Exploration of IAA Producing Bacteria And Amylolitic Bacteria From Several East Java Lakes, and Their Potency For Microbial Consortium To Accelerate Chlorella Vulgaris Growth

Imroatun Naf’ah
Department of Biology, Faculty of mathematics and science, Universitas Negeri Malang

Sitoresmi Prabaningtyas (sitoresmi.prabaningtyas.fmipa@um.ac.id)
Department of Biology, Faculty of mathematics and science, Universitas Negeri Malang

Agung Witjoro
Department of Biology, Faculty of mathematics and science, Universitas Negeri Malang

Yanis Kurnia Basitoh
Department of Biology, Faculty of mathematics and science, Universitas Negeri Malang

Achmad Rodiansyah
Department of Biology, Faculty of mathematics and science, Universitas Negeri Malang

Dhiyauddin Aridhowi
Department of Biology, Faculty of mathematics and science, Universitas Negeri Malang

Research

Keywords: amylolytic bacteria, Chlorella vulgaris, IAA producing bacteria, microbial consortium, microalgae

DOI: https://doi.org/10.21203/rs.3.rs-520439/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

The consortium of various types of bacteria from lakes in East Java has the potency to stimulate microalgae *Chlorella vulgaris* growth. Increased microalgae density from co-culture has an excellent potency for sources of biomass that can be developed for renewable energy. Several stages conducted of this research started from an exploration of IAA producing bacteria and amylolytic bacteria from several East Java Lakes; then, the highest bacterial isolates were identified with morphological and genotypical characteristics. The well-characterized bacterial isolates were used for the microbial consortium in co-culture with *C. vulgaris*. The treatment used in this study as follows: I) *C. vulgaris* without bacteria culture as a control, II) amylolytic bacteria + *C. vulgaris*, III) IAA-producing bacteria + *C. vulgaris*, IV) potential amylolytic bacteria and IAA-producing bacteria + *C. vulgaris*. The exploration result of potential bacteria from Ranu Pani, Ranu Regulo, Telaga Ngebel, and Ranu Grati lakes was found 53 amylolytic bacterial isolates, and 90 isolates IAA-producing bacteria. The highest amylolytic bacteria (isolate L) is related to *Bacillus amyloliquefaciens*, while the most elevated IAA-producing bacteria (isolate C) is related to *Bacillus paramycoides*. The highest cell density was produced in treatment III, reaching $2.7 \times 10^6$ cells/mL on day 50th. The treatments with supplement bacteria showed a significant effect for accelerating the growth of microalgae compared to control.

Introduction

Energy dependence and continuous energy consumption could cause an energy crisis. Global demand for petroleum is expected to increase by 40% in 2025, so environmentally friendly alternative fuels are needed (Hirsch et al., 2005). Biofuel from biological sources (biomass) has a potential resource for renewable energy playing an essential role in the future global energy infrastructure (Cheah et al., 2020; Tandon et al., 2017). Biomass from microalgae has been considered one of the most promising raw materials as a renewable energy source because not producing emissions in their process to generate biofuels (Cheah et al., 2020; Chisti, 2007; Tandon et al., 2017). The recent studies with culture *C. vulgaris* showed the advantages of biomass production compared with plants (Tandon et al., 2017). Besides, *C. vulgaris* has rapid growth correlated with numerous lipid production, low cost, and does not consume large scale tracts of land (Chisti, 2007; Tandon et al., 2017).

The problem in the monoculture of microalgae is how the high biomass with low cost and a short period can be obtained. Single-species cultivation for microalgae is challenging for biomass production on a large scale because it is contrary to the natural tendency of an ecosystem to become increasingly complex (Kazamia et al., 2012). Hence, the use of a microbial consortium consisting of several kinds of bacteria and microalgae can optimize the growth of microalgae in co-culture (Kazamia et al., 2012). This microbial consortium with various types of bacteria from lakes in East Java has a great potential to optimize culture condition; this condition is essential for large-scale cultivation. Co-culture between microalgae and bacteria can increase the microalgae growth because those bacteria are providing essential inorganic mineral elements for microalgae (Marañón et al., 2005). The blooms of algae and aquatic plants are inseparable from the influence of decomposing bacteria in the water environment.
because decomposing bacteria plays as decomposers of organic components into simpler components as nutrients.

Ranu Pani, Ranu Regulo, Ranu Grati, and Telaga Ngebel are favourite lakes in East Java for vacation. Ranu Pani and Ranu Regulo have an average organic substrate content of water, about 18.18% and 26.06%, respectively (Gazali et al., 2014). Ranu Grati has a pH of 7.67 with average temperatures of 29.07°C and a fertility value of 52.41. Based on the TSI value, Ranu Grati classified it as freshwater that had experienced mild eutrophication. Organic materials in the four lakes come from microalgae, plant parts, micro-tricks, agriculture, and waste. The high content of dissolved organic matter (DOM) is an indicator of the abundance and richness of microbes (Gazali et al., 2014).

Amylolysis bacteria can stimulate the microalgae growth by producing amylase enzymes to break down amylum into simple sugars, like glucose, through several stages (Gazali et al., 2014; Silaban et al., 2020). Those simpler molecules can be directly used for supporting microalgae growth. Amylase enzymes from bacteria are widely used in various industries because the strain has rapid growth and require a short amount of time to produce amylases (Keeney & College, 2007). In general, those enzymes were used in textile industries, food industries, and pulp industries. However, those enzymes can also be used in microalgae culture because this type of bacteria can degrade substrates efficiently then be utilized by algae for its growth (Keeney & College, 2007; Souza & Magalhães, 2010).

On the other hand, bacteria producing indole-3-Acetic Acid (IAA hormone) can provide growth factors for responses to light stimulation in plants, including microalgae (Zhao, 2010). IAA is the exogenous auxin class produced by bacteria through the dependent pathway with the addition of tryptophan and independent pathway without tryptophan (Nonhebel, 2015). Most bacteria produce IAA with dependent pathways, notably in the indole-3-acetamide (IAM) pathway and the indole-3-pyruvate (IPA) pathway (Ozdal et al., 2017). IAA hormone is essential for the growth of microalgae, especially *C. vulgaris* (L. De-Bashan, 2008) due to that hormone regulates the growth process and induces the production of metabolites. The utilization of a controlled consortium of bacteria and microalgae could increase microalgae biomass production (Chisti, 2007; Croft et al., 2005; L. E. De-Bashan et al., 2008; Fuentes, Garbayo, Cuaresma, Montero, González-Del-Valle, et al., 2016). This study aims to determine the effect of the amylolytic bacteria and IAA-producing bacteria on the growth of *C. vulgaris* microalgae in co-culture.

**Materials And Methods**

**Samples collection**

Water samples were collected from several lakes in East Java, Indonesia (listed in Table 1) with five stations from each lake. Approximately 1000 mL water samples were stored in a sterile bottle; then, placed into the ice-box to protect the pieces from degradation until the laboratory works.
Table 1
List of sampling location.

| No | Lakes      | Location                | Altitude |
|----|------------|-------------------------|----------|
| 1  | Ranu Pani  | Lumajang regency        | 2.114 masl |
| 2  | Ranu Regulo | Lumajang regency        | 2.100 masl |
| 3  | Ranu Grati  | Pasuruan regency        | 100 masl  |
| 4  | Telaga Ngebel | Ponorogo              | 745 masl  |

Abiotic factors were also measured in this study. Those factors include light intensity using Lux meter SANFIX (LX-1330 B), water temperature, and pH were measured using Refractometer ATAGO S-28E, dissolved oxygen (DO) using oxygen meter (Lutron DO-5510), pH meter Lutron 5509, respectively. Water turbidity was measured using a turbidity meter (TOA-TB 25A).

**Isolation of IAA producing bacteria and quantification IAA contents**

Single colonies that grew with different characteristics were moved into a new plate that contains TSA media with additional tryptophan (25:1). The positive bacterial isolates to produce IAA were tested qualitatively by the colourimetric method using the Salkowski reagent. This reagent was made by 1 mL of 0.5 M FeCl$_3$ (1.35 g / 10 mL) and 50 mL of 50% HClO$_4$ (25 mL HClO$_4$ + 25 mL H$_2$O) then the reagent was stored in an untranslucent bottle at room temperature. Single isolates presenting a pink colour after dropped Salkowski reagent indicated they have the activity to produce IAA. Those positive isolates were used for quantitative tests using UV-vis spectrophotometry.

Single isolates from TSA media were cultured on *Tryptic Soy Broth* (TSB) media with an additional tryptophan (200 ppm) with a ratio (25:1). The spectrophotometric method was used for quantifying IAA contents produced by the isolates. The concentration of IAA was analyzed based on the absorbance value absorbed by UV-Visible spectrophotometer compared to the IAA standard curve. The absorbance value was directly proportional to the IAA standard curve and IAA produced by the isolates. The standard curve was made using pure IAA. The wavelength used was 530 nm; this wavelength was chosen based on the colour produced by the interaction between the Salkowski reagent and the IAA (Glickmann & Dessaux, 1995).

**Amylolitic bacteria selection and semiquantitative assay of amylolytic bacteria activity**

The growth medium was used for the single colonies selection is peptone media 0.1% and Plate Count Agar (PCA) media (instant PCA powder 23.5g / L from Merck, USA). The bacteria from water samples were cultured using the serial dilution method with 0.1% peptone buffer then spread into PCA media. Colonies that grew in PCA media were selected based on different colony morphologies to get the single isolates. NA-modified media that was composed of instant nutrient agar (NA) powder 20g / L (Merck, USA) with an additional 2g of amylum (Sigma-Aldrich, USA) was used for selecting and calculating the hydrolysis index from amylolytic bacteria. These bacterial isolates will produce a clear zone around the
colony after dropped the iodine. The bacterium sample was aseptically inoculated into modified-NA media using the quadrant method. The amylum hydrolysis index of each isolate can be determined based on the diameter of the clear zone formed on the amylum agar medium.

The formula for calculating the amylum hydrolysis index is as follows:

\[
\text{IHA} = \frac{d \text{ of bacterial colony} + d \text{ of clear zone}}{d \text{ of bacterial colony}}
\]

\[d = \text{Diameter}\]

Amylum hydrolysis index data obtained were then analyzed using ANOVA.

**Modified-Gusrina Media preparation**

The Gusrina media that has been modified was made by 45 ppm urea, 30 ppm TSP (Triple Super Phosphate), amount of 1 ppm FeCl\(_3\), and 25 µg / L of vitamin B12 (Hadiyanto et al., 2012). Those components dissolved with distilled water up to 1000 mL into an Erlenmeyer flask then heated with a hot plate. This media stock was made with 10× concentration then it can be stored in the refrigerator.

**Bacterial stock culture and algae stock culture for microbial consortium**

The culture of the highest amylolytic bacteria and the highest ability of IAA-producing bacteria were cultured in 15 mL of nutrient broth (NB) medium at 20\(^0\)C for 24 h. These bacteria were measured the cell density using the 0.5 Mac. Farland solution scale that has equivalent to \(1.5 \times 10^8\) cfu/mL. These bacterial cultures conditions were used as a starter in co-culture.

The microalgae strain that was used in this study is *C. vulgaris* (Purchased from LIPI, Indonesia). The stock of *C. vulgaris* was made with a ratio of 1:4 between the cultures and the medium. The 2,000 mL of the *C. vulgaris* stocks, which have optimal growth, were moved into a plastic tank with capacity up to 15,000 mL, then 10 mL of the Gusrina medium and distilled water up to 10,000 mL were added. After in exponential phase, 1,000 mL of stocks were divided into 20 tank glasses. These tank glasses were used for the consortium in co-culture *C. vulgaris*.

**Consortium condition in microbial consortium with co-culture C. vulgaris**

The medium used for co-culture *C. vulgaris* contains 0.5 mL of Gusrina modified media, 50 mL stock of microalgae, and 400 mL of sterile water. Those solutions for the co-culture medium were placed into a closed-tank glass that has a capacity of 1,000 mL. The treatments were used in co-culture can be seen in Table 2.
Table 2
The treatments used in this study.

| Treatments | Composition |
|------------|-------------|
| I (control) | 50 mL *C. vulgaris* without the bacteria |
| II         | 50 mL *C. vulgaris* + 50 mL amylolytic bacteria |
| III        | 50 mL *C. Vulgaris* + 50 mL IAA-producing bacteria |
| IV         | 50 mL *C. vulgaris* + 25 mL amylolytic bacteria + 25 mL IAA-producing bacteria |

The co-culture was given an aerator set (12 h aerator: 12 h without aerator) and lighting time (16 h lighting: 8 h without light exposure) (Hadiyanto et al., 2012; Lirofiatillah et al., 2020). Those treatments were repeated five times then placed in a closed room at a light intensity of 100–200 µE sec$^{-1}$ m$^{-2}$, 25°C.

**Chlorella vulgaris cells density calculation and data analysis**

Calculation of *C. vulgaris* cells density was using a hemocytometer. The observation was conducted every two days until the 50th days. The bacterial growth was observed every three days. The 50 µL sample was taken from the tank; then, using a dilution technique, the samples were diluted to reach a dilution level of $10^{-6}$. At each dilution of $10^{-4}$, $10^{-5}$, and $10^{-6}$, was taken 100 µL, then put into a petri dish containing PCA media, incubated for 24 h at 20$^0$ C. The collected data were presented in a graphic, and they were analyzed with one-way ANOVA. A 5% LSD followed the significance test to determine the best treatment.

**Results**

**Environmental conditions from Lakes**

The measured abiotic factors from each lake and station were already listed in Table 3.
| Location     | Stations | Abiotic factors |
|--------------|----------|----------------|
|              |          | DO (mg/mL)     |
|              |          | pH             |
|              |          | Salinity       |
|              |          | Water Brightness (cm) |
| Ranu Regulo  | 1        | 8.9            |
|              |          | 9.38           |
|              |          | 0.3            |
|              |          | 75             |
|              | 2        | 8.0            |
|              |          | 8.65           |
|              |          | 0.2            |
|              |          | 75             |
|              | 3        | 8.4            |
|              |          | 8.65           |
|              |          | 0.2            |
|              |          | 80             |
|              | 4        | 11.3           |
|              |          | 9.3            |
|              |          | 0              |
|              |          | 75             |
|              | 5        | 11.6           |
|              |          | 9.4            |
|              |          | 0.2            |
|              |          | 75             |
| Ranu Pani    | 1        | 4.2            |
|              |          | 7.24           |
|              |          | 0.4            |
|              |          | 80             |
|              | 2        | 5.0            |
|              |          | 6.83           |
|              |          | 0.3            |
|              |          | 82             |
|              | 3        | 6.0            |
|              |          | 6.95           |
|              |          | 0.2            |
|              |          | 100            |
|              | 4        | 11.6           |
|              |          | 7.3            |
|              |          | 0.4            |
|              |          | 50             |
|              | 5        | 6.7            |
|              |          | 6.88           |
|              |          | 0.4            |
|              |          | 50             |
| Ranu Grati   | 1        | 13.4           |
|              |          | 8.7            |
|              |          | 0.2            |
|              |          | 163            |
|              | 2        | 12.3           |
|              |          | 8.4            |
|              |          | 0.1            |
|              |          | 165            |
|              | 3        | 13.5           |
|              |          | 8.6            |
|              |          | 0.1            |
|              |          | 368            |
|              | 4        | 13.0           |
|              |          | 8.71           |
|              |          | 0              |
|              |          | 757            |
|              | 5        | 13.5           |
|              |          | 8.84           |
|              |          | 0              |
|              |          | 757            |
| Telaga Ngebel| 1        | 2.5            |
|              |          | 6.98           |
|              |          | 0.2            |
|              |          | 676            |
|              | 2        | 4.3            |
|              |          | 7.25           |
|              |          | 0.1            |
|              |          | 517            |
|              | 3        | 8.5            |
|              |          | 7.69           |
|              |          | 0.1            |
|              |          | 383            |
|              | 4        | 6.0            |
|              |          | 7.17           |
|              |          | 0.1            |
|              |          | 450            |

**Table 3**
**Abiotic factors measured from each lake**

The total isolates of IAA-producing bacteria and produced IAA content

Ninety isolates of bacteria that positively produce IAA from Ranu Grati, Ranu Pani, Ranu Regulo, and Telaga Ngebel were successfully isolated. As a result, the ten highest bacterial isolates based on UV-Visible spectrophotometer measurements were recorded in Table 4, and complete data were listed in (supplementary files 1). The bacterial isolate C from Ranu Pani was able to produce the highest IAA hormone concentration of 158.11 ppm.
Table 4
Ten highest isolates of IAA-producing bacteria to produce IAA

| Isolate | Absorbance | Concentration IAA (ppm) | Source               |
|---------|------------|-------------------------|----------------------|
| C       | 0.653      | 158.11                  | Ranu Pani, Regulo    |
| G13     | 0.453      | 104.05                  | Ranu Grati           |
| G28     | 0.298      | 62.16                   | Ranu Grati           |
| G33     | 0.212      | 38.92                   | Ranu Grati           |
| G1      | 0.210      | 38.38                   | Ranu Grati           |
| G18     | 0.176      | 29.19                   | Ranu Grati           |
| J       | 0.168      | 27.03                   | Ranu Pani, Regulo    |
| G7      | 0.161      | 25.14                   | Ranu Grati           |
| TN      | 0.161      | 25.14                   | Telaga Ngebel        |
| G4      | 0.152      | 22.70                   | Ranu Grati           |

The total amylolytic bacteria and their activity

Fifty-three bacterial isolates from Ranu Pani, Ranu Regulo, Ranu Grati, and Telaga Ngebel had been known to be amylolytic. The average results of calculating the most extensive ten amylum hydrolysis index can be seen in Table 5, and full data can be seen in (supplementary files 2). Table 5 shows that the L bacterial isolate from Ranu Pani had the highest hydrolysis index value of 5.89.

Table 5
Ten highest isolates to hydrolyze amylum

| Isolates | Hydrolysis Index | Source               |
|----------|------------------|----------------------|
| L        | 5.9              | Ranu Pani, Regulo    |
| G33      | 4.7              | Ranu Grati           |
| D        | 4.65             | Ranu Pani, Regulo    |
| KN       | 4.63             | Lake of Ngebel       |
| VN       | 4.15             | Lake of Ngebel       |
| IN       | 4.08             | Lake of Ngebel       |
| P        | 4.02             | Ranu Pani, Regulo    |
| G13      | 3.77             | Ranu Grati           |
| V        | 3.43             | Ranu Pani, Regulo    |
| NN       | 3.12             | Lake of Ngebel       |
**Bacterial isolate C and L identification**

The morphological characteristics of isolate C and isolate L can be seen in Table 6.

| Characteristics      | Isolate C | Isolate L                |
|----------------------|-----------|--------------------------|
| Colony color         | White     | White                    |
| Shape of colony      | Circular  | Irregular                |
| Colony side          | Smooth, gloomy | Choppy, gloomy,       |
| Colony diameter      | 0.1 cm    | 0.285 cm                 |
| Cell shape           | Basil     | Basil                    |
| Spores               | -         | Terminal                 |
| Gram                 | +         | +                        |

Based on those morphological characteristics, bacterial isolate C and bacterial isolate L could be described as genus of Bacilli; but, the annotation to level species cannot be determined.

To inform identification results based on barcoding DNA with 16S rRNA sequence, in this paper, we were re-analysis 16S sequence from (Rodiansyah et al., 2021) with additional reference sequences specific on *Bacillus cereus* Group and *Bacillus substilis* Group. The maximum likelihood (ML) tree showed that both genus Bacilli separated into two groups with a confidence value of 100. The bacterial isolate C is located in one clade with *Bacillus paramycoides* with a confidence value of 66. However, the bacterial isolate L was situated in the same clade with *Bacillus amyloliquefaciens* with a bootstrap value of 36 (Fig. 1).

The genetic distance analysis from genus Bacillus shows a significant difference in sequences between-group similarity, about 94%, but within-group similarity shows they are homogeneity (Similarity 99%) (Table 7).

| Groups                               | p-distance | Similarity | SE  |
|--------------------------------------|------------|------------|-----|
| *Bacillus cereus* Group (Isolate C)  | 0.0033     | 99.67%     | 0.0009 |
| *Bacillus substilis* Group (Isolate L) | 0.0061     | 99.39%     | 0.0011 |
| Out of the Group                     | 0.0036     | 99.64%     | 0.0016 |

**Chlorella vulgaris cells density and data analysis**
The graph in Fig. 2 below shows the influence of the treatments on the microalgae growth from day 0 to 50. The growth data was measured with the calculation of cell density using haemocytometer. In general, microbial consortium treatments have significantly different growth of microalgae compared to control.

In treatment I (control treatment), the pattern of the growth of \textit{C. vulgaris} start from the lag phase (day 0 to 6), then the log phase (day 6 to 12), and finally, the death phase (day 12 to 50) (purple line in Fig. 2). The graph of the microalgae growth in this treatment has a moderate increase from day 2 to 12 that has the highest average cell density reaching $3.5 \times 10^5$ cells/mL. After that, the growth of microalgae decreased gradually on day 14 and then levelled off until day 50 that has an average cell density of $3 \times 10^3$ cells/mL.

In treatment II, the growth of \textit{C. vulgaris} starts from the lag phase (day 0 to 10), log phase (day 10 to 38), and death phase (day 40 to 50) (green line in Fig. 2). The growth of microalgae in the lag phase decreased slightly from day 0 to 10, in the log phase rose gradually and then climbed sharply, with an average cell density of $2.4 \times 10^6$ cells/mL. After reaching the death phase, the growth of microalgae dropped moderately, with an average cell density of $1.9 \times 10^6$ cells/mL. In treatment III, the development of \textit{C. vulgaris} start from the lag phase (day 0 to 8); after that, the growth of microalgae fell steadily until day ten, then boom dramatically at the log phase (day 12 to 50) with cells density reaching at $2.7 \times 10^6$ cells/mL (red line in Fig. 2). In treatment IV, the growth of \textit{C. vulgaris} starts from the lag phase (day 0 to 10), log phase (day 12 to 38), and death phase (day 40 to 50) (blue line in Fig. 2). At the lag phase, the growth of microalgae decreased slowly then climb dramatically at the log phase with a cell density of $2.1 \times 10^6$ cells/mL; finally, there was a fall gradual in the death phase with an average cell density of $1.4 \times 10^6$ cells/mL.

The hypothesis calculated with ANOVA t-test shows that te F value > F Table (Table 8); as a result, the hypothesis is accepted, indicated that there is an influence of a consortium of amylolytic bacteria and IAA producing bacteria on the \textit{C. vulgaris} growth.

|                      | Sum of Squares | df | Mean Square | F     | Sig. |
|----------------------|----------------|----|-------------|-------|------|
| Between Groups       | 12.468         | 3  | 4.156       | 16.88 | .000 |
| Within Groups        | 23.136         | 94 | .246        |       |      |
| Total                | 35.604         | 97 |             |       |      |

The treatments other than control gives a significant influence on the development of the density of \textit{C. vulgaris} cells. The further test with the Least Significant Difference Test (LSD) significant 5% can be seen in Table 8. Treatment III showed the best treatment for increasing \textit{C. vulgaris}, which microbial consortium treatments are significantly different compared to the control treatment (Table 9).
Table 9
Results of LSD 5% from 4 treatments

| Treatment | N  | Subset for alpha = 0.05 |
|-----------|----|------------------------|
|           | 1  | 2                      |
| LSD\(^a\) |    |                         |
| C         | 20 | .904                   |
| C + A     | 26 | 1.7417                 |
| C + I + A | 26 | 1.7993                 |
| C + I     | 26 | 1.8178                 |
| Sig.      | 1.00 | .619                  |

Discussion

Heterotrophic bacteria are available in various ecosystems, including lake waters (K. Liu et al., 2016). The presence of heterotrophic bacteria in lake waters is influenced by organic material in lake waters that can support bacterial growth (Yuningsih et al., 2014). Heterotrophic bacteria can decompose complex organic compounds containing elements C, H, and N into simpler compounds because they can produce extracellular enzymes (K. Liu et al., 2016). Some bacterial isolates that were successfully isolated in this study prove that the waters from four lakes can supply the nutrition needed by bacteria for supporting their life.

Amylolytic bacteria can gradually degrade amylum in lake waters because amylum can not directly convert amylum into glucose (Rahmansyah & Sudiana, 2010). Initially, amylum will be hydrolyzed into the dextrin compound in the form of polysaccharides. The dextrin is re-hydrolyzed again into oligosaccharides in the form of maltose. Maltose is subsequently converted into monosaccharides in the form of glucose (Brewster, 1953). Our highest amylolytic bacteria from Ranu Pani, based on phenotypic and genotypic characteristics, was identified as *Bacillus amyloliquefaciens*. This species is an amylolytic bacteria that play an essential role as a remodel of organic materials into simpler inorganic components in nature (Waluyo, 2009). Amylolytic bacteria can produce extracellular enzymes to degrading amylum in water (Brown et al., 2008). Amylum that has been degraded into simpler compounds can be utilized by *C. vulgaris* microalgae as a raw material for the photosynthesis process (Brown et al., 2008). The carbon element from carbohydrate and protein decomposition by bacteria in waters can increase the biomass of microalgae *C. vulgaris* (Brown et al., 2008). The essence of carbon can be assimilated in waters in the form of CO\(_2\), which plays a role in the photosynthetic process of microalgae *C. vulgaris* (Hopkin, 2020).

On the other hand, this study also uses the highest potential bacteria producing IAA from Ranu Pani, identified as *Bacillus paramycoides*. The analysis results of variants obtained that IAA-producing bacteria significantly influence the growth of *C. vulgaris*. Further tests explained that the treatment of a consortium of IAA-producing bacteria had the most significant influence on the cell density of *C. vulgaris*.
as the indicator from rate growth. Bacteria produce IAA hormones through gene regulation and tryptophan biosynthesis in microalgae as a precursor for bacteria regulation. Amino acids, such as tryptophan, are easily detected by pairs of organisms in the culture to act as nutritional signals for synthesis IAA with indole 3-glycerol phosphate as the primary substrate (Hopkin, 2020; Vessey, 2016). They live together in waters as mutualism symbiotic. Auxin produced from these bacteria could encourage microalgae cell division processes to increase microalgae biomass (Cooper & Smith, 2015).

*Bacillus paramycoides* play a vital role in the decomposition of organic compounds that have a function in the growth of microalgae (Vessey, 2016). This species can hydrolyze amylum, produce H$_2$S, urease, tryptophan deaminase, indole production, and acid production from mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, and arabinose (Y. Liu et al., 2017). These multiple potencies from *Bacillus paramycoides* showed probably more efficient for stimulating the *C. vulgaris* growth because that bacterium continues to produce IAA in a long period at culture media (Pratt, 1938).

In the microbial consortium, bacteria can reduce the oxygen pressure in the culture, and they can convert complex elements (N, S, P, and C) to become elements ready consumed for algae photosynthesis (Ramanan et al., 2016)(Mujtaba & Lee, 2016). Those elements reduced by bacteria can be used as source components for microalgae photosynthesis; after that, the oxygen as photosynthesis products can be used for bacteria to grow and produce some valuable enzymes and metabolites like auxin as a growth factor. Microalgae can consume this auxin to increase the productivity and quality of biomass *C. vulgaris* (Fuentes, Garbayo, Cuaresma, Montero, Valle, et al., 2016). This complex interaction between bacteria and microalgae has tremendous benefits for cultivating the most appropriate, efficient, and environmentally friendly microbe (Dao et al., 2018). Besides on the biological factor, optimalization of microalgae growth on bioreactor also depends on abiotic factors, like flashing light exposure (Lirofatiillah et al., 2020; Yustinadiar et al., 2020), temperatures (Chakraborty et al., 2016), and optimal nutrients used in the medium (Chandra et al., 2019). In the future, this microbial consortium combined with optimal bioreactor could be considered for the new method for culturing microalgae on the industrial scale that has the potency for biodiesel at a low cost and environment friendly (Allen et al., 2018).

**Conclusions**

The most potential IAA-producing bacteria identified as *Bacillus paramycoides* from Ranu Pani with an IAA concentration of 158.11 ppm, while the highest amylolytic bacteria has a hydrolyze index of 5.9 identified as *Bacillus amyloliquefaciens*. The microbial consortium shows the effectiveness of stimulating *C. vulgaris* growth compared to control. The microbial consortium with IAA-producing bacteria supplementation was the most effective to enhance microalgae growth, reaching up to $2.7 \times 10^6$ cells/mL of cell density on day 50. Future research with broad-scale culture in bioreactor and exploring the specific interaction in co-culture are needed for obtaining high biomass.

**Declarations**

**Acknowledgements**
The authors are grateful to the laboratory of microbiology and laboratory biology molecular for provides place and instruments for this study.

Funding

This research was funded by PNBP, Universitas Negeri Malang 2019 with the CAMRY PUI scheme.

Availability of data and materials

The complete data was available in supplementary files

Ethics approval and consent to participate

Not applicable

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable

Authors’ contributions

IN carried out the co-culture C. vulgaris with microbial consortium, tabulated all data and wrote the manuscript, SP designed this study, SP and AW supervised and reviewed the manuscript, YKB carried out the potential test for IAA-producing bacteria and amylolytic bacteria, AR analyzed the bacterial identification, wrote, and reviewed the manuscript, SP, YKB, and DA collected samples for this study.

Author details

1Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang, Malang 65145, Indonesia

References

1. Allen, J., Unlu, S., Demirel, Y., Black, P., & Riekhof, W. (2018). Integration of biology, ecology and engineering for sustainable algal-based biofuel and bioproduct biorefinery. In Bioresources and Bioprocessing (Vol. 5, Issue 1). https://doi.org/10.1186/s40643-018-0233-5
2. Brewster, R. . (1953). Organic Chemistry (2th ed.). Prentice Hall, Inc.
3. Brown, C. S., Anderson, V. J., Claassen, V. P., Stannard, M. E., Wilson, L. M., Atkinson, S. Y., Bromberg, J. E., lli, T. A. G., Munis, M. D., Brown, C. S., Anderson, V. J., Claassen, V. P., Stannard, M. E., Wilson, L. M., Atkinson, S. Y., Bromberg, J. E., lli, T. A. G., & Munis, M. D. (2008). Restoration Ecology and
Invasive Plants in the Semiarid West. *Invasive Plant Science and Management, 1*(4), 399–413. https://doi.org/10.1614/IPSM-08-082.1

4. Chakraborty, S., Mohanty, D., Ghosh, S., & Das, D. (2016). Improvement of lipid content of Chlorella minutissima MCC 5 for biodiesel production. *Journal of Bioscience and Bioengineering, 122*(3), 294–300. https://doi.org/10.1016/j.jbiosc.2016.01.015

5. Chandra, R., Amit, & Ghosh, U. K. (2019). Effects of various abiotic factors on biomass growth and lipid yield of Chlorella minutissima for sustainable biodiesel production. *Environmental Science and Pollution Research, 26*(4), 3848–3861. https://doi.org/10.1007/s11356-018-3696-1

6. Cheah, W. Y., Show, P. L., Yap, Y. J., Fatimah, H., Zaid, M., Lam, M. K., Lim, J. W., Ho, Y., & Tao, Y. (2020). CY-1 biomass and lipid production in palm oil mill effluent (POME) using novel-designed photobioreactor. *Bioengineered, 11*(1), 61–69. https://doi.org/10.1080/21655979.2019.1704536

7. Chisti, Y. (2007). Biodiesel from microalgae. In *Biotechnology Advances* (Vol. 25, Issue 3, pp. 294–306). https://doi.org/10.1016/j.biortech.2017.09.079

8. Cooper, M. B., & Smith, A. G. (2015). Exploring mutualistic interactions between microalgae and bacteria in the omics age. In *Current Opinion in Plant Biology* (Vol. 26, pp. 147–153). Elsevier Ltd. https://doi.org/10.1016/j.pbi.2015.07.003

9. Croft, M. T., Lawrence, A. D., Raux-Deery, E., Warren, M. J., & Smith, A. G. (2005). Algae acquire vitamin B12 through a symbiotic relationship with bacteria. *Nature, 438*(7064), 90–93. https://doi.org/10.1038/nature04056

10. Dao, G., Wu, G., Wang, X., Zhuang, L., Zhang, T., & Hu, H.-Y. (2018). Bioresource Technology Enhanced Growth and Fatty Acid Accumulation of Microalgae Scenedesmus sp. LX1 by Two Types of Auxin. *Bioresource Technology, 247*(July 2017), 561–567. https://doi.org/10.1016/j.biortech.2017.09.079

11. De-Bashan, L. (2008). INVOLVEMENT OF INDOLE-3-ACETIC ACID PRODUCED BY THE GROWTH-PROMOTING BACTERIUM AZOSPIRILLUM SPP. IN PROMOTING GROWTH OF CHLORELLA VULGARIS. *Phycol, 947*, 938–947. https://doi.org/10.1111/j.1529-8817.2008.00533.x

12. De-Bashan, L. E., Antoun, H., & Bashan, Y. (2008). Involvement of indole-3-acetic acid produced by the growth-promoting bacterium Azospirillum spp. in promoting growth of Chlorella vulgaris. *Journal of Phycology, 44*(4), 938–947. https://doi.org/10.1111/j.1529-8817.2008.00533.x

13. Fuentes, J. L., Garbayo, I., Cuaresma, M., Montero, Z., González-Del-Valle, M., & Vílchez, C. (2016). Impact of microalgae-bacteria interactions on the production of algal biomass and associated compounds. In *Marine Drugs* (Vol. 14, Issue 5, p. 100). MDPI AG. https://doi.org/10.3390/md14050100

14. Fuentes, J. L., Garbayo, I., Cuaresma, M., Montero, Z., Valle, M. G., & Vílchez, C. (2016). Impact of Microalgae-Bacteria Interactions on the Production of Algal Biomass and Associated Compounds. *Marine Drugs, 4*(100), 2–16. https://doi.org/10.3390/md14050100

15. Gazali, A., Suheriyanto, D., & Romaidi. (2014). *Macrozoobenthos Biodiversity as Bioindicator of Water Quality in Ranu Pani-Ranu Regulo, Bromo Tengger Semeru National Park.* 86–91.
16. Glickmann, E., & Dessaux, Y. (1995). A Critical Examination of the Specificity of the Salkowski Reagent for Indolic Compounds Produced by Phytopathogenic Bacteria. 61(2), 793–796.

17. Hadiyanto, H., Azimatun Nur, M. M., & Hartanto, G. D. (2012). Cultivation of chlorella sp. As biofuel sources in palm oil mill effluent (POME). International Journal of Renewable Energy Development, 7(2), 45–49. https://doi.org/10.14710/ijred.1.2.45-49

18. Hirsch, R., Bedzek, R., & Wendling, R. (2005). Peaking of World Oil Production: Impacts, Mitigation and Risk Management. https://doi.org/10.2172/939271

19. Hopkin, M. (2020). Ecological Society of America. In Nature (Vol. 12, Issue 4, pp. 937–947). https://doi.org/10.1038/news050808-1

20. Kazamia, E., Aldridge, D. C., & Smith, A. G. (2012). Synthetic Ecology – A Way Forward for Sustainable Algal Biofuel Production? Journal of Biotechnology, 162(1), 163–169. https://doi.org/10.1016/j.jbiotec.2012.03.022

21. Keeney, J. B., & College, J. (2007). Microorganisms: Applications in Molecular Biology Bacteria: Prokaryotic Unicellular. https://doi.org/10.1002/9780470015902.a0000971.pub2

22. Lirotillah, Prabaningtyas, S., Saptasari, M., Aridowi, D., Marisahaniulfah, M., Listyorini, D., & Suyono, E. A. (2020). Effect of differences in the form of photobioreactor prototypes and aeration period on Chlorella sp. Cell growth in co-culture with bacteria. AIP Conference Proceedings, 2231(1), 40014. https://doi.org/10.1063/5.0002527

23. Liu, K., Liu, Y., Jiao, N., Zhu, L., Wang, J., Hu, A., & Liu, X. (2016). Vertical variation of bacterial community in Nam Co, a large stratified lake in central Tibetan Plateau. Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology, 109(10), 1323–1335. https://doi.org/10.1007/s10482-016-0731-4

24. Liu, Y., Du, J., Lai, Q., Zeng, R., Ye, D., Xu, J., & Shao, Z. (2017). Proposal of nine novel species of the bacillus cereus group. International Journal of Systematic and Evolutionary Microbiology, 67(8), 2499–2508. https://doi.org/10.1099/ijsem.0.001821

25. Marañón, E., Cermeño, P., & Pérez, V. (2005). Continuity in the Photosynthetic Production of Dissolved Organic Carbon from Eutrophic to Oligotrophic Waters. Marine Ecology Progress Series, 299, 7–17.

26. Mujtaba, G., & Lee, K. (2016). Advanced treatment of wastewater using symbiotic co-culture of microalgae and bacteria. In Applied Chemistry for Engineering (Vol. 27, Issue 1, pp. 1–9). Korean Society of Industrial Engineering Chemistry. https://doi.org/10.14478/ace.2016.1002

27. Nonhebel, H. M. (2015). Tryptophan-independent indole-3-acetic acid synthesis: Critical evaluation of the evidence. In Plant Physiology (Vol. 169, Issue 2, pp. 1001–1005). American Society of Plant Biologists. https://doi.org/10.1104/pp.15.01091

28. Ozdal, M., Ozdal, O. G., Sezen, A., Algur, O. F., & Kurbanoglu, E. B. (2017). Continuous production of indole-3-acetic acid by immobilized cells of Arthrobacter agilis. 3 Biotech, 7(1). https://doi.org/10.1007/s13205-017-0605-0

29. Pratt, R. (1938). Influence of Auxins on the Growth of Chlorella vulgaris. American Journal of Botany, 25(7), 498. https://doi.org/10.2307/2436677
30. Rahmansyah, M., & Sudiana, I. M. (2010). Soil microbial enzymatic activity relate to role of methanotrophic bacteria in the tropical forest soil of Gunung Salak National Park. *ARPN Journal of Agricultural and Biological Science, 5*(2), 51–57.

31. Ramanan, R., Kim, B., Cho, D., Oh, H., & Kim, H. (2016). Algae – Bacteria Interactions: Evolution, Ecology and Emerging Applications. *Biotechnology Advances, 34*(1), 14–29. https://doi.org/10.1016/j.biotechadv.2015.12.003

32. Rodiansyah, A., Mahmuda, A. F., Ulfah, M. M., Rohmawati, U., Listyorini, D., Suyono, E. A., & Prabaningtyas, S. (2021). Identification of Potential Bacteria on Several Lakes in East Java, Indonesia Based on 16S rRNA Sequence Analysis. *Hayati J Biosci, 28*(2), In Press.

33. Silaban, S., Marika, D. B., & Simorangkir, M. (2020). Isolation and characterization of amylase-producing amylolytic bacteria from rice soil samples. *Journal of Physics: Conference Series, 1485*(1), 12006. https://doi.org/10.1088/1742-6596/1485/1/012006

34. Souza, P. M. de, & Magalhães, P. de O. e. (2010). Application of Microbial a-Amylase in Industri-A Review. *Brazilian Journal of Microbiology, 41*, 850–861.

35. Tandon, P., Jin, Q., & Huang, L. (2017). A promising approach to enhance microalgae productivity by exogenous supply of vitamins. In *Microbial Cell Factories* (Vol. 16, Issue 1, p. 219). https://doi.org/10.1186/s12934-017-0834-2

36. Vessey, J. K. (2016). *Plant Growth Promoting Rhizobacteria as Biofertilizers Growth Promoting Rhizobacteria as Biofertilizers*. 255(2), 571–586.

37. (2009). *Mikrobiologi Lingkungan*.

38. Yuningsih, H. D., Anggoro, S., & Soedarsono, P. (2014). HUBUNGAN BAHAN ORGANIK DENGAN PRODUKTIVITAS PERAIRAN PADA KAWASAN TUTUPAN ECENG GONDOK, PERAIRAN TERBUKA DAN KERAMBA JARING APUNG DI RAWA PENING KABUPATEN SEMARANG JAWA TENGAH. *Management of Aquatic Resources Journal (MAQUARES), 3*(1), 37–43. https://doi.org/10.14710/marj.v3i1.4284

39. Yustinadiar, N., Manurung, R., & Suantika, G. (2020). Enhanced biomass productivity of microalgae Nannochloropsis sp. in an airlift photobioreactor using low-frequency flashing light with blue LED. *Bioprocess, 7*, 43. https://doi.org/10.1186/s40643-020-00331-9

40. Zhao, Y. (2010). Auxin biosynthesis and its role in plant development. *Annual Review of Plant Biology, 61*, 49–64. https://doi.org/10.1146/annurev-arplant-042809-112308

Figures
Figure 1

Phylogenetic tree of C isolate, and L isolate with ML method. Salmonella enterica subs. enterica Strain LT2 and Ty2 set as out of the group.
Figure 2

C. vulgaris cell growth curve in each treatment. The treatment I (purple line), Treatment II (green line), Treatment III (red line), and Treatment IV (blue line).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GraphicalAbstractrevisedFinal.png
- SupplementaryFinal.docx