

Logan, N.A. & De Vos, P., 2009. Genus I. Bacillus Cohn 1872. In *Bergey's Manual of Systematic Bacteriology Second Edition Volume Three The Firmicutes*. Springer, Springer Dordrecht Heidelberg London New York. pp.21-127. doi: 10.1007/b92997.

Luang-In, V. et al., 2019. Isolation and identification of amylase-producing bacteria from soil in Nasinuan community forest, Maha Sarakham, Thailand. *Biomedical & Pharmacology Journal*, 12(3), pp.1061-1068.

Luo, J-c. et al., 2021. Characterization of a deep sea *Bacillus toyonensis* isolate: genomic and pathogenic features. *Frontiers in Cellular and Infection Microbiology,* 11, 629116. doi: 10.3389/fcimb.2021.629116.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry,* 31(3), pp.426–428. doi: 10.1021/ac60147a030

Moradi, M. et al., 2014. Screening and isolation of powerful amylolytic bacterial strains. *International Journal of Current Microbiology and Applied Sciences*, 3(2), pp.758–768.

Naidu, K. et al., 2019. Purification and characterization of α-amylase from *Paenibacillus* sp. D9 and *Escherichia coli* recombinants. *Biocatalysis and Biotransformation*, 38(1), pp.24-34. doi: 10.1080/10242422.2019.1628738.

Nisa, I.K. et al., 2021. The potential of amylase enzyme activity against bacteria isolated from several lakes in East Java, Indonesia. *Biodiversitas,* 22(1), pp.42-49.

Niu, D. et al., 2017. Highly efficient enzymatic preparation of isomalto-oligosaccharides from starch using an enzyme cocktail. *Electronic Journal of Biotechnology,* 26, pp.46–51. doi: 10.1016/j.ejbt.2016.12.002.

496 Ochoa-Solano, J.L. & Olmos-Soto, J., 2006. The functional property of Bacillus for Shrimp
497 feeds. *Food Microbiology* 23(6), pp.519–525, doi: 10.1016/j.fm.2005.10.004.
498 Ozturk, H.U. et al., 2014. A *maltooligosaccharides* producing α-amylase from *Bacillus*
499 *subtilis* SDP1 isolated from rhizosphere of *Acacia cyanophylla* Lindley. *Food*
500 *Biotechnology*, 28(4), pp.309-332. doi: 10.1080/08905436.2014.963600.
501 Pan, S. et al., 2017. Maltooligosaccharide-forming amylase: Characteristics, preparation, and
502 application. *Biotechnology Advances*, 35(5), pp.619–632. doi:
503 10.1016/j.biotechadv.2017.04.004.
504 Putri, W.D.R. et al., 2012. Isolation and characterization of amylolytic lactic acid bacteria
505 during growol fermentation, an Indonesian traditional food. *Jurnal Teknologi*
506 *Pertanian*, 13(1), pp.52-60.
507 Rahmani, N. et al., 2013. Production of maltooligosaccharides from black potato (*Coleus*
508 *tuberosusi)* starch by α-amylase from a marine bacterium (*Brevibacterium* sp.).
509 *Microbiology Indonesia*, 7(3), pp.129-136. doi: 10.5454/mi.7.3.6.
510 Rahmani, N. et al., 2018. Xylanase and feruloyl esterase from actinomycetes cultures could
511 enhance sugarcane bagasse hydrolysis in the production of fermentable sugars.
512 *Bioscience Biotechnology and Biochemistry*, 82(5), pp.904–915. doi:
513 10.1080/09168451.2018.1438169.
514 Santos, F.D.S. et al., 2018. *Bacillus toyonensis* improves immune response in the mice
515 vaccinated with recombinant antigen of bovine herpesvirus type 5. *Benef. Microbes*,
516 9(1), pp.133–142. doi: 10.3920/BM2017.0021.
517 Sivaramakrishnan, S. et al., 2006. α-Amylase from microbial sources: An overview on recent
518 developments. *Food Technol. Biotechnol.*, 44 (2), pp.173–184.
519 Smibert, R.M. & Krieg, N.R., 1981. General Characterization. In *Manual Methods for*
520 *General Bacteriology*. American Society for Microbiology, Washington.

521 Subagiyo, Djarod, M.S.R. & Setyati, W.A., 2017. Potensi ekosistem mangrove sebagai
522 sumber bakteri untuk produksi protease, amilase, dan selulase. *Jurnal Kelautan*
523 *Tropis*, 20(2), pp.106–111.
524 Vaseekaran, S., Balakumar, S. & Arasaratnam, V., 2010. Isolation and identification of a
525 bacterial strain producing thermostable α- amylase. *Tropical Agricultural Research*,
526 22 (1), pp.1-11. doi: 10.4038/tar.v22i1.2603.
527 Vijayalakshmi, et al., 2012. Isolation and characterization of *Bacillus subtilis* KC3 for
528 amylolytic activity. *International Journal of Bioscience, Biochemistry and*
529 *Bioinformatics*, 2(5), pp.336-341. doi: 10.7763/IJBBB.2012.V2.128.
530 Wang, J. et al., 2021. Toyoncin, a novel leaderless bacteriocin that is produced by *Bacillus*
531 *toyonensis* XIN-YC13 and specifically targets *B. cereus* and *Listeria*
532 *monocytogenes. Applied and Environmental Microbiology*, 87(12), e00185-21. doi:
533 10.1128/AEM.00185-21.
534 Yopi, et al., 2017. Isolation and characterization of mannanase, xylanase, and cellulase from
535 marine bacteria *Bacillus* sp. *Biofarmasi Journal of Natural Product Biochemistry*,
536 15(1), pp.15–20. doi: 10.13057/biofar/f150103.
537 Yufinta, C.P., Julyantoro, P.G.S. & Pratiwi, M.A., 2018. Pengaruh penambahan *Bacillus* sp.
538 terhadap kelulushidupan pasca larva udang Vannamei (*Litopenaeus vannamei*) yang
539 terinfeksi vibriosis. *Current Trends in Aquatic Science*, 1(1), pp.89-95.
540 Zhao, C. et al., 2017. Functional properties, structural studies and chemo-enzymatic synthesis
541 of oligosaccharides. *Trends in Food Science and Technology*, 66, pp.135–145. doi:
542 10.1016/j.tifs.2017.06.008.
543 Zhuang, Y., Zhou, X. & Wang, S., 2012. Gapped BLAST and PSI-BLAST: a new generation
544 of protein database search programs. *Plant Systematics and Evolution*, 298(7),
545 pp.3389–3402. doi: 10.2503/jjshs.58.977.

546 Zubaidah, A. et al., 2019. Screening bakteri selulolitik dan amilolitik pada rumen sapi sebagai
547 kandidat probiotik pada budidaya ikan secara *in vitro. Jurnal Riset Akuakultur*,
548 14(4), pp.261-271.

Journal of Tropical Biodiversity and Biotechnology
Volume 08, Issue 02 (2023): jtbb78346
DOI: 10.22146/jtbb.78346

Research Article

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

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

Keywords:
Amylolytic bacteria
Canna
Maltooligosaccharides
16S rDNA gene
Submitted:
13 October 2022
Accepted:
30 January 2023
Published:

Editor:
Miftahul Ilmi

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

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

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

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

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

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

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

MATERIALS AND METHODS

Sample Collection and Location of Sampling

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

Isolation, Screening, and Morphological Characterization of Amylolytic Bacteria

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

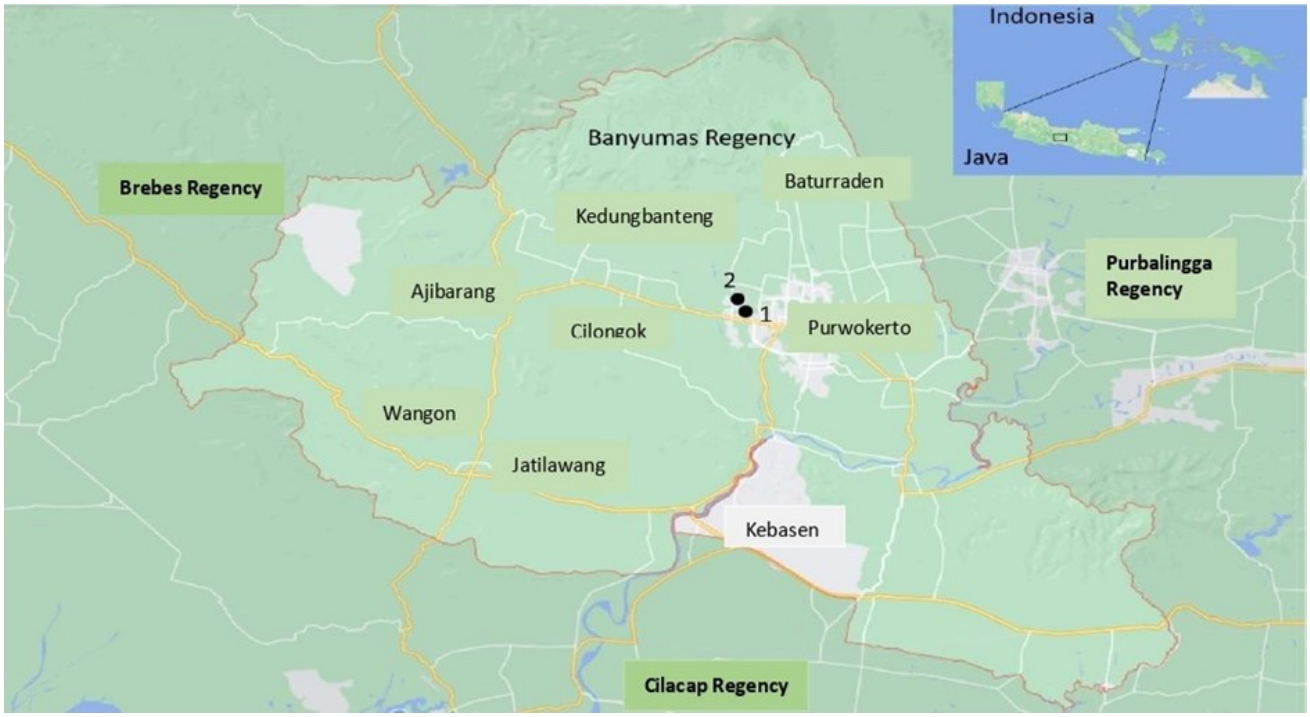


Figure 1. A map of Banyumas Regency and sampling sites: (1) the Perhutani Forest KPH West Banyumas, Central Java, (2) Community gardens around the Perhutani Forest KPH West Banyumas, Central Java.

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

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

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

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

Phenetic and Phylogenetic Characterizations of the Selected Bacterium

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

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

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

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

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

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

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

Crude Enzyme Production and Amylase Activity at Different Fermentation Time

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

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

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

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

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

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

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

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

RESULTS AND DISCUSSION

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

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

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

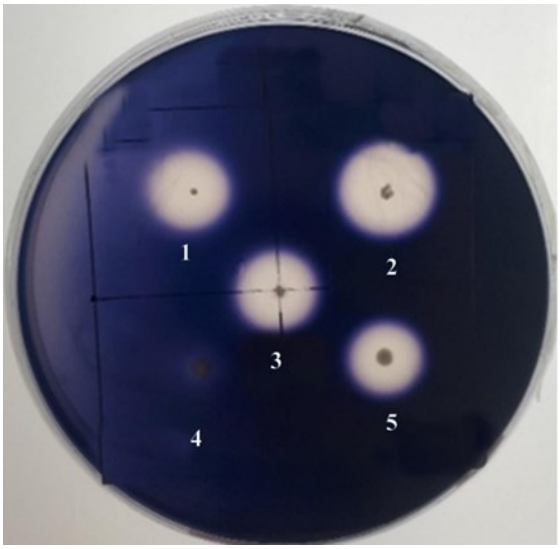


Figure 2. The amylolytic zones produced by bacterial isolates of: (1) T10, (2) D3, (3) A3, (4) TH6, and (5) S1 on a NA medium + 1% soluble starch.

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

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

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

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

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

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

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

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

Based on the ability of the selected isolate 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).

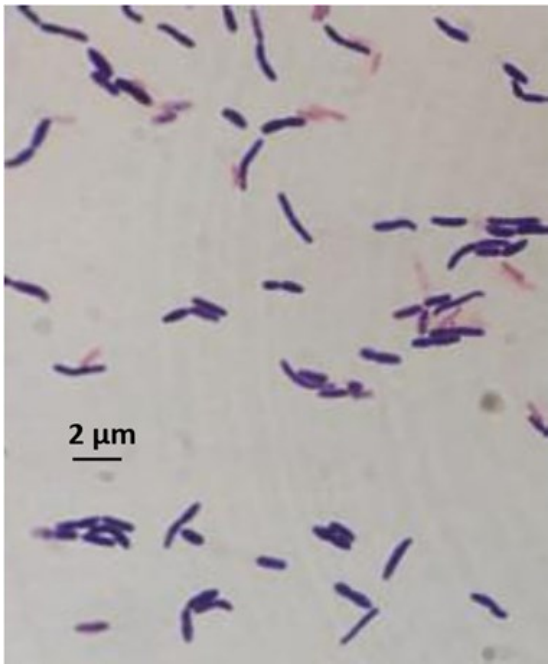


Figure 3. The appearance of bacterial cells isolate T10 under a microscope with a magnification of 1000x. The cells appear single or in chains.

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.mlm.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-7112, and *B. toyonensis* l3aM to form a separate cluster (Figure 5). Hence, isolate T10 was identified as the species member of *B. toyonensis* based on the phenetic and phylogenetic characteristics.

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

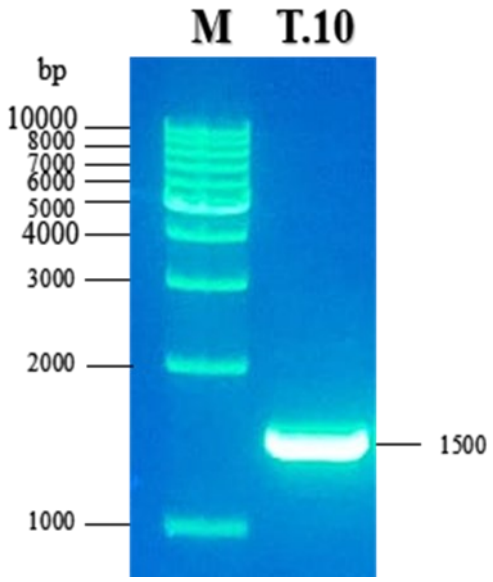


Figure 4. An electropherogram of the amplified 16S rRNA gene of isolate T10 with a size of 1500 bp. Marker (M): 1 kb DNA ladder.

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

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

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

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

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

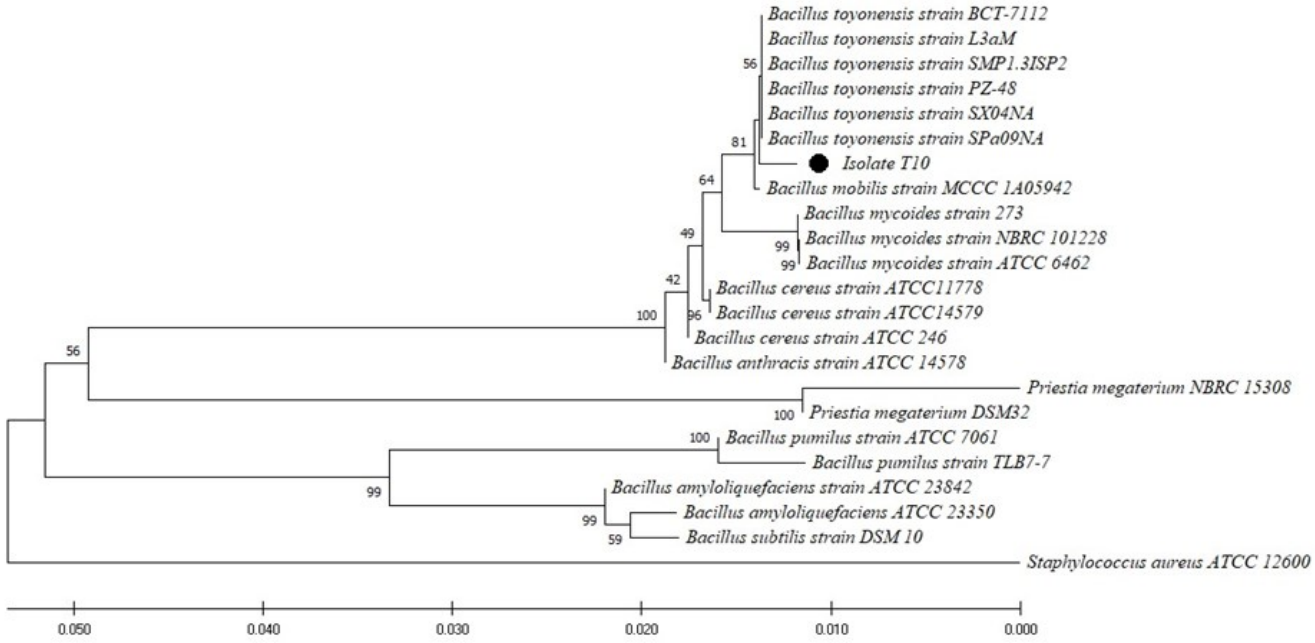


Figure 5. A phylogenetic tree showing the relationship between strain T10 isolated from rhizospheres of Canna (*C. edulis*) and several species members of the genus *Bacillus* on the basis of 16S rRNA gene sequence reconstructed based on Neighbor-Joining, Model Maximum Composite Likelihood, and 1000x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

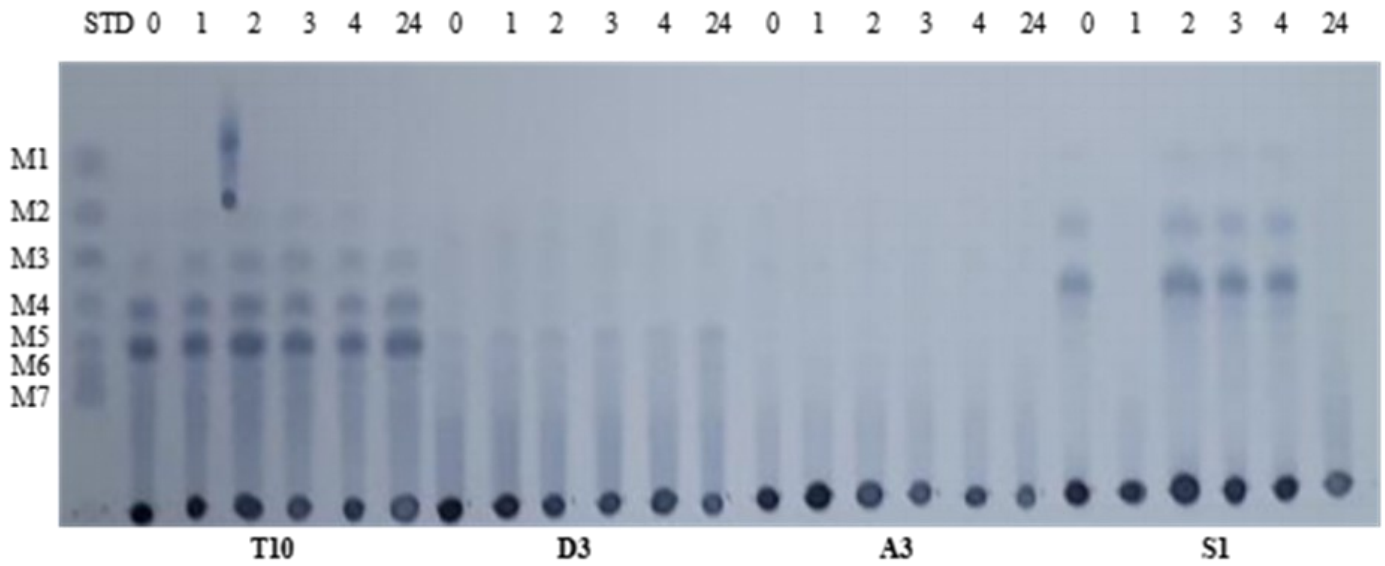


Figure 6. The product profile of starch hydrolyzed by amylase of the amylolytic bacteria (T10, D3, A3, and S1) using a TLC method with reaction times (hours): of 0, 1, 2, 3, 4, and 24 at 30°C. The Standards (STD): monosaccharide (M1), maltose (M2), maltotriose (M3), maltotetraose (M4), maltopentaose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

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

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

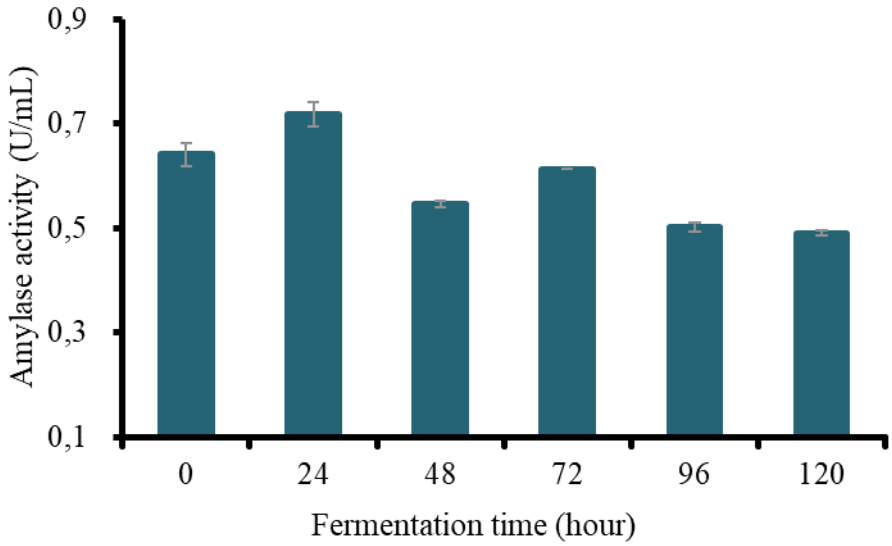


Figure 7. Amylase activity of T10 at different incubation times.

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

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

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

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

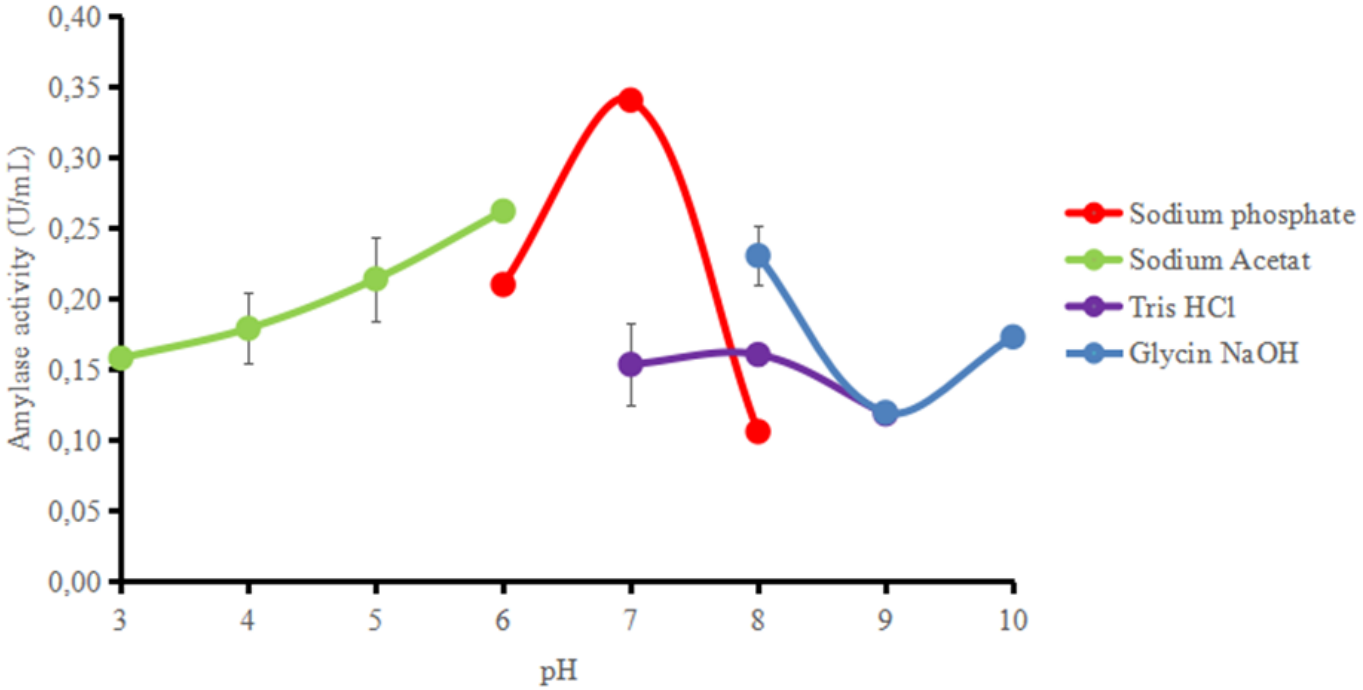


Figure 8. Amylase activity of T10 at different pH and buffers.

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

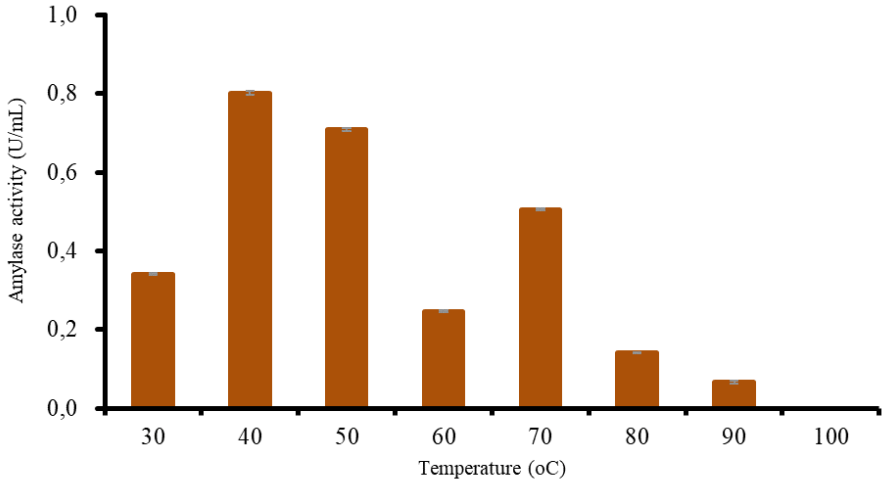


Figure 9. Amylase activity of isolate T10 at different temperatures.

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

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

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

CONCLUSIONS

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

AUTHORS CONTRIBUTION

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

ACKNOWLEDGMENTS

We would like to thank the Research Center for Biotechnology (BRIN) and International Women University (IWU) for funding this research.

CONFLICT OF INTEREST

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

REFERENCES

Abdalla, M. et al., 2021. One-pot production of maltoheptaose (DP7) from starch by sequential addition of cyclodextrin glucotransferase and cyclomaltodextrinase. *Enzyme and Microbial Technology*, 149 (6), 109847. doi: 10.1016/j.enzmictec.2021.109847

Abdul-Manas, N.H. et al., 2014. The characterization of an alkali-stable maltogenic amylase from *Bacillus lehensis* G1 and improved maltooligosaccharide production by hydrolysis suppression. *PLoS ONE* 9(9). doi: 10.1371/journal.pone.0106481

Afifah, N., Putri, D.H. & Irdawati, 2018. Isolation and identification of endophytic bacteria from the Andalas plant stem (*Morus macroura* Miq.). *Bioscience*, 2(1), pp.72-75.

Agamennone, V. et al., 2019. Genome annotation and antimicrobial properties of *B. toyonensis* VU-DES13, isolated from the *Folsomia candida* gut. *Entomologia Experimentalis et Aplicata* 167, pp:269-285. doi: 10.1111/eea.12763.

Asgher, M. et al., 2007. A thermostable α-amylase from moderately thermophilic *Bacillus subtilis* strain for starch processing. *J Food Eng.*, 79, pp.950-955.

Bajpai, B., Chaudhary, M. & Saxena, J., 2015. Production and characterization of α-Amylase from an extremely halophilic archaeon, *Haloferax* sp. HA10. *Food Technol Biotechnol.*, 53(1), pp.11-17, doi: 10.17113/ftb.53.01.15.3824

Ben-Ali, M. et al., 2006. Thermostability enhancement and change in starch hydrolysis profile of the maltohexaose-forming amylase of *Bacillus stearothermophilus* US100 strain. *Biochemical Journal*, 394(1), pp.51–56. doi: 10.1042/BJ20050726

Behal, A. et al., 2016. Characterization of alkaline α- amylase from *Bacillus* sp. AB 04. *IJAB*, 8(1), pp.80-83.

De-Moraes-Russo, C.A. & Selvatti, A.P., 2018. Bootstrap and rogue identification tests for phylogenetic analyses. *Molecular Biology and Evolution*, 35(9), pp.2327–2333. doi: 10.1093/molbev/msy118.

Dey, G. et al., 2002. Purification and characterization of maltooligosaccharide-forming amylase from *Bacillus circulans* GRS 313. *Journal of Industrial Microbiology and Biotechnology*, 28(4), pp.193-200. DOI: 10.1038/sj/jim/7000220.

Ding, N. et al., 2021. Carbohydrate-binding module and linker allow cold adaptation and salt tolerance of maltopentaose-forming amylase from marine bacterium *Saccharophagus degradans* 2-40T. *Frontiers in Microbiology*, 12(7), pp.1–14. doi: 10.3389/fmicb.2021.708480.

Divakaran, D., Chandran, A. & Pratap-Chandran, R., 2011. Comparative study on production of α-amylase from *Bacillus licheniformis* strains. *Brazilian Journal of Microbiology*, 42(4), pp.1397–1404. doi: 10.1590/S1517-83822011000400022.

Duan, Y. et al., 2021. Isolation, identification, and antibacterial mechanisms of *Bacillus amyloliquefaciens* QSB-6 and its effect on plant roots. *Frontiers in Microbiology*, 12:746799. doi:10.3389/fmicb.2021.746799.

El-Fallal, A. et al., 2012. Starch and microbial α-amylases: from concepts to biotechnological applications. In *Carbohydrates-Comprehensive Studies on Glycobiology and Glycotechnology*, Intech Open Science, pp.459-488. doi: 10.5772/51571

Gebreyohannes, G., 2015. Isolation and optimization of amylase producing bacteria and actinomycetes from soil samples of Maraki and Tewedros campus, University of Gondar, North West Ethiopia. *African Journal of Microbiology Research*, 9(31), pp.1877-1882.

Ginting, E.L. et al., 2021. Isolation and identification of thermophilic amylolytic bacteria from Likupang Marine Hydrothermal, North Sulawesi, Indonesia. Biodiversitas, 22(6), pp.3326-3332. doi: 10.13057/biodiv/d220638.

Gupta, R. et al., 2003. Microbial α-amylases: A biotechnological perspective. *Process Biochemistry*, 38(11), pp.1599–1616. doi: 10.1016/S0032-9592(03)00053-0.

Hasanah, U. et al., 2020. Amylolytic activity of bacterial strains isolated from sago pulp of the traditional sago industry in Palopo, South Sulawesi. *AIP Conference Proceedings* 2231, 040073 (2020), https://doi.org/10.1063/5.0002487.

Hellmuth, K. & van-den Brink, J.M., 2013. Microbial production of enzymes used in food applications. In *Microbial Production of Food Ingredients, Enzymes and Nutraceuticals.* Woodhead Publishing Limited. doi: 10.1533/9780857093547.2.262.

Jang, E.Y. et al., 2020. Amylase-producing maltooligosaccharide provides potential relief in rats with loperamide-induced constipation. *Evidence-Based Complementary and Alternative Medicine*. doi: 10.1155/2020/5470268.

Jiménez, G. et al., 2013. Description of *Bacillus toyonensis* sp. nov., a novel species of the *Bacillus cereus* group, and pairwise genome comparisons of the species of the group by means of ANI calculations. *Systematic and Applied Microbiology* 36(6), pp.383-391. doi: 10.1016/j.syapm.2013.04.008.

Lim, S.J. & Oslan, S.N., 2021. Native to designed: Microbial α-Amylases for industrial applications. *PeerJ*, 9, pp.1–30. doi: 10.7717/peerj.11315.

Logan, N.A. & De Vos, P., 2009. Genus I. Bacillus Cohn 1872. In *Bergey's Manual of Systematic Bacteriology Second Edition Volume Three The Firmicutes*. Springer, Springer Dordrecht Heidelberg London New York. pp.21-127. doi: 10.1007/b92997.

Luang-In, V. et al., 2019. Isolation and identification of amylase-producing bacteria from soil in Nasinuan community forest, Maha Sarakham, Thailand. *Biomedical & Pharmacology Journal*, 12(3), pp.1061-1068.

Luo, J-c. et al., 2021. Characterization of a deep sea *Bacillus toyonensis* isolate: genomic and pathogenic features. *Frontiers in Cellular and Infection Microbiology*, 11, 629116. doi: 10.3389/fcimb.2021.629116.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31(3), pp.426–428. doi: 10.1021/ac60147a030

Moradi, M. et al., 2014. Screening and isolation of powerful amylolytic bacterial strains. *International Journal of Current Microbiology and Applied Sciences*, 3(2), pp.758–768.

Naidu, K. et al., 2019. Purification and characterization of α-amylase from *Paenibacillus* sp. D9 and *Escherichia coli* recombinants. *Biocatalysis and Biotransformation*, 38(1), pp.24-34. doi: 10.1080/10242422.2019.1628738.

Nisa, I.K. et al., 2021. The potential of amylase enzyme activity against bacteria isolated from several lakes in East Java, Indonesia. *Biodiversitas*, 22(1), pp.42-49.

Ochoa-Solano, J.L. & Olmos-Soto, J., 2006. The functional property of Bacillus for Shrimp feeds. *Food Microbiology* 23(6), pp.519–525, doi: 10.1016/j.fm.2005.10.004.

Ozturk, H.U. et al., 2014. A *maltooligosaccharides* producing α-amylase from *Bacillus subtilis* SDP1 isolated from rhizosphere of *Acacia cyanophylla* Lindley. *Food Biotechnology*, 28(4), pp.309-332. doi: 10.1080/08905436.2014.963600.

Pan, S. et al., 2017. Maltooligosaccharide-forming amylase: Characteristics, preparation, and application. *Biotechnology Advances*, 35(5), pp.619–632. doi: 10.1016/j.biotechadv.2017.04.004.

Putri, W.D.R. et al., 2012. Isolation and characterization of amylolytic lactic acid bacteria during growol fermentation, an Indonesian traditional food. *Jurnal Teknologi Pertanian*, 13(1), pp.52-60.

Rahmani, N. et al., 2013. Production of maltooligosaccharides from black potato (*Coleus tuberosusi)* starch by α-amylase from a marine bacterium (*Brevibacterium* sp.). *Microbiology Indonesia*, 7(3), pp.129-136. doi: 10.5454/mi.7.3.6.

Rahmani, N. et al., 2018. Xylanase and feruloyl esterase from actinomycetes cultures could enhance sugarcane bagasse hydrolysis in the production of fermentable sugars. *Bioscience Biotechnology and Biochemistry*, 82(5), pp.904–915. doi: 10.1080/09168451.2018.1438169.

Santos, F.D.S. et al., 2018. *Bacillus toyonensis* improves immune response in the mice vaccinated with recombinant antigen of bovine herpesvirus type 5. *Benef. Microbes*, 9(1), pp.133–142. doi: 10.3920/BM2017.0021.

Sivaramakrishnan, S. et al., 2006. α-Amylase from microbial sources: An overview on recent developments. *Food Technol. Biotechnol.*, 44 (2), pp.173–184.

Smibert, R.M. & Krieg, N.R., 1981. General Characterization. In *Manual Methods for General Bacteriology*. American Society for Microbiology, Washington.

Subagiyo, Djarod, M.S.R. & Setyati, W.A., 2017. Potensi ekosistem mangrove sebagai sumber bakteri untuk produksi protease, amilase, dan selulase. *Jurnal Kelautan Tropis*, 20(2), pp.106–111.

Vaseekaran, S., Balakumar, S. & Arasaratnam, V., 2010. Isolation and identification of a bacterial strain producing thermostable α- amylase. *Tropical Agricultural Research*, 22 (1), pp.1-11. doi: 10.4038/tar.v22i1.2603.

Vijayalakshmi, et al., 2012. Isolation and characterization of *Bacillus subtilis* KC3 for amylolytic activity. *International Journal of Bioscience, Biochemistry and Bioinformatics*, 2(5), pp.336-341. doi: 10.7763/IJBBB.2012.V2.128.

Wang, J. et al., 2021. Toyoncin, a novel leaderless bacteriocin that is produced by *Bacillus toyonensis* XIN-YC13 and specifically targets *B. cereus* and *Listeria monocytogenes*. *Applied and Environmental Microbiology*, 87(12), e00185-21. doi: 10.1128/AEM.00185-21.

Yopi, et al., 2017. Isolation and characterization of mannanase, xylanase, and cellulase from marine bacteria *Bacillus* sp. *Biofarmasi Journal of Natural Product Biochemistry*, 15(1), pp.15–20. doi: 10.13057/biofar/f150103.

Yufinta, C.P., Julyantoro, P.G.S. & Pratiwi, M.A., 2018. Pengaruh penambahan *Bacillus* sp. terhadap kelulushidupan pasca larva udang Vannamei (*Litopenaeus vannamei*) yang terinfeksi vibriosis. *Current Trends in Aquatic Science*, 1(1), pp.89-95.

Zhao, C. et al., 2017. Functional properties, structural studies and chemo -enzymatic synthesis of oligosaccharides. *Trends in Food Science and Technology*, 66, pp.135–145. doi: 10.1016/j.tifs.2017.06.008.

Zhuang, Y., Zhou, X. & Wang, S., 2012. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Plant Systematics and Evolution*, 298(7), pp.3389–3402. doi: 10.2503/jjshs.58.977.

Zubaidah, A. et al., 2019. Screening bakteri selulolitik dan amilolitik pada rumen sapi sebagai kandidat probiotik pada budidaya ikan secara *in vitro*. *Jurnal Riset Akuakultur*, 14(4), pp.261-271.

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

Rina Dwi Agustiani[1,3], Oedjijono[1]*, Nanik Rahmani[2], Nuraeni Ekowati[1]

[1]Faculty of Biology, University of Jenderal Soedirman, Jalan dr. Soeparno 63 Purwokerto 53122, Indonesia.

[2]Center for Applied Microbiology Research, Research Institute for Life and Environmental Sciences, National Research and Innovation Agency (BRIN), Cibinong, Bogor 16911, Indonesia.

[3]Department of Biology, Faculty of Science and Technology, International Women University, Bandung 40173, Indonesia.

* Corresponding author, Hp: +62 811261256, Email: oedjijono@unsoed.ac.id

Abstract

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

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

Keywords:
Amylolytic bacteria, Canna, Maltooligosaccharides, 16S rDNA gene.

1. 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.

2. Materials and methods

2.1. 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).

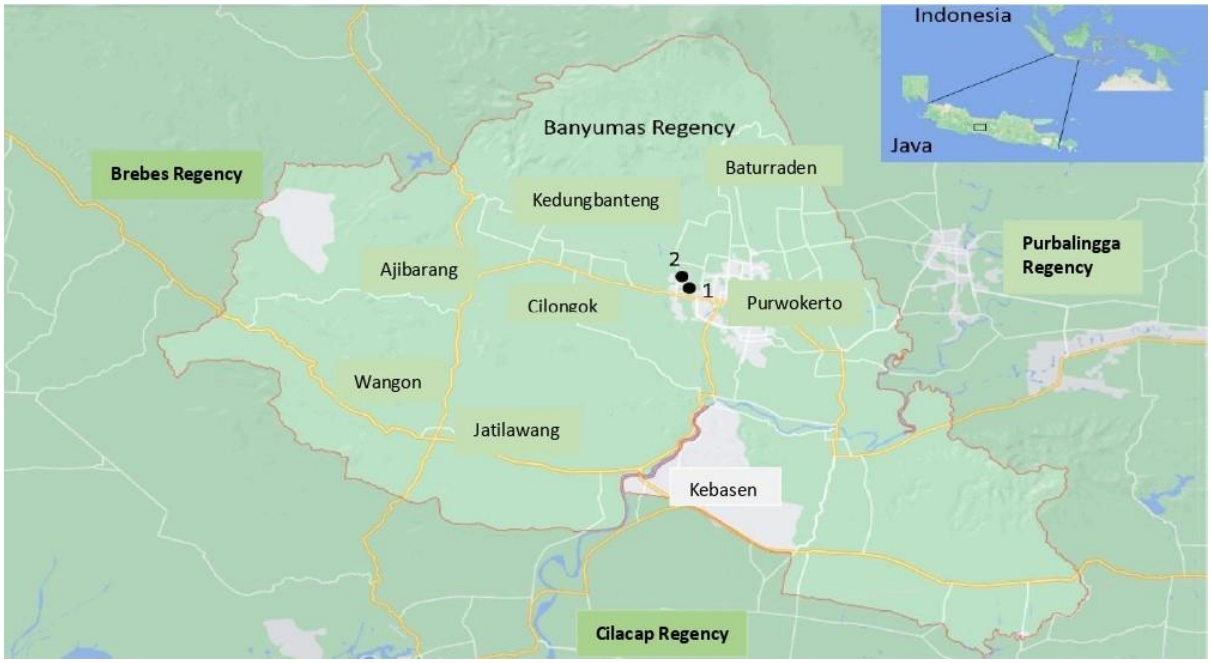


Figure 1. A map of Banyumas Regency and sampling sites: (1) the Perhutani Forest KPH West Banyumas, Central Java, (2) Community gardens around the Perhutani Forest KPH West Banyumas, Central Java.

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 µL 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

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

2.3. Phenetic and phylogenetic characterizations of the selected bacterium

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

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

2.4 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.

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 $60F_{254}$ 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 µL). 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 $$= \frac{\text{c x d x 1000}}{\text{t x mw}} \text{ U/mL}$$
165 c: amylase concentration; d: dilution; t: incubation time; mw: molecular weight.

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

2.6. 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 sodium 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).

3. Results and Discussion

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

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

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

clear zones are produced due to the degradation of amylum in the medium, resulting in enhancing the amylolytic index (Ginting et al. 2021). The research results showed that eight isolates of TH6, TH7, T5, T10, D2, D3, A3, and S1 showed high total clear zone diameters (mm) of 18, 18, 18, 20, 18, 30, 18, and 18, respectively (Table 1). The consistency of the bacterial isolates, resulting in the total clear zone diameter, was shown by the isolates TH6, T10, D3, A3, and S1, while the other isolates tended to reduce or lose their amylolytic activity (Figure 2). Based on the ability of isolates to produce a clear zone diameter $\geq$ 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 amylolytic index of more than 9 mm were potentials to produce amylase. According to Ochoa-Solano & Olmos–Soto (2006), bacterial isolates produce clear zones two or three times the diameter of the colony are potential enzyme producers.

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

206

207

208 **Figure 2.** The amylolytic 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.

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 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.

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	+	+	+	+	+

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).

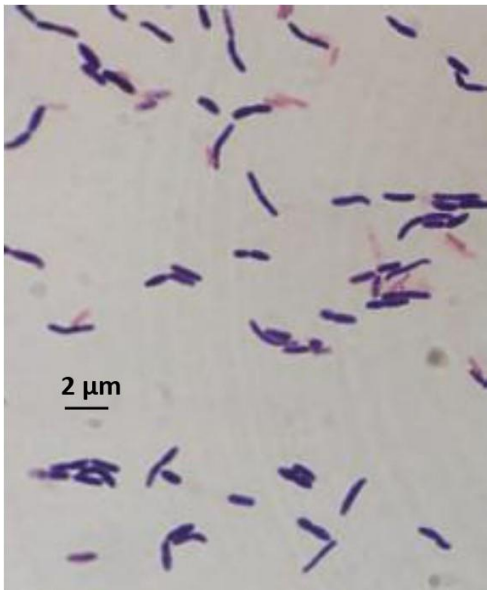


Figure 3. The appearance of bacterial cells isolate T10 under a microscope with a magnification of 1000x. The cells appear single or in chains.

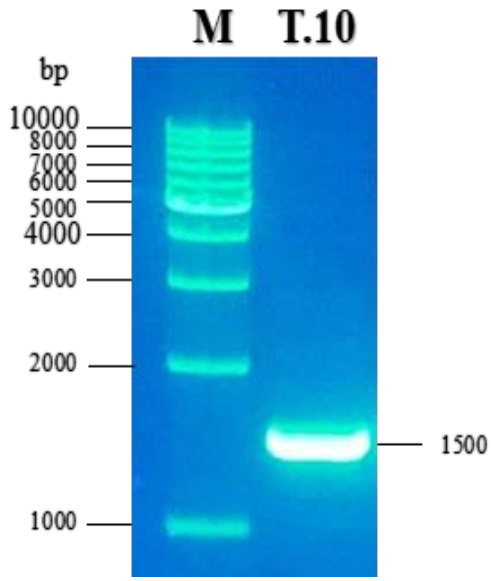
Table 3. Phenotypic characterization of the isolate T10

Characteristics	Isolate T10
Cell length (µm)	3.00 - 4.00
Egg-yolk lecithinase	+
Anaerobic growth	+
Rhizoid colony	-
Parasporal crystal	-
Growth temperature range (°C)	10 - 45
Optimal growth temperature (°C)	35
Salinity tolerance range (%NaCl)	≤ 4
API 50CHB	
Glycerol	-
D-Ribose	+

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

The electrophoresis visualization of the PCR product showed that the DNA of T10 produced a single band with a size of 1500 kb (Figure 4). The results of comparing the 16S rRNA gene sequence of isolate T10 and nucleotide sequences in the GeneBank (http://blast.ncbi.mlm.nih.gov/) showed that the bacterium is closely related to species members of the genus *Bacillus.* The BLAST analysis showed that 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-7112, and *B. toyonensis* l3aM to form a separate cluster (Figure 5). Hence, isolate T10 was identified as the species member of *B. toyonensis* based on the phenetic and phylogenetic characteristics.

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.
275



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

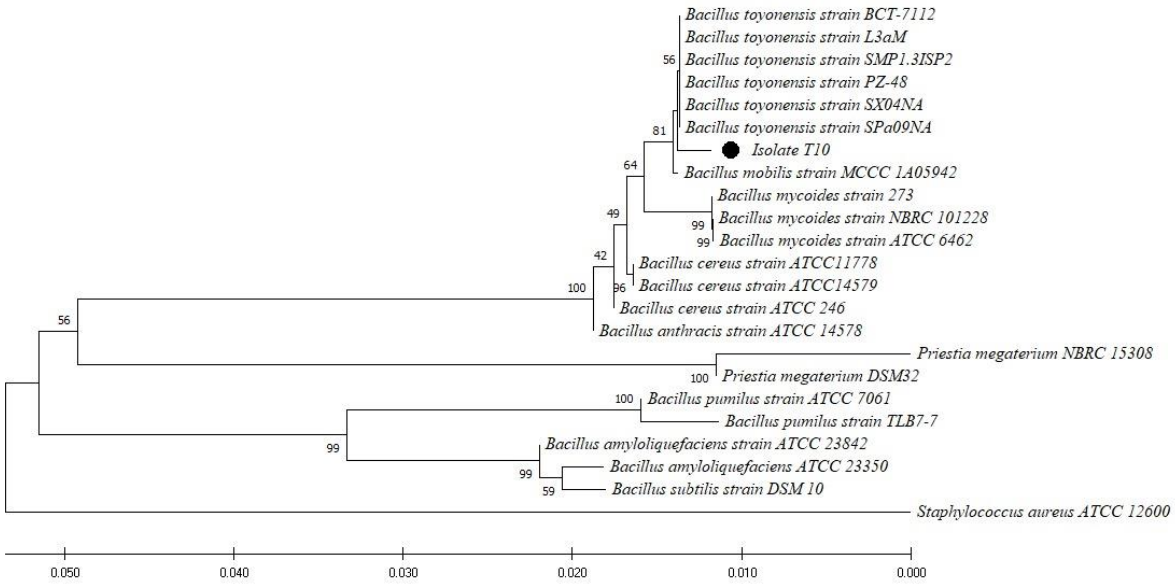


Figure 5. A phylogenetic tree showing the relationship between strain T10 isolated from rhizospheres of Canna (*C. edulis*) and several species members of the genus ***Bacillus*** on the basis of 16S rRNA gene sequence reconstructed based on Neighbor-Joining, Model Maximum Composite Likelihood, and 1000x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

3.3. 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).

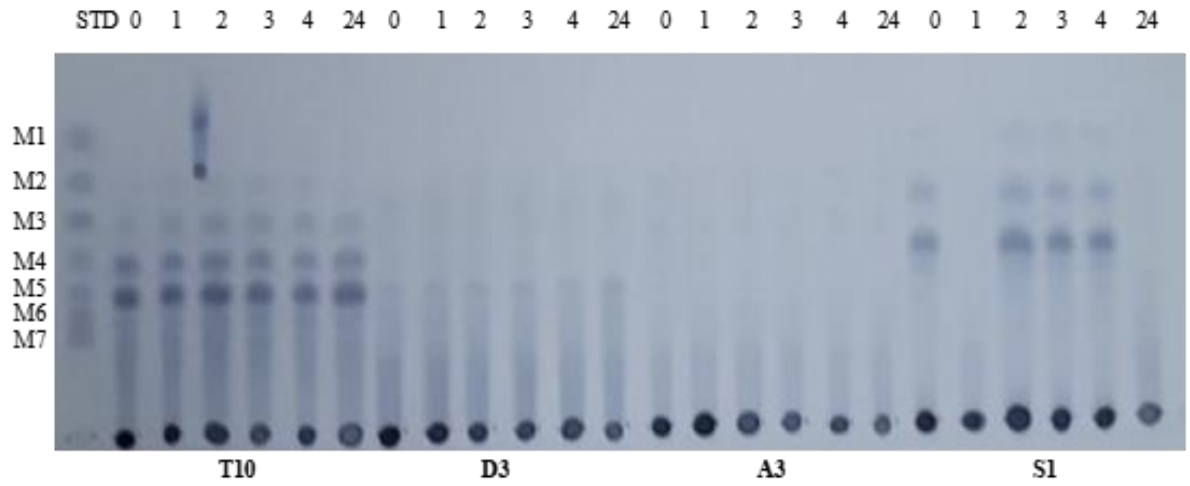


Figure 6. The product profile of starch hydrolyzed by amylase of the amylolytic bacteria (T10, D3, A3, and S1) using a TLC method with reaction times (hours): of 0, 1, 2, 3, 4, and 24 at 30°C. The Standards (STD): monosaccharide (M1), maltose (M2), maltotriose (M3), maltotetraose (M4), maltopentaose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

Based on the TLC chromatogram, the starch degraded by T10 amylase resulted in malto-oligosaccharides of maltotriose, maltotetraose, and maltopentaose. Amylases can break down starch polymer bonds into shorter oligosaccharides or simple sugar molecules (Putri et al. 2012). The results showed that amylolytic bacteria with high amylolytic indexes (AI) did not correlate with their ability to degrade amylum. The isolate T10, with its total diameter lower than isolate D3, showed a higher ability to break down starch polymer bonds into shorter or oligosaccharides. The results of this study proved that a high AI value is only sometimes accompanied by the 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.

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

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

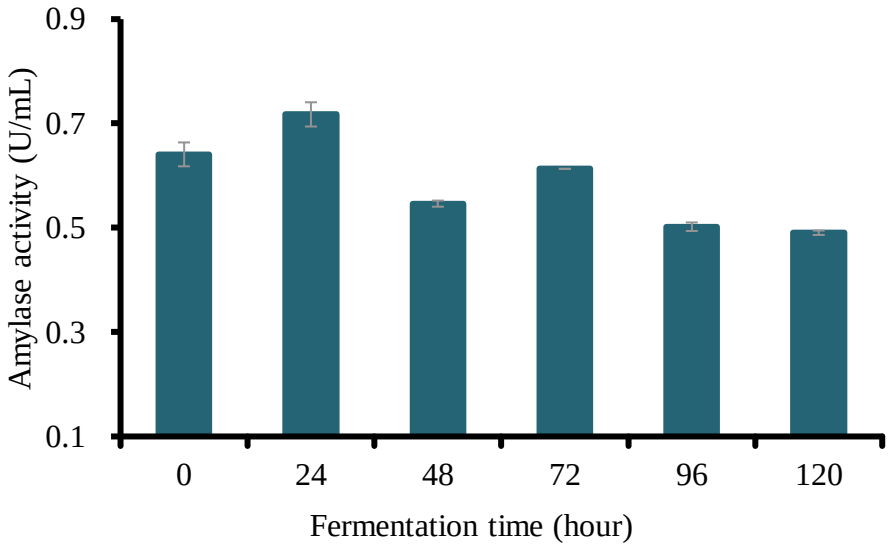


Figure 7. Amylase activity of T10 at different incubation times.

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.

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 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).

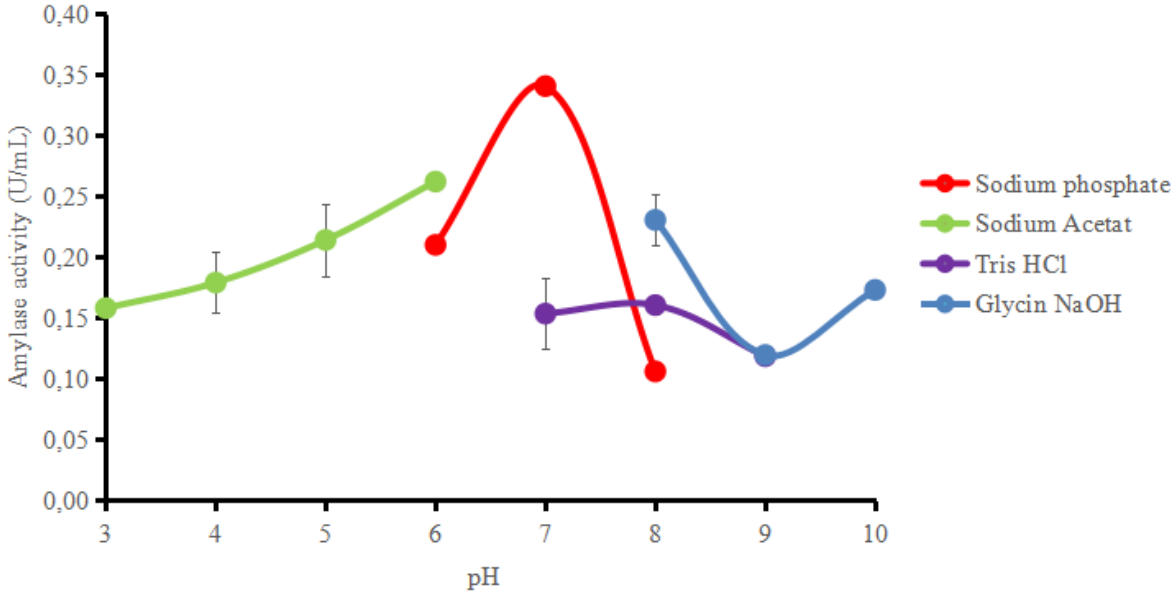


Figure 8. Amylase activity of T10 at different pH and buffers.

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.*

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.

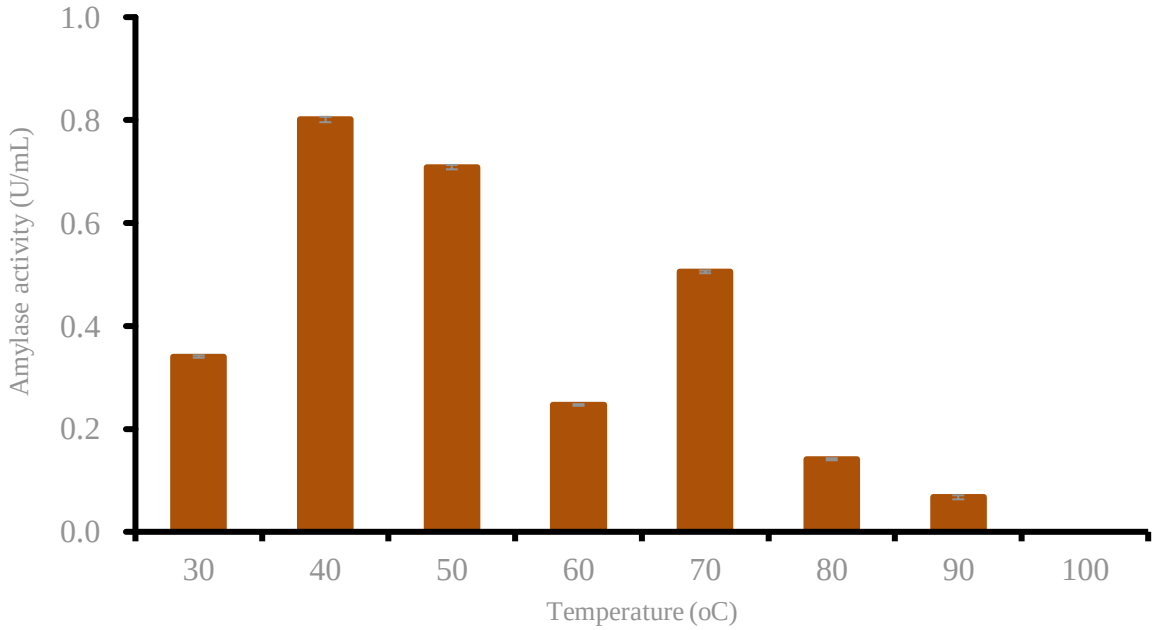


Figure 9. Amylase activity of isolate T10 at different temperatures.

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 decreased. 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).

4. 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.

Acknowledgments

We would like to thank the Research Center for Biotechnology (BRIN) and International Women University (IWU) for funding this research.

Conflict of interest

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

References

Abdalla, M. et al., 2021. One-pot production of maltoheptaose (DP7) from starch by sequential addition of cyclodextrin glucotransferase and cyclomaltodextrinase. *Enzyme and Microbial Technology*, 149 (6), 109847. doi: 10.1016/j.enzmictec.2021.109847

Abdul-Manas, N.H. et al., 2014. The characterization of an alkali-stable maltogenic amylase from *Bacillus lehensis* G1 and improved maltooligosaccharide production by hydrolysis suppression. *PLoS ONE* 9(9). doi: 10.1371/journal.pone.0106481

Afifah, N., Putri, D.H. & Irdawati, 2018. Isolation and identification of endophytic bacteria from the Andalas plant stem (*Morus macroura* Miq.). *Bioscience*, 2(1), pp.72-75.

Agamennone, V. et al., 2019. Genome annotation and antimicrobial properties of *B. toyonensis* VU-DES13, isolated from the *Folsomia candida* gut. *Entomologia Experimentalis et Aplicata* 167, pp:269-285. doi: 10.1111/eea.12763.

Asgher, M. et al., 2007. A thermostable α-amylase from moderately thermophilic *Bacillus subtilis* strain for starch processing. *J Food Eng.*, 79, pp.950-955.

Bajpai, B., Chaudhary, M. & Saxena, J., 2015. Production and characterization of α-Amylase from an extremely halophilic archaeon, *Haloferax* sp. HA10. *Food Technol Biotechnol.*, 53(1), pp.11-17, doi: 10.17113/ftb.53.01.15.3824

435 Ben-Ali, M. et al., 2006. Thermostability enhancement and change in starch hydrolysis
436 profile of the maltohexaose-forming amylase of *Bacillus stearothermophilus* US100
437 strain. *Biochemical Journal*, 394(1), pp.51–56. doi: 10.1042/BJ20050726
438 Behal, A. et al., 2016. Characterization of alkaline α- amylase from *Bacillus* sp. AB 04.
439 *IJAB*, 8(1), pp.80-83.
440 De-Moraes-Russo, C.A. & Selvatti, A.P., 2018. Bootstrap and rogue identification tests for
441 phylogenetic analyses. *Molecular Biology and Evolution*, 35(9), pp.2327–2333. doi:
442 10.1093/molbev/msy118.
443 Dey, G. et al., 2002. Purification and characterization of maltooligosaccharide-forming
444 amylase from *Bacillus circulans* GRS 313. *Journal of Industrial Microbiology and*
445 *Biotechnology*, 28(4), pp.193-200. DOI: 10.1038/sj/jim/7000220.
446 Ding, N. et al., 2021. Carbohydrate-binding module and linker allow cold adaptation and salt
447 tolerance of maltopentaose-forming amylase from marine bacterium
448 *Saccharophagus degradans* 2-40T. *Frontiers in Microbiology*, 12(7), pp.1–14. doi:
449 10.3389/fmicb.2021.708480.
450 Divakaran, D., Chandran, A. & Pratap-Chandran, R., 2011. Comparative study on production
451 of α-amylase from *Bacillus licheniformis* strains. *Brazilian Journal of*
452 *Microbiology*, 42(4), pp.1397–1404. doi: 10.1590/S1517-83822011000400022.
453 Duan, Y. et al., 2021. Isolation, identification, and antibacterial mechanisms of *Bacillus*
454 *amyloliquefaciens* QSB-6 and its effect on plant roots. *Frontiers in Microbiology*,
455 12:746799. doi:10.3389/fmicb.2021.746799.
456 El-Fallal, A. et al., 2012. Starch and microbial α-amylases: from concepts to biotechnological
457 applications. In *Carbohydrates-Comprehensive Studies on Glycobiology and*
458 *Glycotechnology*, Intech Open Science, pp.459-488. doi: 10.5772/51571

459 Gebreyohannes, G., 2015. Isolation and optimization of amylase producing bacteria and
460 actinomycetes from soil samples of Maraki and Tewedros campus, University of
461 Gondar, North West Ethiopia. *African Journal of Microbiology Research,* 9(31),
462 pp.1877-1882.
463 Ginting, E.L. et al., 2021. Isolation and identification of thermophilic amylolytic bacteria
464 from Likupang Marine Hydrothermal, North Sulawesi, Indonesia. Biodiversitas,
465 22(6), pp.3326-3332. doi: 10.13057/biodiv/d220638.
466 Gupta, R. et al., 2003. Microbial α-amylases: A biotechnological perspective. *Process*
467 *Biochemistry*, 38(11), pp.1599–1616. doi: 10.1016/S0032-9592(03)00053-0.
468 Hasanah, U. et al., 2020. Amylolytic activity of bacterial strains isolated from sago pulp of
469 the traditional sago industry in Palopo, South Sulawesi. *AIP Conference*
470 *Proceedings* 2231, 040073 (2020), https://doi.org/10.1063/5.0002487.
471 Hellmuth, K. & van-den Brink, J.M., 2013. Microbial production of enzymes used in food
472 applications. In *Microbial Production of Food Ingredients, Enzymes and*
473 *Nutraceuticals*. Woodhead Publishing Limited. doi:
474 10.1533/9780857093547.2.262.
475 Jang, E.Y. et al., 2020. Amylase-producing maltooligosaccharide provides potential relief in
476 rats with loperamide-induced constipation. *Evidence-Based Complementary and*
477 *Alternative Medicine*. doi: 10.1155/2020/5470268.
478 Jiménez, G. et al., 2013. Description of *Bacillus toyonensis* sp. nov., a novel species of
479 the *Bacillus cereus* group, and pairwise genome comparisons of the species of the
480 group by means of ANI calculations. *Systematic and Applied Microbiology* 36(6),
481 pp.383-391. doi: 10.1016/j.syapm.2013.04.008.
482 Lim, S.J. & Oslan, S.N., 2021. Native to designed: Microbial α-Amylases for industrial
483 applications. *PeerJ*, 9, pp.1–30. doi: 10.7717/peerj.11315.

Logan, N.A. & De Vos, P., 2009. Genus I. Bacillus Cohn 1872. In *Bergey's Manual of Systematic Bacteriology Second Edition Volume Three The Firmicutes*. Springer, Springer Dordrecht Heidelberg London New York. pp.21-127. doi: 10.1007/b92997.

Luang-In, V. et al., 2019. Isolation and identification of amylase-producing bacteria from soil in Nasinuan community forest, Maha Sarakham, Thailand. *Biomedical & Pharmacology Journal*, 12(3), pp.1061-1068.

Luo, J-c. et al., 2021. Characterization of a deep sea *Bacillus toyonensis* isolate: genomic and pathogenic features. *Frontiers in Cellular and Infection Microbiology*, 11, 629116. doi: 10.3389/fcimb.2021.629116.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31(3), pp.426–428. doi: 10.1021/ac60147a030

Moradi, M. et al., 2014. Screening and isolation of powerful amylolytic bacterial strains. *International Journal of Current Microbiology and Applied Sciences*, 3(2), pp.758–768.

Naidu, K. et al., 2019. Purification and characterization of α-amylase from *Paenibacillus* sp. D9 and *Escherichia coli* recombinants. *Biocatalysis and Biotransformation*, 38(1), pp.24-34. doi: 10.1080/10242422.2019.1628738.

Nisa, I.K. et al., 2021. The potential of amylase enzyme activity against bacteria isolated from several lakes in East Java, Indonesia. *Biodiversitas*, 22(1), pp.42-49.

Ochoa-Solano, J.L. & Olmos-Soto, J., 2006. The functional property of Bacillus for Shrimp feeds. *Food Microbiology* 23(6), pp.519–525, doi: 10.1016/j.fm.2005.10.004.

Ozturk, H.U. et al., 2014. A *maltooligosaccharides* producing α-amylase from *Bacillus subtilis* SDP1 isolated from rhizosphere of *Acacia cyanophylla* Lindley. *Food Biotechnology*, 28(4), pp.309-332. doi: 10.1080/08905436.2014.963600.

509 Pan, S. et al., 2017. Maltooligosaccharide-forming amylase: Characteristics, preparation, and
510 application. *Biotechnology Advances,* 35(5), pp.619–632. doi:
511 10.1016/j.biotechadv.2017.04.004.
512 Putri, W.D.R. et al., 2012. Isolation and characterization of amylolytic lactic acid bacteria
513 during growol fermentation, an Indonesian traditional food. *Jurnal Teknologi*
514 *Pertanian*, 13(1), pp.52-60.
515 Rahmani, N. et al., 2013. Production of maltooligosaccharides from black potato (*Coleus*
516 *tuberosusi)* starch by α-amylase from a marine bacterium (*Brevibacterium* sp.).
517 *Microbiology Indonesia*, 7(3), pp.129-136. doi: 10.5454/mi.7.3.6.
518 Rahmani, N. et al., 2018. Xylanase and feruloyl esterase from actinomycetes cultures could
519 enhance sugarcane bagasse hydrolysis in the production of fermentable sugars.
520 *Bioscience Biotechnology and Biochemistry,* 82(5), pp.904–915. doi:
521 10.1080/09168451.2018.1438169.
522 Santos, F.D.S. et al., 2018. *Bacillus toyonensis* improves immune response in the mice
523 vaccinated with recombinant antigen of bovine herpesvirus type 5. *Benef. Microbes*,
524 9(1), pp.133–142. doi: 10.3920/BM2017.0021.
525 Sivaramakrishnan, S. et al., 2006. α-Amylase from microbial sources: An overview on recent
526 developments. *Food Technol. Biotechnol.*, 44 (2), pp.173–184.
527 Smibert, R.M. & Krieg, N.R., 1981. General Characterization. In *Manual Methods for*
528 *General Bacteriology*. American Society for Microbiology, Washington.
529 Subagiyo, Djarod, M.S.R. & Setyati, W.A., 2017. Potensi ekosistem mangrove sebagai
530 sumber bakteri untuk produksi protease, amilase, dan selulase. *Jurnal Kelautan*
531 *Tropis*, 20(2), pp.106–111.

532 Vaseekaran, S., Balakumar, S. & Arasaratnam, V., 2010. Isolation and identification of a
533 bacterial strain producing thermostable α- amylase. *Tropical Agricultural Research,*
534 22 (1), pp.1-11. doi: 10.4038/tar.v22i1.2603.
535 Vijayalakshmi, et al., 2012. Isolation and characterization of *Bacillus subtilis* KC3 for
536 amylolytic activity. *International Journal of Bioscience, Biochemistry and*
537 *Bioinformatics,* 2(5), pp.336-341. doi: 10.7763/IJBBB.2012.V2.128.
538 Wang, J. et al., 2021. Toyoncin, a novel leaderless bacteriocin that is produced by *Bacillus*
539 *toyonensis* XIN-YC13 and specifically targets *B. cereus* and *Listeria monocytogenes.*
540 *Applied and Environmental Microbiology,* 87(12), e00185-21. doi:
541 10.1128/AEM.00185-21.
542 Yopi, et al., 2017. Isolation and characterization of mannanase, xylanase, and cellulase from
543 marine bacteria *Bacillus* sp. *Biofarmasi Journal of Natural Product Biochemistry,*
544 15(1), pp.15–20. doi: 10.13057/biofar/f150103.
545 Yufinta, C.P., Julyantoro, P.G.S. & Pratiwi, M.A., 2018. Pengaruh penambahan *Bacillus* sp.
546 terhadap kelulushidupan pasca larva udang Vannamei (*Litopenaeus vannamei*) yang
547 terinfeksi vibriosis. *Current Trends in Aquatic Science,* 1(1), pp.89-95.
548 Zhao, C. et al., 2017. Functional properties, structural studies and chemo-enzymatic synthesis
549 of oligosaccharides. *Trends in Food Science and Technology,* 66, pp.135–145. doi:
550 10.1016/j.tifs.2017.06.008.
551 Zhuang, Y., Zhou, X. & Wang, S., 2012. Gapped BLAST and PSI-BLAST: a new generation
552 of protein database search programs. *Plant Systematics and Evolution,* 298(7),
553 pp.3389–3402. doi: 10.2503/jjshs.58.977.
554 Zubaidah, A. et al., 2019. Screening bakteri selulolitik dan amilolitik pada rumen sapi sebagai
555 kandidat probiotik pada budidaya ikan secara *in vitro. Jurnal Riset Akuakultur,*
556 14(4), pp.261-271.

Copyright Transfer Agreement

Manuscript title:

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

Author(s):

Rina Dwi Agustiani, Oedjijono, Nanik Rahmani, Nuraeni Ekowati

The author(s) hereby agree to transfer the copyright of the entitled manuscript to the Journal of Tropical Biodiversity and Biotechnology upon publication. The copyright will be held by the journal under Creative Commons Attribution (**CC-BY-SA**).

JTBB requires the Corresponding Author(s) to sign a copyright transfer agreement on behalf of all the authors.

Date: 14-3-2023 **Name:** Oedjijono **Signature:**

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

Rina Dwi Agustiani[1,3], Oedjijono[1]*, Nanik Rahmani[2], Nuraeni Ekowati[1]

[1]Faculty of Biology, University of Jenderal Soedirman, Jalan dr. Soeparno 63 Purwokerto 53122, Indonesia.

[2]Center for Applied Microbiology Research, Research Institute for Life and Environmental Sciences, National Research and Innovation Agency (BRIN), Cibinong, Bogor 16911, Indonesia.

[3]Department of Biology, Faculty of Science and Technology, International Women University, Bandung 40173, Indonesia.

* Corresponding author, Hp: +62 811261256, Email: oedjijono@unsoed.ac.id

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 7 in an amount of 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%.

Keywords:
Amylolytic bacteria, Canna, Maltooligosaccharides, 16S rDNA gene.

1. 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 hydrolised 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 the production of 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 habitats 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 protein 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 be able 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 hydrolised by the bacterial enzymes, and to identify the selected amylolytic bacteria based on 16S rRNA gene sequences.

2. Materials and methods

2.1. 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).

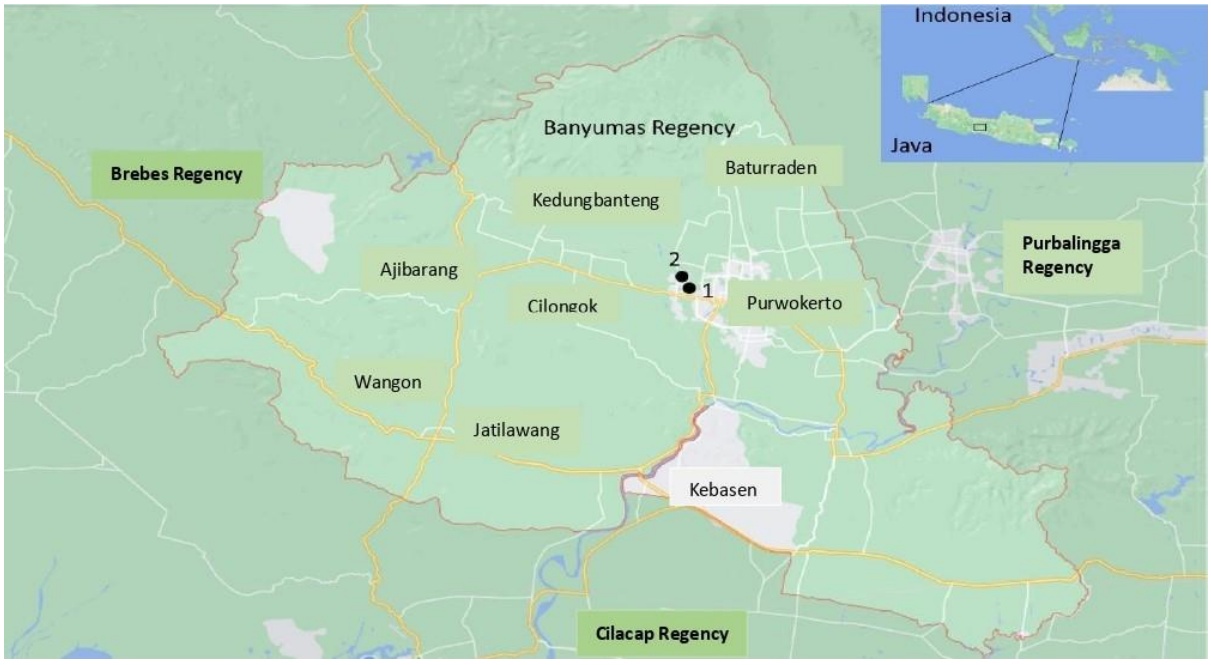


Figure 1. A map of Banyumas Regency and sampling sites: (1) the Perhutani Forest KPH West Banyumas, Central Java, (2) Community gardens around the Perhutani Forest KPH West Banyumas, Central Java.

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 colonies 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 µL 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

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

2.3. Phenetic and phylogenetic characterizations of the selected bacterium

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

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

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

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

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

2.5. 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 sampling every 24 hour and then centrifuge and the supernatant obtained was tested for its amylase activity.

The enzyme reaction was conducted as above when measuring of 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:

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

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

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

2.6. 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 sodium 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).

3. Results and Discussion

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

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

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

clear zones are produced due to the degradation of amylum in the medium, resulting in enhancing the amylolytic index (Ginting et al. 2021). The research results showed that eight isolates of TH6, TH7, T5, T10, D2, D3, A3, and S1 showed high total clear zone diameters (mm) of 18, 18, 18, 20, 18, 30, 18, and 18, respectively (Table 1). The consistency of the bacterial isolates, resulting in the total clear zone diameter, was shown by the isolates TH6, T10, D3, A3, and S1, while the other isolates tended to reduce or lose their amylolytic activity (Figure 2). Based on the ability of isolates to produce a clear zone diameter $\geq$ 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 amylolytic index of more than 9 mm were potentials for producing 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.

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

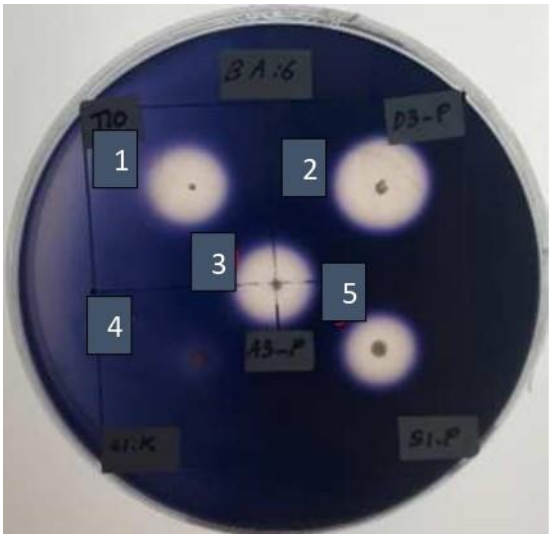


Figure 2. The amylolytic zones produced by bacterial isolates of: (1) T10, (2) D3, (3) A3, (4) TH6, and (5) S1 on a NA medium + 1% soluble starch.

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 of 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 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.

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	+	+	+	+	+

3.2. 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, indicated 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).

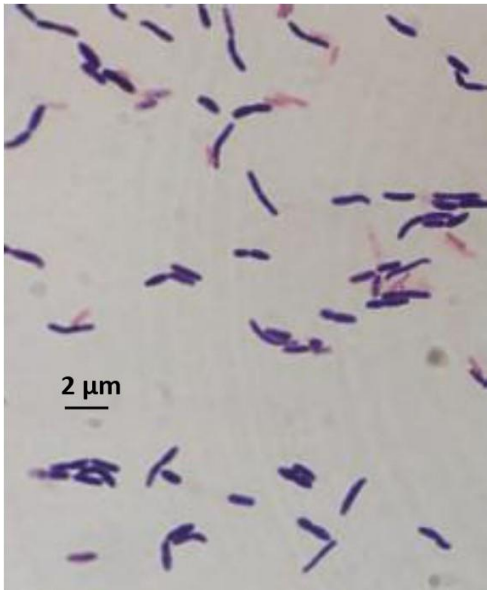


Figure 3. The appearance of bacterial cells isolate T10 under a microscope with a magnification of 1000x. The cells appear single or in chains.

Table 3. Phenotypic characterization of the isolate T10

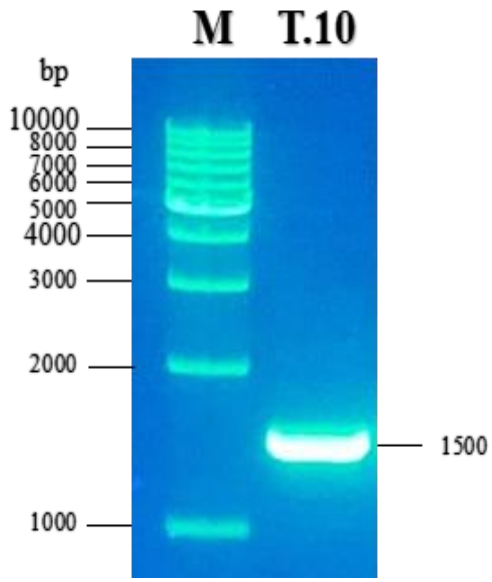
Characteristics	Isolate T10
Cell length (µm)	3.00 - 4.00
Egg-yolk lecithinase	+

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 the comparison between the 16S rRNA gene sequence of isolate T10 and nucleotide sequences in the GeneBank (http://blast.ncbi.mlm.nih.gov/) showed that the bacterium is closely related to species

members of the genus *Bacillus*. The BLAST analysis results 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 constructed using Neighbor-Joining, Model Maximum Composite Likelihood, and 1000x Bootstrapping. A dendrogram resulted from MEGA10 program showed that isolate T10 joined *B. toyonensis* SX04NA, *B. toyonensis* Spa09NA, *B. toyonensis* SMP1, *B. toyonensis* PZ-48, *B. toyonensis* BCT-7112, and *B. toyonensis* l3aM to form a separate cluster (Figure 5). Hence, isolate T10 was identified as the species member of *B. toyonensis* based on the phenetic and phylogenetic characteristics.

B. toyonensis strain BCT-7112^{T} was first isolated in 1966 in Japan from a survey designed to obtain naturally occurring microorganisms as pure cultures in the laboratory for use as probiotics in animal nutrition (Jiménez et al. 2013). This strain was first identified as *B. cereus* var. *toyoi*, and it has been used as the active ingredient of the preparation TOYOCERIN, is 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.

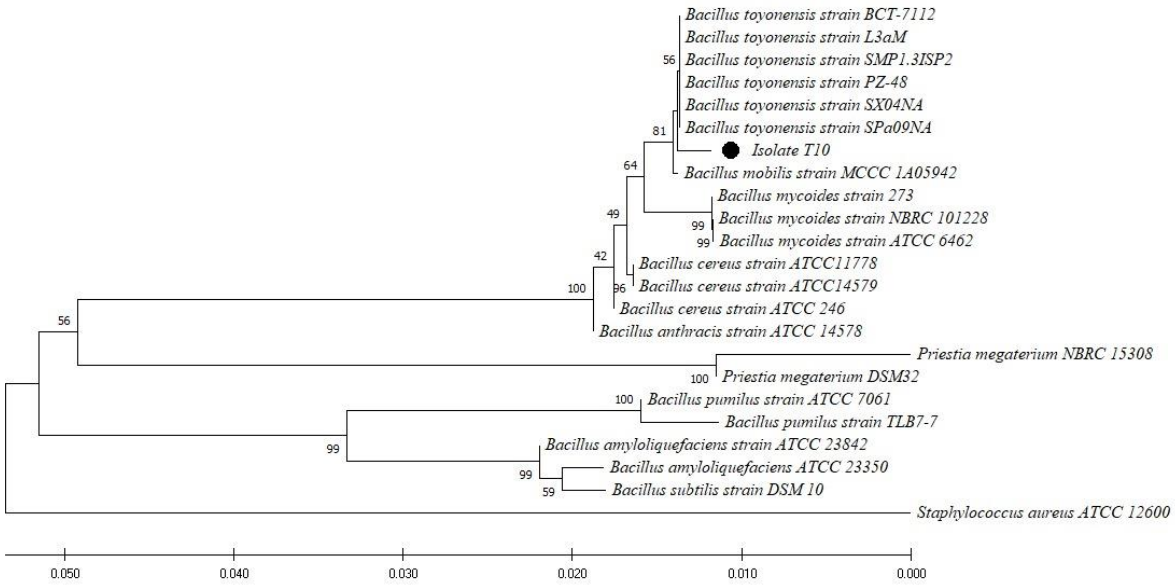


276

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

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

3.3. 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).

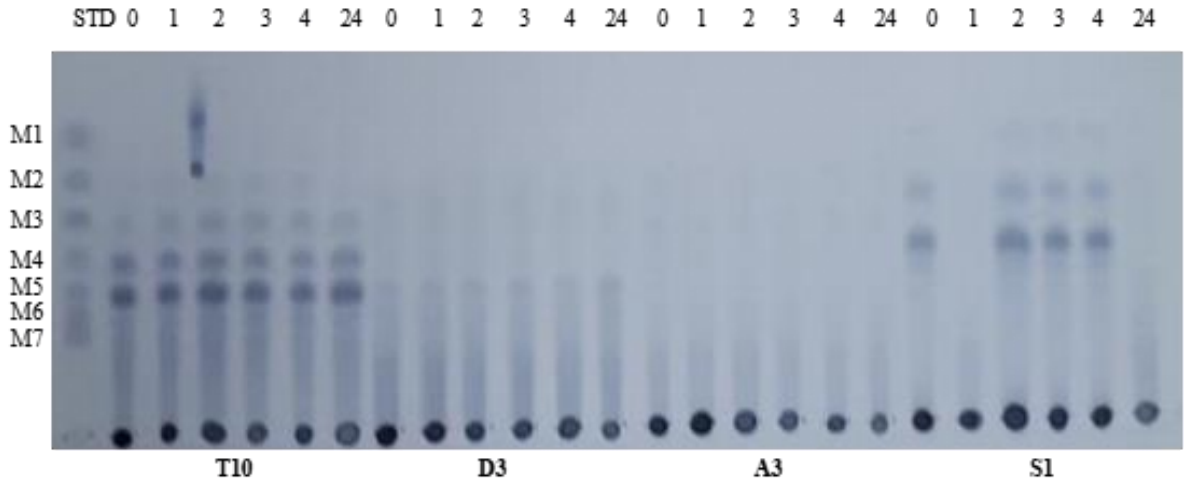


Figure 6. The product profile of starch hydrolyzed by amylase of the amylolytic bacteria (T10, D3, A3, and S1) using a TLC method with reaction times (hours): of 0, 1, 2, 3, 4, and 24 at 30°C. The Standards (STD): monosaccharide (M1), maltose (M2), maltotriose (M3), maltotetraose (M4), maltopentaose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

Based on the TLC chromatogram, the starch degraded by T10 amylase resulted in malto-oligosaccharides 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 amylolytic bacteria with high amylolytic indexes (AI) did not correlate with their ability to degrade amylum. The isolate T10, with its total diameter lower than isolate D3, showed a higher ability to break down starch polymer bonds into shorter or oligosaccharides. The results of this study proved that a high AI value is only sometimes accompanied by the 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 maltotriosa 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.

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

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

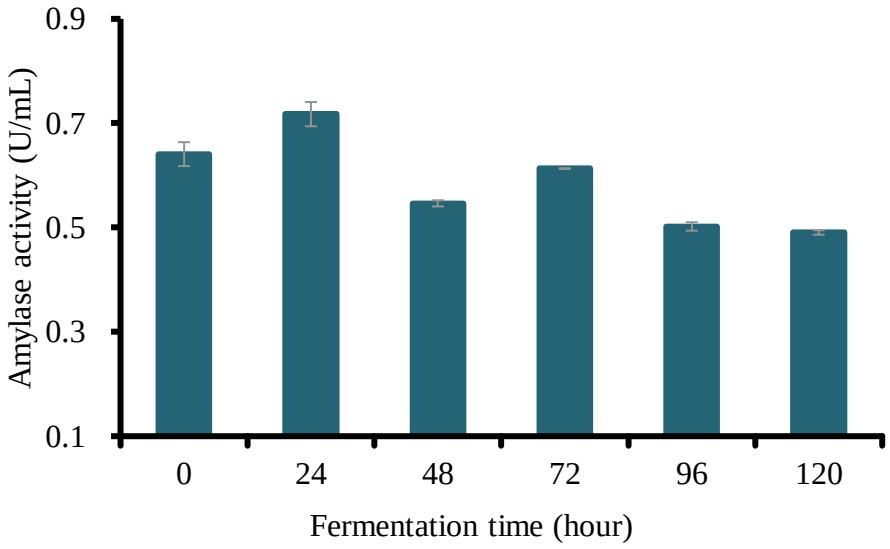


Figure 7. Amylase activity of T10 at different incubation times.

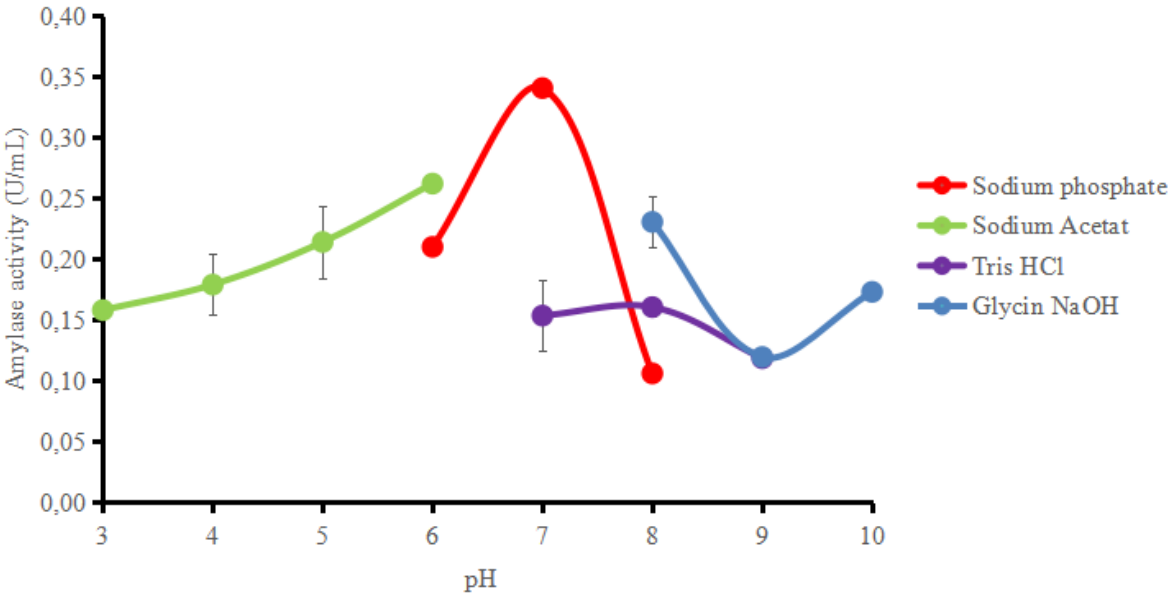
The amylase activity of *Bacillus cereus* KN isolated from Ranu Ngebel and incubated for three days was 0.016 U/mL, while strain G20 isolated from Ranu Grati was lower at about 0.0001 U/mL (Nisa et al. 2021). Luang et al. (2019) found *Bacillus* sp. 3.5AL2 isolated from soils of the unexplored Nasinuan 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. it decreased after 48 h incubations due to the suppression and accumulation of other byproducts in the fermentation medium and also 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

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

The amylase activity of isolate T10 was observed at temperatures ranging from 30-90ºC 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. 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 40º C with more than 75% activity in range of 50 - 80º 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 40ºC.

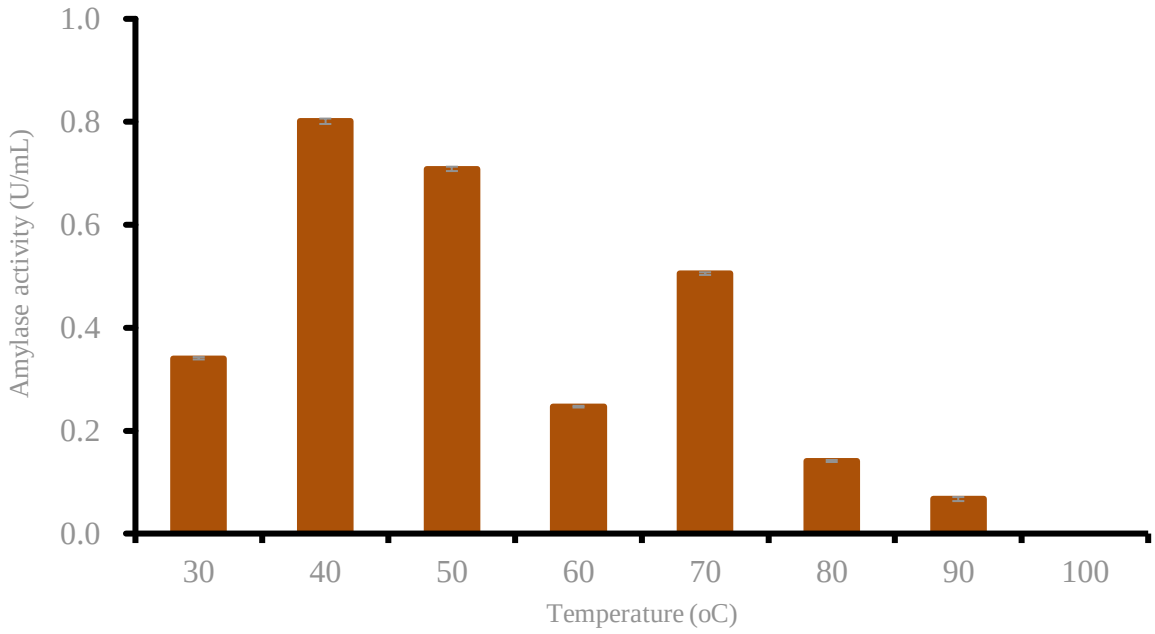


Figure 9. Amylase activity of isolate T10 at different temperatures.

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 decreased. 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).

4. 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.

Acknowledgments

We would like to thank the Research Center for Biotechnology (BRIN) and International Women University (IWU) for funding this research.

Conflict of interest

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

References

Abdalla, M. et al., 2021. One-pot production of maltoheptaose (DP7) from starch by sequential addition of cyclodextrin glucotransferase and cyclomaltodextrinase. *Enzyme and Microbial Technology,* 149 (6), 109847. doi: 10.1016/j.enzmictec.2021.109847

Abdul-Manas, N.H. et al., 2014. The characterization of an alkali-stable maltogenic amylase from *Bacillus lehensis* G1 and improved maltooligosaccharide production by hydrolysis suppression. *PLoS ONE* 9(9). doi: 10.1371/journal.pone.0106481

Afifah, N., Putri, D.H. & Irdawati, 2018. Isolation and identification of endophytic bacteria from the Andalas plant stem (*Morus macroura* Miq.). *Bioscience*, 2(1), pp.72-75.

427 Agamennone, V. et al., 2019. Genome annotation and antimicrobial properties of *B.*
428 *toyonensis* VU-DES13, isolated from the *Folsomia candida* gut. *Entomologia*
429 *Experimentalis et Aplicata* 167, pp:269-285. doi: 10.1111/eea.12763.
430 Asgher, M. et al., 2007. A thermostable α-amylase from moderately thermophilic *Bacillus*
431 *subtilis* strain for starch processing. *J Food Eng.*, 79, pp.950-955.
432 Bajpai, B., Chaudhary, M. & Saxena, J., 2015. Production and characterization of α-Amylase
433 from an extremely halophilic archaeon, *Haloferax* sp. HA10. *Food Technol*
434 *Biotechnol.*, 53(1), pp.11-17, doi: 10.17113/ftb.53.01.15.3824
435 Ben-Ali, M. et al., 2006. Thermostability enhancement and change in starch hydrolysis
436 profile of the maltohexaose-forming amylase of *Bacillus stearothermophilus* US100
437 strain. *Biochemical Journal*, 394(1), pp.51–56. doi: 10.1042/BJ20050726
438 Behal, A. et al., 2016. Characterization of alkaline α- amylase from *Bacillus* sp. AB 04.
439 *IJAB*, 8(1), pp.80-83.
440 De-Moraes-Russo, C.A. & Selvatti, A.P., 2018. Bootstrap and rogue identification tests for
441 phylogenetic analyses. *Molecular Biology and Evolution*, 35(9), pp.2327–2333. doi:
442 10.1093/molbev/msy118.
443 Dey, G. et al., 2002. Purification and characterization of maltooligosaccharide-forming
444 amylase from *Bacillus circulans* GRS 313. *Journal of Industrial Microbiology and*
445 *Biotechnology*, 28(4), pp.193-200. DOI: 10.1038/sj/jim/7000220.
446 Ding, N. et al., 2021. Carbohydrate-binding module and linker allow cold adaptation and salt
447 tolerance of maltopentaose-forming amylase from marine bacterium
448 *Saccharophagus degradans* 2-40T. *Frontiers in Microbiology*, 12(7), pp.1–14. doi:
449 10.3389/fmicb.2021.708480.

Divakaran, D., Chandran, A. & Pratap-Chandran, R., 2011. Comparative study on production of α-amylase from *Bacillus licheniformis* strains. *Brazilian Journal of Microbiology*, 42(4), pp.1397–1404. doi: 10.1590/S1517-83822011000400022.

Duan, Y. et al., 2021. Isolation, identification, and antibacterial mechanisms of *Bacillus amyloliquefaciens* QSB-6 and its effect on plant roots. *Frontiers in Microbiology*, 12:746799. doi:10.3389/fmicb.2021.746799.

El-Fallal, A. et al., 2012. Starch and microbial α-amylases: from concepts to biotechnological applications. In *Carbohydrates-Comprehensive Studies on Glycobiology and Glycotechnology*, Intech Open Science, pp.459-488. doi: 10.5772/51571

Gebreyohannes, G., 2015. Isolation and optimization of amylase producing bacteria and actinomycetes from soil samples of Maraki and Tewedros campus, University of Gondar, North West Ethiopia. *African Journal of Microbiology Research*, 9(31), pp.1877-1882.

Ginting, E.L. et al., 2021. Isolation and identification of thermophilic amylolytic bacteria from Likupang Marine Hydrothermal, North Sulawesi, Indonesia. Biodiversitas, 22(6), pp.3326-3332. doi: 10.13057/biodiv/d220638.

Gupta, R. et al., 2003. Microbial α-amylases: A biotechnological perspective. *Process Biochemistry*, 38(11), pp.1599–1616. doi: 10.1016/S0032-9592(03)00053-0.

Hasanah, U. et al., 2020. Amylolytic activity of bacterial strains isolated from sago pulp of the traditional sago industry in Palopo, South Sulawesi. *AIP Conference Proceedings* 2231, 040073 (2020), https://doi.org/10.1063/5.0002487.

Hellmuth, K. & van-den Brink, J.M., 2013. Microbial production of enzymes used in food applications. In *Microbial Production of Food Ingredients, Enzymes and Nutraceuticals*. Woodhead Publishing Limited. doi: 10.1533/9780857093547.2.262.

475 Jang, E.Y. et al., 2020. Amylase-producing maltooligosaccharide provides potential relief in
476 rats with loperamide-induced constipation. *Evidence-Based Complementary and*
477 *Alternative Medicine*. doi: 10.1155/2020/5470268.
478 Jiménez, G. et al., 2013. Description of *Bacillus toyonensis* sp. nov., a novel species of
479 the *Bacillus cereus* group, and pairwise genome comparisons of the species of the
480 group by means of ANI calculations. *Systematic and Applied Microbiology* 36(6),
481 pp.383-391. doi: 10.1016/j.syapm.2013.04.008.
482 Lim, S.J. & Oslan, S.N., 2021. Native to designed: Microbial α-Amylases for industrial
483 applications. *PeerJ*, 9, pp.1–30. doi: 10.7717/peerj.11315.
484 Logan, N.A. & De Vos, P., 2009. Genus I. Bacillus Cohn 1872. In *Bergey's Manual of*
485 *Systematic Bacteriology Second Edition Volume Three The Firmicutes*. Springer,
486 Springer Dordrecht Heidelberg London New York. pp.21-127. doi:
487 10.1007/b92997.
488 Luang-In, V. et al., 2019. Isolation and identification of amylase-producing bacteria from soil
489 in Nasinuan community forest, Maha Sarakham, Thailand. *Biomedical &*
490 *Pharmacology Journal*, 12(3), pp.1061-1068.
491 Luo, J-c. et al., 2021. Characterization of a deep sea *Bacillus toyonensis* isolate: genomic and
492 pathogenic features. *Frontiers in Cellular and Infection Microbiology*, 11, 629116.
493 doi: 10.3389/fcimb.2021.629116.
494 Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar.
495 *Analytical Chemistry*, 31(3), pp.426–428. doi: 10.1021/ac60147a030
496 Moradi, M. et al., 2014. Screening and isolation of powerful amylolytic bacterial strains.
497 *International Journal of Current Microbiology and Applied Sciences*, 3(2), pp.758–
498 768.

499 Naidu, K. et al., 2019. Purification and characterization of α-amylase from *Paenibacillus* sp.
500 D9 and *Escherichia coli* recombinants. *Biocatalysis and Biotransformation,* 38(1),
501 pp.24-34. doi: 10.1080/10242422.2019.1628738.
502 Nisa, I.K. et al., 2021. The potential of amylase enzyme activity against bacteria isolated
503 from several lakes in East Java, Indonesia. *Biodiversitas,* 22(1), pp.42-49.
504 Niu, D. et al., 2017. Highly efficient enzymatic preparation of isomalto-oligosaccharides
505 from starch using an enzyme cocktail. *Electronic Journal of Biotechnology,* 26,
506 pp.46–51. doi: 10.1016/j.ejbt.2016.12.002.
507 Ochoa-Solano, J.L. & Olmos-Soto, J., 2006. The functional property of Bacillus for Shrimp
508 feeds. *Food Microbiology* 23(6), pp.519–525, doi: 10.1016/j.fm.2005.10.004.
509 Ozturk, H.U. et al., 2014. A *maltooligosaccharides* producing α-amylase from *Bacillus subtilis*
510 SDP1 isolated from rhizosphere of *Acacia cyanophylla* Lindley. *Food Biotechnology,*
511 28(4), pp.309-332. doi: 10.1080/08905436.2014.963600.
512 Pan, S. et al., 2017. Maltooligosaccharide-forming amylase: Characteristics, preparation, and
513 application. *Biotechnology Advances,* 35(5), pp.619–632. doi:
514 10.1016/j.biotechadv.2017.04.004.
515 Putri, W.D.R. et al., 2012. Isolation and characterization of amylolytic lactic acid bacteria
516 during growol fermentation, an Indonesian traditional food. *Jurnal Teknologi*
517 *Pertanian*, 13(1), pp.52-60.
518 Rahmani, N. et al., 2013. Production of maltooligosaccharides from black potato (*Coleus*
519 *tuberosusi)* starch by α-amylase from a marine bacterium (*Brevibacterium* sp.).
520 *Microbiology Indonesia*, 7(3), pp.129-136. doi: 10.5454/mi.7.3.6.
521 Rahmani, N. et al., 2018. Xylanase and feruloyl esterase from actinomycetes cultures could
522 enhance sugarcane bagasse hydrolysis in the production of fermentable sugars.

Bioscience Biotechnology and Biochemistry, 82(5), pp.904–915. doi: 10.1080/09168451.2018.1438169.

Santos, F.D.S. et al., 2018. *Bacillus toyonensis* improves immune response in the mice vaccinated with recombinant antigen of bovine herpesvirus type 5. *Benef. Microbes*, 9(1), pp.133–142. doi: 10.3920/BM2017.0021.

Sivaramakrishnan, S. et al., 2006. α-Amylase from microbial sources: An overview on recent developments. *Food Technol. Biotechnol.*, 44 (2), pp.173–184.

Smibert, R.M. & Krieg, N.R., 1981. General Characterization. In *Manual Methods for General Bacteriology*. American Society for Microbiology, Washington.

Subagiyo, Djarod, M.S.R. & Setyati, W.A., 2017. Potensi ekosistem mangrove sebagai sumber bakteri untuk produksi protease, amilase, dan selulase. *Jurnal Kelautan Tropis*, 20(2), pp.106–111.

Vaseekaran, S., Balakumar, S. & Arasaratnam, V., 2010. Isolation and identification of a bacterial strain producing thermostable α- amylase. *Tropical Agricultural Research*, 22 (1), pp.1-11. doi: 10.4038/tar.v22i1.2603.

Vijayalakshmi, et al., 2012. Isolation and characterization of *Bacillus subtilis* KC3 for amylolytic activity. *International Journal of Bioscience, Biochemistry and Bioinformatics*, 2(5), pp.336-341. doi: 10.7763/IJBBB.2012.V2.128.

Wang, J. et al., 2021. Toyoncin, a novel leaderless bacteriocin that is produced by *Bacillus toyonensis* XIN-YC13 and specifically targets *B. cereus* and *Listeria monocytogenes. Applied and Environmental Microbiology*, 87(12), e00185-21. doi: 10.1128/AEM.00185-21.

Yopi, et al., 2017. Isolation and characterization of mannanase, xylanase, and cellulase from marine bacteria *Bacillus* sp. *Biofarmasi Journal of Natural Product Biochemistry*, 15(1), pp.15–20. doi: 10.13057/biofar/f150103.

548 Yufinta, C.P., Julyantoro, P.G.S. & Pratiwi, M.A., 2018. Pengaruh penambahan *Bacillus* sp.
549 terhadap kelulushidupan pasca larva udang Vannamei (*Litopenaeus vannamei*) yang
550 terinfeksi vibriosis. *Current Trends in Aquatic Science*, 1(1), pp.89-95.
551 Zhao, C. et al., 2017. Functional properties, structural studies and chemo-enzymatic synthesis
552 of oligosaccharides. *Trends in Food Science and Technology*, 66, pp.135–145. doi:
553 10.1016/j.tifs.2017.06.008.
554 Zhuang, Y., Zhou, X. & Wang, S., 2012. Gapped BLAST and PSI-BLAST: a new generation
555 of protein database search programs. *Plant Systematics and Evolution*, 298(7),
556 pp.3389–3402. doi: 10.2503/jjshs.58.977.
557 Zubaidah, A. et al., 2019. Screening bakteri selulolitik dan amilolitik pada rumen sapi sebagai
558 kandidat probiotik pada budidaya ikan secara *in vitro*. *Jurnal Riset Akuakultur*,
559 14(4), pp.261-271.

Journal of Tropical Biodiversity and Biotechnology
Volume 08, Issue 02 (2023): jtbb78346
DOI: 10.22146/jtbb.78346

Research Article

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

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

Keywords:
Amylolytic bacteria
Canna
Maltooligosaccharides
16S rDNA gene
Submitted:
13 October 2022
Accepted:
30 January 2023
Published:

Editor:
Miftahul Ilmi

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

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

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

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

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

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

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

MATERIALS AND METHODS

Sample Collection and Location of Sampling

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

Isolation, Screening, and Morphological Characterization of Amylolytic Bacteria

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

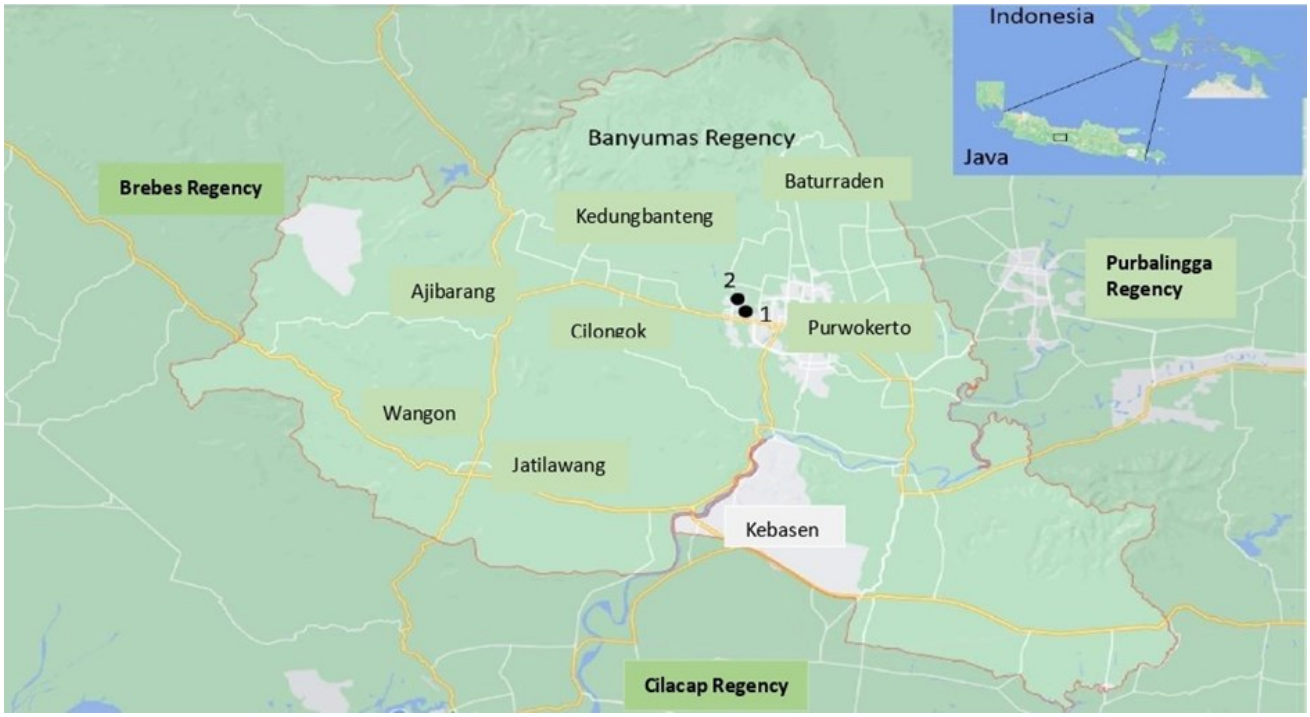


Figure 1. A map of Banyumas Regency and sampling sites: (1) the Perhutani Forest KPH West Banyumas, Central Java, (2) Community gardens around the Perhutani Forest KPH West Banyumas, Central Java.

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

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

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

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

Phenetic and Phylogenetic Characterizations of the Selected Bacterium

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

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

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

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

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

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

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

Crude Enzyme Production and Amylase Activity at Different Fermentation Time

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

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

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

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

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

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

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

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

RESULTS AND DISCUSSION

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

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

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

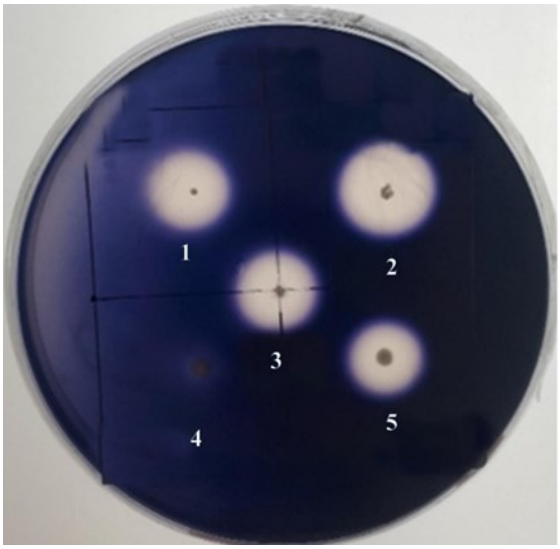


Figure 2. The amylolytic zones produced by bacterial isolates of: (1) T10, (2) D3, (3) A3, (4) TH6, and (5) S1 on a NA medium + 1% soluble starch.

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

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

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

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

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

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

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

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

Based on the ability of the selected isolate 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).

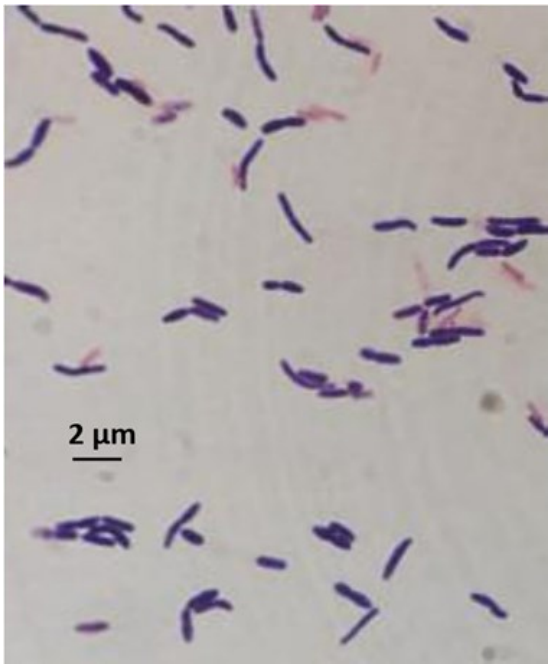


Figure 3. The appearance of bacterial cells isolate T10 under a microscope with a magnification of 1000x. The cells appear single or in chains.

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.mlm.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-7112, and *B. toyonensis* l3aM to form a separate cluster (Figure 5). Hence, isolate T10 was identified as the species member of *B. toyonensis* based on the phenetic and phylogenetic characteristics.

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

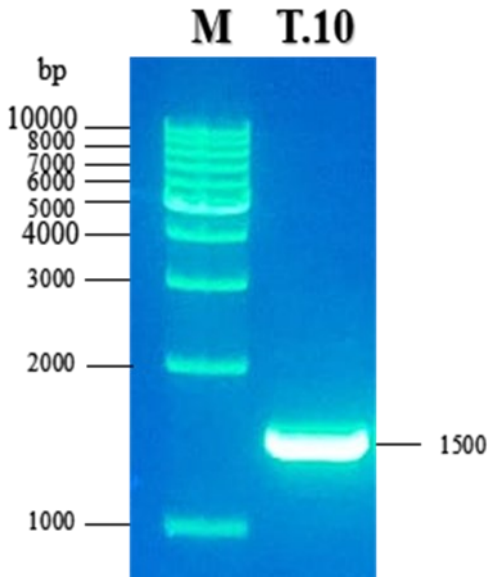


Figure 4. An electropherogram of the amplified 16S rRNA gene of isolate T10 with a size of 1500 bp. Marker (M): 1 kb DNA ladder.

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

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

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

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

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

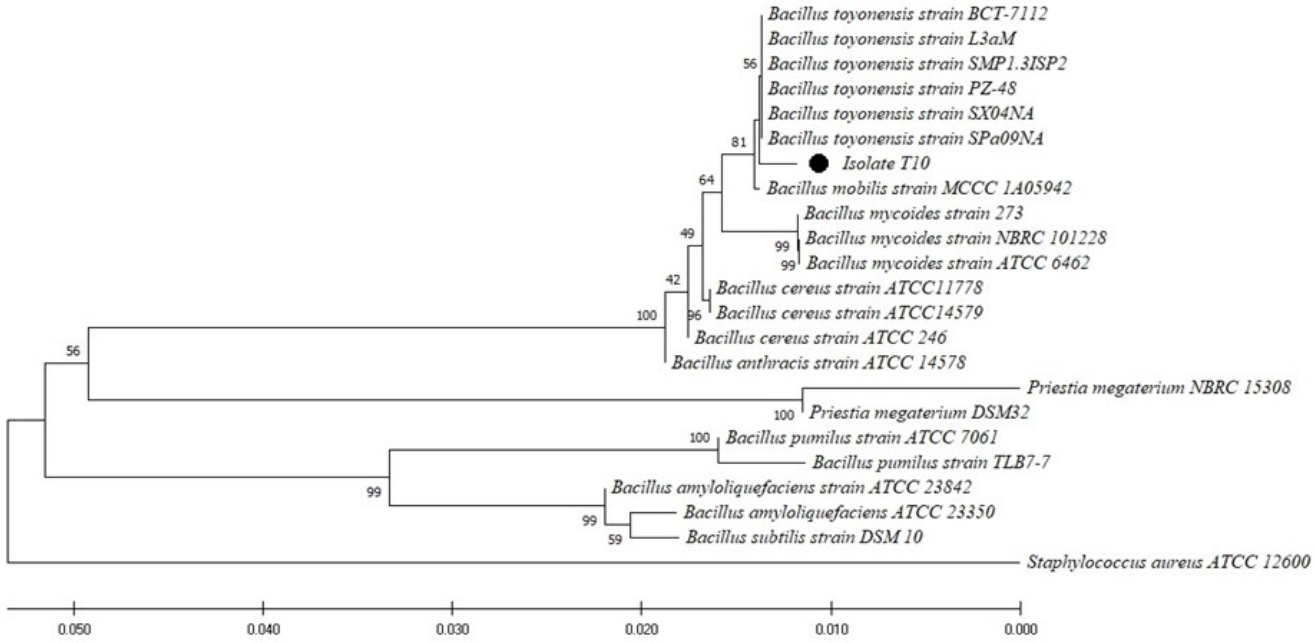


Figure 5. A phylogenetic tree showing the relationship between strain T10 isolated from rhizospheres of Canna (*C. edulis*) and several species members of the genus *Bacillus* on the basis of 16S rRNA gene sequence reconstructed based on Neighbor-Joining, Model Maximum Composite Likelihood, and 1000x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

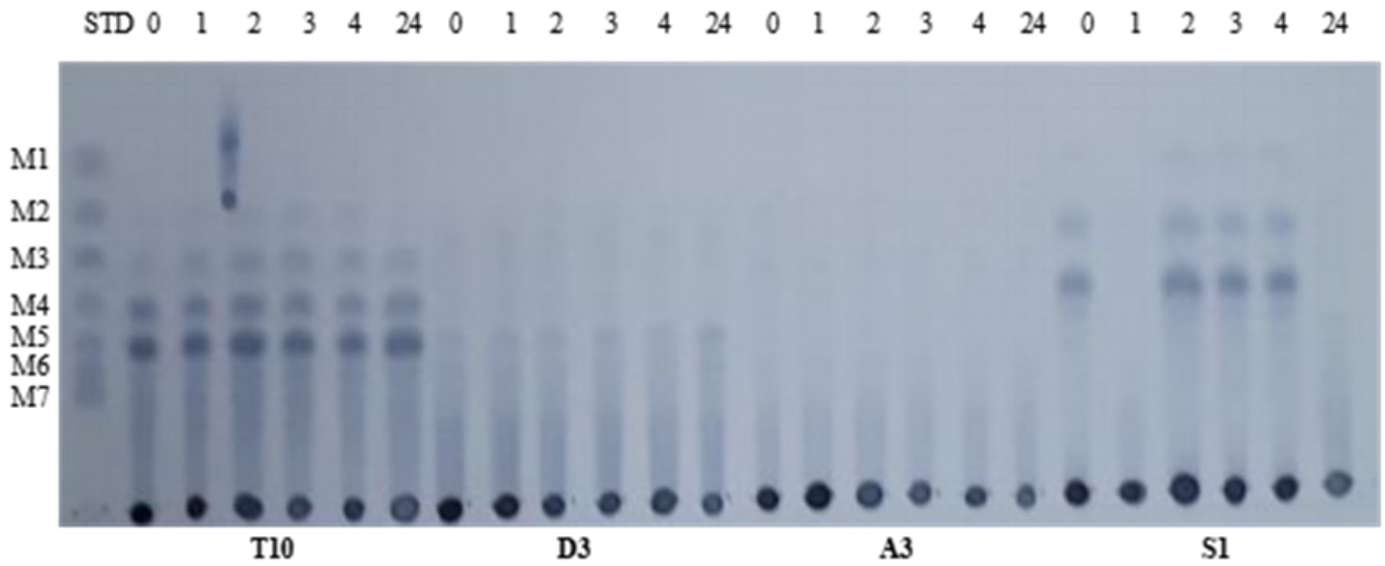


Figure 6. The product profile of starch hydrolyzed by amylase of the amylolytic bacteria (T10, D3, A3, and S1) using a TLC method with reaction times (hours): of 0, 1, 2, 3, 4, and 24 at 30°C. The Standards (STD): monosaccharide (M1), maltose (M2), maltotriose (M3), maltotetraose (M4), maltopentaose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

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

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

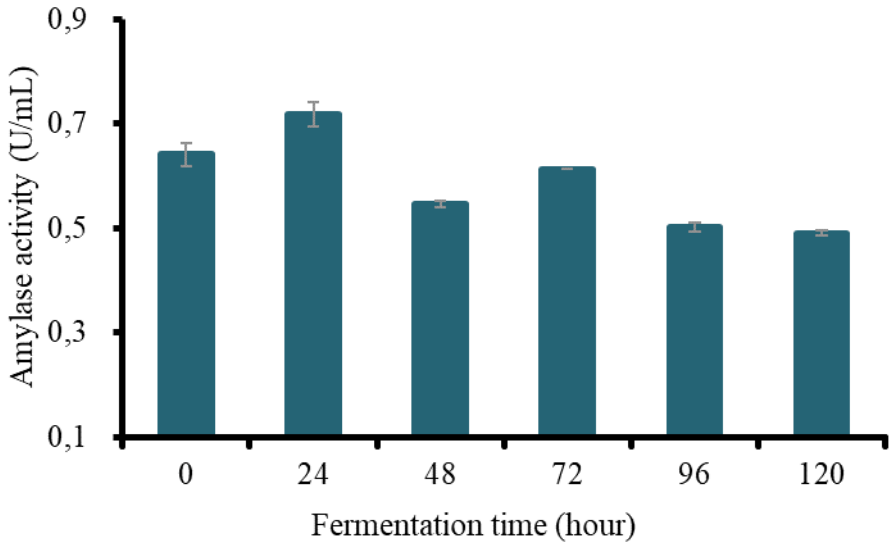


Figure 7. Amylase activity of T10 at different incubation times.

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

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

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

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

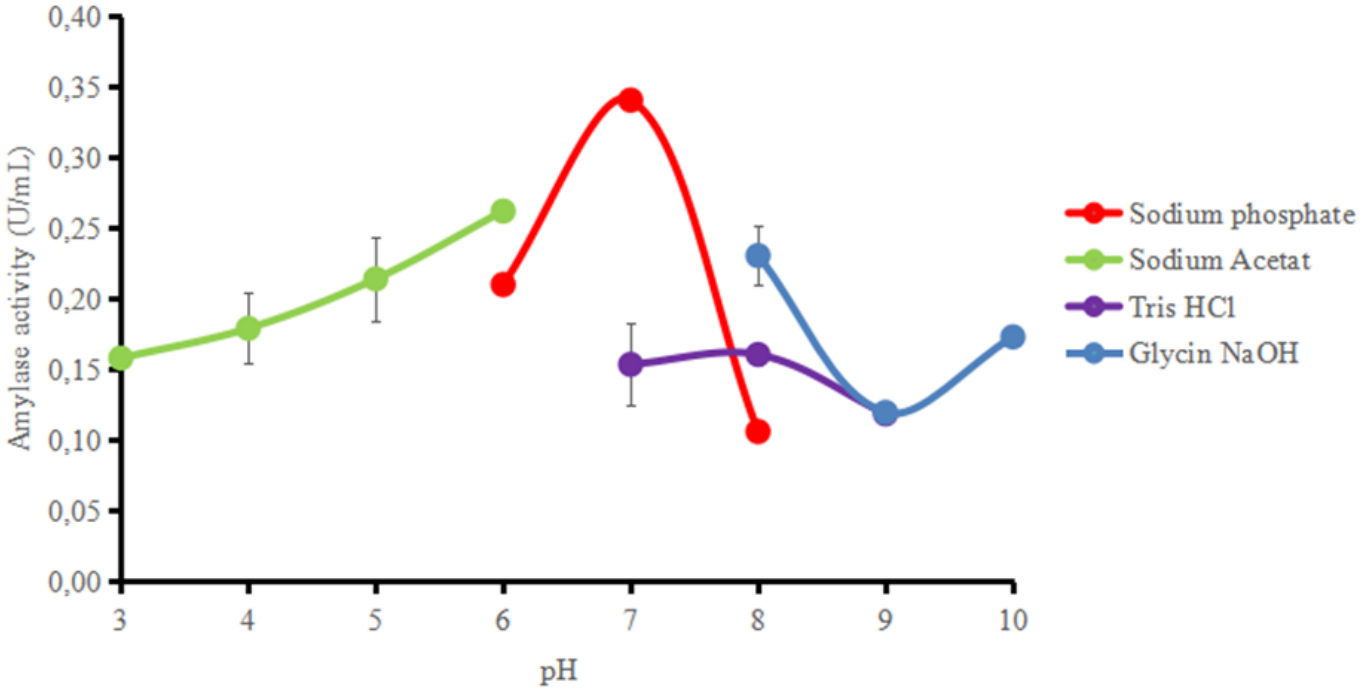


Figure 8. Amylase activity of T10 at different pH and buffers.

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

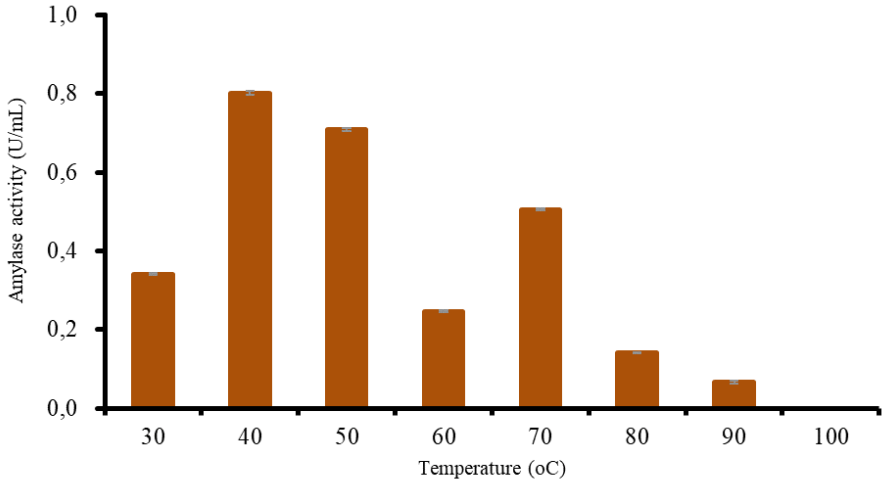


Figure 9. Amylase activity of isolate T10 at different temperatures.

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

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

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

CONCLUSIONS

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

AUTHORS CONTRIBUTION

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

ACKNOWLEDGMENTS

We would like to thank the Research Center for Biotechnology (BRIN) and International Women University (IWU) for funding this research.

CONFLICT OF INTEREST

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

REFERENCES

Abdalla, M. et al., 2021. One-pot production of maltoheptaose (DP7) from starch by sequential addition of cyclodextrin glucotransferase and cyclomaltodextrinase. *Enzyme and Microbial Technology*, 149 (6), 109847. doi: 10.1016/j.enzmictec.2021.109847

Abdul-Manas, N.H. et al., 2014. The characterization of an alkali-stable maltogenic amylase from *Bacillus lehensis* G1 and improved maltooligosaccharide production by hydrolysis suppression. *PLoS ONE* 9(9). doi: 10.1371/journal.pone.0106481

Afifah, N., Putri, D.H. & Irdawati, 2018. Isolation and identification of endophytic bacteria from the Andalas plant stem (*Morus macroura* Miq.). *Bioscience*, 2(1), pp.72-75.

Agamennone, V. et al., 2019. Genome annotation and antimicrobial properties of *B. toyonensis* VU-DES13, isolated from the *Folsomia candida* gut. *Entomologia Experimentalis et Aplicata* 167, pp:269-285. doi: 10.1111/eea.12763.

Asgher, M. et al., 2007. A thermostable α-amylase from moderately thermophilic *Bacillus subtilis* strain for starch processing. *J Food Eng.*, 79, pp.950-955.

Bajpai, B., Chaudhary, M. & Saxena, J., 2015. Production and characterization of α-Amylase from an extremely halophilic archaeon, *Haloferax* sp. HA10. *Food Technol Biotechnol.*, 53(1), pp.11-17, doi: 10.17113/ftb.53.01.15.3824

Ben-Ali, M. et al., 2006. Thermostability enhancement and change in starch hydrolysis profile of the maltohexaose-forming amylase of *Bacillus stearothermophilus* US100 strain. *Biochemical Journal*, 394(1), pp.51–56. doi: 10.1042/BJ20050726

Behal, A. et al., 2016. Characterization of alkaline α- amylase from *Bacillus* sp. AB 04. *IJAB*, 8(1), pp.80-83.

De-Moraes-Russo, C.A. & Selvatti, A.P., 2018. Bootstrap and rogue identification tests for phylogenetic analyses. *Molecular Biology and Evolution*, 35(9), pp.2327–2333. doi: 10.1093/molbev/msy118.

Dey, G. et al., 2002. Purification and characterization of maltooligosaccharide-forming amylase from *Bacillus circulans* GRS 313. *Journal of Industrial Microbiology and Biotechnology*, 28(4), pp.193-200. DOI: 10.1038/sj/jim/7000220.

Ding, N. et al., 2021. Carbohydrate-binding module and linker allow cold adaptation and salt tolerance of maltopentaose-forming amylase from marine bacterium *Saccharophagus degradans* 2-40T. *Frontiers in Microbiology*, 12(7), pp.1–14. doi: 10.3389/fmicb.2021.708480.

Divakaran, D., Chandran, A. & Pratap-Chandran, R., 2011. Comparative study on production of α-amylase from *Bacillus licheniformis* strains. *Brazilian Journal of Microbiology*, 42(4), pp.1397–1404. doi: 10.1590/S1517-83822011000400022.

Duan, Y. et al., 2021. Isolation, identification, and antibacterial mechanisms of *Bacillus amyloliquefaciens* QSB-6 and its effect on plant roots. *Frontiers in Microbiology*, 12:746799. doi:10.3389/fmicb.2021.746799.

El-Fallal, A. et al., 2012. Starch and microbial α-amylases: from concepts to biotechnological applications. In *Carbohydrates-Comprehensive Studies on Glycobiology and Glycotechnology*, Intech Open Science, pp.459-488. doi: 10.5772/51571

Gebreyohannes, G., 2015. Isolation and optimization of amylase producing bacteria and actinomycetes from soil samples of Maraki and Tewedros campus, University of Gondar, North West Ethiopia. *African Journal of Microbiology Research*, 9(31), pp.1877-1882.

Ginting, E.L. et al., 2021. Isolation and identification of thermophilic amylolytic bacteria from Likupang Marine Hydrothermal, North Sulawesi, Indonesia. Biodiversitas, 22(6), pp.3326-3332. doi: 10.13057/biodiv/d220638.

Gupta, R. et al., 2003. Microbial α-amylases: A biotechnological perspective. *Process Biochemistry*, 38(11), pp.1599–1616. doi: 10.1016/S0032-9592(03)00053-0.

Hasanah, U. et al., 2020. Amylolytic activity of bacterial strains isolated from sago pulp of the traditional sago industry in Palopo, South Sulawesi. *AIP Conference Proceedings* 2231, 040073 (2020), https://doi.org/10.1063/5.0002487.

Hellmuth, K. & van-den Brink, J.M., 2013. Microbial production of enzymes used in food applications. In *Microbial Production of Food Ingredients, Enzymes and Nutraceuticals.* Woodhead Publishing Limited. doi: 10.1533/9780857093547.2.262.

Jang, E.Y. et al., 2020. Amylase-producing maltooligosaccharide provides potential relief in rats with loperamide-induced constipation. *Evidence-Based Complementary and Alternative Medicine*. doi: 10.1155/2020/5470268.

Jiménez, G. et al., 2013. Description of *Bacillus toyonensis* sp. nov., a novel species of the *Bacillus cereus* group, and pairwise genome comparisons of the species of the group by means of ANI calculations. *Systematic and Applied Microbiology* 36(6), pp.383-391. doi: 10.1016/j.syapm.2013.04.008.

Lim, S.J. & Oslan, S.N., 2021. Native to designed: Microbial α-Amylases for industrial applications. *PeerJ*, 9, pp.1–30. doi: 10.7717/peerj.11315.

Logan, N.A. & De Vos, P., 2009. Genus I. Bacillus Cohn 1872. In *Bergey's Manual of Systematic Bacteriology Second Edition Volume Three The Firmicutes*. Springer, Springer Dordrecht Heidelberg London New York. pp.21-127. doi: 10.1007/b92997.

Luang-In, V. et al., 2019. Isolation and identification of amylase-producing bacteria from soil in Nasinuan community forest, Maha Sarakham, Thailand. *Biomedical & Pharmacology Journal*, 12(3), pp.1061-1068.

Luo, J-c. et al., 2021. Characterization of a deep sea *Bacillus toyonensis* isolate: genomic and pathogenic features. *Frontiers in Cellular and Infection Microbiology*, 11, 629116. doi: 10.3389/fcimb.2021.629116.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31(3), pp.426–428. doi: 10.1021/ac60147a030

Moradi, M. et al., 2014. Screening and isolation of powerful amylolytic bacterial strains. *International Journal of Current Microbiology and Applied Sciences*, 3(2), pp.758–768.

Naidu, K. et al., 2019. Purification and characterization of α-amylase from *Paenibacillus* sp. D9 and *Escherichia coli* recombinants. *Biocatalysis and Biotransformation*, 38(1), pp.24-34. doi: 10.1080/10242422.2019.1628738.

Nisa, I.K. et al., 2021. The potential of amylase enzyme activity against bacteria isolated from several lakes in East Java, Indonesia. *Biodiversitas*, 22(1), pp.42-49.

Ochoa-Solano, J.L. & Olmos-Soto, J., 2006. The functional property of Bacillus for Shrimp feeds. *Food Microbiology* 23(6), pp.519–525, doi: 10.1016/j.fm.2005.10.004.

Ozturk, H.U. et al., 2014. A *maltooligosaccharides* producing α-amylase from *Bacillus subtilis* SDP1 isolated from rhizosphere of *Acacia cyanophylla* Lindley. *Food Biotechnology*, 28(4), pp.309-332. doi: 10.1080/08905436.2014.963600.

Pan, S. et al., 2017. Maltooligosaccharide-forming amylase: Characteristics, preparation, and application. *Biotechnology Advances*, 35(5), pp.619–632. doi: 10.1016/j.biotechadv.2017.04.004.

Putri, W.D.R. et al., 2012. Isolation and characterization of amylolytic lactic acid bacteria during growol fermentation, an Indonesian traditional food. *Jurnal Teknologi Pertanian*, 13(1), pp.52-60.

Rahmani, N. et al., 2013. Production of maltooligosaccharides from black potato (*Coleus tuberosusi)* starch by α-amylase from a marine bacterium (*Brevibacterium* sp.). *Microbiology Indonesia*, 7(3), pp.129-136. doi: 10.5454/mi.7.3.6.

Rahmani, N. et al., 2018. Xylanase and feruloyl esterase from actinomycetes cultures could enhance sugarcane bagasse hydrolysis in the production of fermentable sugars. *Bioscience Biotechnology and Biochemistry*, 82(5), pp.904–915. doi: 10.1080/09168451.2018.1438169.

Santos, F.D.S. et al., 2018. *Bacillus toyonensis* improves immune response in the mice vaccinated with recombinant antigen of bovine herpesvirus type 5. *Benef. Microbes*, 9(1), pp.133–142. doi: 10.3920/BM2017.0021.

Sivaramakrishnan, S. et al., 2006. α-Amylase from microbial sources: An overview on recent developments. *Food Technol. Biotechnol.*, 44 (2), pp.173–184.

Smibert, R.M. & Krieg, N.R., 1981. General Characterization. In *Manual Methods for General Bacteriology*. American Society for Microbiology, Washington.

Subagiyo, Djarod, M.S.R. & Setyati, W.A., 2017. Potensi ekosistem mangrove sebagai sumber bakteri untuk produksi protease, amilase, dan selulase. *Jurnal Kelautan Tropis*, 20(2), pp.106–111.

Vaseekaran, S., Balakumar, S. & Arasaratnam, V., 2010. Isolation and identification of a bacterial strain producing thermostable α- amylase. *Tropical Agricultural Research*, 22 (1), pp.1-11. doi: 10.4038/tar.v22i1.2603.

Vijayalakshmi, et al., 2012. Isolation and characterization of *Bacillus subtilis* KC3 for amylolytic activity. *International Journal of Bioscience, Biochemistry and Bioinformatics*, 2(5), pp.336-341. doi: 10.7763/IJBBB.2012.V2.128.

Wang, J. et al., 2021. Toyoncin, a novel leaderless bacteriocin that is produced by *Bacillus toyonensis* XIN-YC13 and specifically targets *B. cereus* and *Listeria monocytogenes*. *Applied and Environmental Microbiology*, 87(12), e00185-21. doi: 10.1128/AEM.00185-21.

Yopi, et al., 2017. Isolation and characterization of mannanase, xylanase, and cellulase from marine bacteria *Bacillus* sp. *Biofarmasi Journal of Natural Product Biochemistry*, 15(1), pp.15–20. doi: 10.13057/biofar/f150103.

Yufinta, C.P., Julyantoro, P.G.S. & Pratiwi, M.A., 2018. Pengaruh penambahan *Bacillus* sp. terhadap kelulushidupan pasca larva udang Vannamei (*Litopenaeus vannamei*) yang terinfeksi vibriosis. *Current Trends in Aquatic Science*, 1(1), pp.89-95.

Zhao, C. et al., 2017. Functional properties, structural studies and chemo -enzymatic synthesis of oligosaccharides. *Trends in Food Science and Technology*, 66, pp.135–145. doi: 10.1016/j.tifs.2017.06.008.

Zhuang, Y., Zhou, X. & Wang, S., 2012. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Plant Systematics and Evolution*, 298(7), pp.3389–3402. doi: 10.2503/jjshs.58.977.

Zubaidah, A. et al., 2019. Screening bakteri selulolitik dan amilolitik pada rumen sapi sebagai kandidat probiotik pada budidaya ikan secara *in vitro*. *Jurnal Riset Akuakultur*, 14(4), pp.261-271.