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MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF *PSEUDOXANTHOMONAS* SPECIES AND ITS OPTIMAL GROWTH KINETICS

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ABSTRACT

Pseudoxanthomonas species are candidate microorganisms with potential for biocontrol, therefore this study aimed to isolate and characterize a strain of *Pseudoxanthomonas* sp. by morphological, physiological and biochemical analysis. In addition, *Pseudoxanthomonas* sp. growth conditions were also optimized. The effects of different culture mediums, carbon sources, nitrogen sources and pH on the growth of *Pseudoxanthomonas* sp. were studied by a combination of univariate and orthogonal experiments to determine its optimal growth kinetics. The results showed that the isolated strains were gram-negative, pale yellow, round, raised, with clean edges, translucent and shiny. One of the best-growing representative strains was selected for further physiological and biochemical characterization and was identified as *Pseudoxanthomonas* sp. The optimal growth conditions for the representative strain were reported. It was found that using LB medium as the base medium, adding 1.5% glucose as the carbon source, 1.5% yeast extract as the nitrogen source, adjusting the medium pH to 7.5, and incubating at a temperature of 37°C yielded the best growth kinetics. These findings hold significance for subsequent research endeavors. Specifically, they are pivotal for advancing the biotechnological development of *Pseudoxanthomonas* sp.

***Keywords*:** Isolation; Characterization; *Pseudoxanthomonas* sp.; Optimization; Culture conditions

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1. INTRODUCTION

There is a growing need for sustainable agriculture to find an environmentally friendly alternative to chemical pesticides and physical methods to control harmful substances in the environment. Biocontrol agents (BCAs) are a relatively safe, effective and environmentally friendly option, so how to effectively use BCAs is an important part of sustainable agriculture today, where screening for microorganisms with biocontrol potential is an important step in the development of biocontrol agents (Droby et al. 2009; Fravel 2005; Havens et al. 2019; Xu and Hu 2020). *Pseudoxanthomonas* sp. is a Gram-negative, non-flagellated, non-spore-producing, rod-shaped bacterium (Finkmann et al. 2000). Nowadays, *Pseudoxanthomonas* sp. is widely used in biocontrol and other applications. First, *Pseudoxanthomonas* sp. can remove contaminants that persist in the environment and pose a risk to humans and animals and can degrade benzo[a]pyrene (B[a]P), the endocrine disruptor nonylphenol (NP), non-steroidal anti-inflammatory drugs (NSAIDs), neonicotinoid insecticides (NNIs), non-steroidal anti-inflammatory drugs (NSAIDs), neonicotinoid insecticides, low molecular weight polycyclic aromatic hydrocarbons (PAHs), aliphatic and aromatic hydrocarbons and other environmental pollutants (Nayak et al. 2009; Nopcharoenkul et al. 2013; Lu et al. 2019; Pang et al. 2020a; 2020b; Mohapatra and Phale, 2021; Bhandari et al. 2021; Lu et al. 2022). *Pseudoxanthomonas* sp. is capable of simultaneously degrading diclofenac (DCF), ibuprofen (IBU), and naproxen (NAP) from the environment, with the potential to remove the threat of NSAIDs from aquatic ecosystems (Lu et al. 2019). *Pseudoxanthomonas suwonensis* stain capable of degrading the organophosphate pesticide profenofos from pesticide-contaminated soil samples. The optimal conditions for profenofos degradation by *P. suwonensis* strain HNM were pH 7 and 30°C (Talwar and Ninnekar 2015). *Pseudoxanthomonas* sp. also reported to degraded diesel, crude oil, n-tetradecane and n-hexadecane from different substrates (Nopcharoenkul et al. 2013). A biosurfactant-producing *Pseudoxanthomonas* sp. exhibits emulsifying activity against both aliphatic and aromatic hydrocarbons (Nayak et al. 2009). *Pseudoxanthomonas* sp. has also ability to degrade benzene, toluene and ethylbenzene compounds (Ruan et

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al. 2022). *Pseudoxanthomonas* sp. was able to degrade the engineering thermoplastic Bisphenol-A polycarbonate (PC), which is a polycarbonate that has been used for the production of polycarbonate (Yue et al. 2021).

Furthermore, *Pseudoxanthomonas* sp. has the ability to degrade cellulose, produce ethanol, act as an antagonist to pathogens, and promote plant growth (Du et al. 2015; Morales-Borrell et al. 2020; Nakkeeran et al. 2021). Examined and described a pear-derived strain, evaluating its capacity for indoleacetic acid (IAA) production, nitrogen fixation, phosphate solubilization and siderophore production. The identified strain, recognized as *Pseudoxanthomonas mexicana* notably enhances plant growth (Liaqat and Eltem 2016). *Pseudoxanthomonas mexicana* S254 exhibited maximum arsenic resistance (225 mM) and produced growth hormone (14.15 µg/mL) to stimulate the growth of *Vigna radiata* (Huda et al. 2022). *Pseudoxanthomonas taiwanensis* isolated from China has a high ethanol production capacity and that *P. taiwanensis* can effectively promote the ethanol production of other bacteria. The application of *P. taiwanensis* to the newly developed bacterial colony resulted in a conversion of 78% of ethanol with ethanol titers up to 2.5g/L. These findings suggest that *P. taiwanensis* has excellent ethanol production ability (Du et al. 2015). *Pseudoxanthomonas suwonensis* strain J1 has the ability to efficiently degrade cellulose (Hou et al. 2015). *Pseudoxanthomonas* sp. R-28 degraded 96% and 95% of filter paper and pure cellulosic waste, respectively, in 5 days of incubation. Additionally, it degraded 60% of non-pretreated rice straw in 7 days (Kumar et al. 2015). The primary aim of this study was to isolate a strain of *Pseudoxanthomonas* sp. from the environment and determine the optimal medium and culture conditions for its growth using one-way and orthogonal experiments.

2. MATERIALS AND METHODS

2.1. Chemicals and Culture Media

Luria−Bertani broth, agar powder, Yeast extract, soya peptone, tryptone and Gram stain were purchased from Huankai Microbial (Guangdong, China). D-(+)-glucose, fructose, lactose, D-sorbitol, soluble starch, urea, beef extract and sodium chloride were purchased from Aladdin (Shanghai, China).

2.2. Strain Isolation and Purification

The samples were collected from soil and water. Each sample weighed 10g and was mixed with 90mL of sterilized 0.85% saline solution. After incubation on a rotary shaker at 37°C for 12h, the samples were centrifuged at 5000 r/min for 5min. The supernatant was then diluted from 10^{-1} to 10^{-7}, fold with sterilized 0.85% saline solution. 200µL of the dilution was spread on LB agar medium and incubated for 24h at 37°C.

The cultured bacteria were picked and purified according to the differences in colony morphology, color and size, and purified on LB agar medium. The purification operation was repeated 5 times to obtain the single colonies. These isolates were ultimately retained as pure cultures on LB agar medium for additional analysis.

2.3. Morphological, Physiological and Biochemical Characterization

The best-growing strain was selected and identified by examining its colony morphology and growth pattern. The stain was placed on LB agar medium and incubated at 37°C for 24h. The characterization of the strain's morphology was recorded, considering factors such as color, shape, size, transparency, surface characteristics, and edge characteristics. Subsequently, Gram staining was employed for further morphological identification of the strain. To investigate the physiological and biochemical characteristics, standard techniques outlined in "Bergey's Manual of Determinative Bacteriology" were employed.

2.4. The Growth Curve Determination of Strain

The strain selected in the previous experiments were used in this experiment. The selected strains were streaked on LB agar medium. The newly grown single colonies were picked out to the LB medium and then was cultivated at 37°C on a rotary shaker at 180 r/min until the OD_{600} value was 1.0. The culture was inoculated into 50mL LB medium at 1% inoculum size and cultivated on a rotary shaker at 37°C and 180 r/min, samples were taken every 2h. The growth curve was drawn after OD_{600} was determined by microplate reader.

2.5. Effects of Different Culture Conditions on the Growth of the Strain

In order to obtain the optimal culture conditions of the representative strain, the culture conditions of the strain were optimized by single factor, including different carbon sources (glucose, fructose, lactose, sorbitol, soluble starch), nitrogen sources (urea, yeast extract, soya peptone, tryptone, beef extract), pH (5, 5.5, 6, 6.5, 7, 7.5, and 8) and temperature (16, 23, 30, 37, 44, and 51°C).

The effects of carbon sources and their concentration on the growth of the strain were studied. The LB medium served as the base medium, and glucose, fructose, lactose, sorbitol, and soluble starch were chosen as carbon sources, each added at a concentration of 3%. The pH value was adjusted to 7. The culture solution was inoculated

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with a 1% inoculum size in the medium containing various carbon sources. The basic medium served as the control, and the culture was sampled after 18 hours of incubation on a rotary shaker at 37°C and 180 r/min. The OD_{600} of each culture was determined. Further, in order to explore the optimal concentration of dominant carbon sources, the dominant carbon source obtained from the above experiments was selected, and the optimal carbon sources were adjusted to six different concentrations of 0.5, 1, 1.5, 2, 2.5 and 3%, respectively.

The effects of nitrogen sources and their concentration on the growth of the strain were studied. LB medium was used as the basic medium, and the dominant carbon source and its optimal concentration were selected according to the experimental results of the previous step. Then urea, yeast extract, soya peptone, tryptone and beef extract were selected as nitrogen sources, with 3% added respectively. The pH was adjusted to 7, The seed solution was inoculated with 1% inoculum size in the medium with different nitrogen sources. The culture was taken after 18 hours cultivated on a rotary shaker at 37°C and 180 r/min. The OD_{600} of each culture was determined. Further, the dominant nitrogen sources obtained from the above experiments were selected, and the optimal nitrogen sources were adjusted to six different concentrations of 0.5, 1, 1.5, 2, 2.5 and 3%, respectively, to explore the optimal concentration of dominant nitrogen sources.

The effects of pH value of culture medium on the growth of strain were studied. LB medium was used as the basic medium. According to the experimental results of the previous step, the optimal concentration of dominant carbon source and nitrogen source was added, and the pH of the medium was adjusted to 5, 5.5, 6, 6.5, 7, 7.5, and 8. The seed solution was inoculated in LB medium with different pH at 1% inoculum size. The culture was taken after 18 hours cultivated on a rotary shaker at 37°C and 180 r/min. The OD_{600} of each culture was determined.

The effect of culture temperature on the growth of the strain was studied. The seed solution was inoculated into LB modified medium obtained in the above experiment according to the inoculation amount of 1%, and the OD_{600} of each medium was determined at 180 r/min for 18h at five different temperatures of 16, 23, 30, 37, 44, and 51°C.

2.6. Orthogonal Experiment

On the basis of single factor experiment, the optimal carbon source concentration, optimal nitrogen source concentration, medium pH and temperature were selected to carry out four factor three-level orthogonal experiment, and the most suitable culture conditions for the growth of the strain were selected. The experimental design is shown in Table 1.

Table 1: The orthogonal test factor level table

Factor/ Level	Glucose (%) A	Yeast extract (%) B	pH value (C)	Temperature (°C) D
1	0.5	1.5	6.5	30
2	1	2	7	37
3	1.5	2.5	7.5	44

2.7. Statistical Analysis

The obtained data were analyzed by using Microsoft Excel (ver. 2016) and SPSS (ver. 27.0) and plots were drawn by using Origin (ver. 2021). The data were compared by one-way analysis of variance (ANOVA) and Duncan's multiple range test to determine the significant difference between the mean values ($P<0.05$). All results were expressed as the mean ± standard deviation of three replicates.

3. RESULTS

3.1. Morphological, Physiological and Biochemical Characterization of *Pseudoxanthomonas* sp. with the best Ability to Growth

After successive isolation, 16 strains of bacteria were isolated from the samples. According to the morphological identification, the colonies were light yellow, round, raised, neat edges, translucent, shiny on LB agar medium (Fig. 1a). The strains were observed as gram-negative bacteria under the microscope, with a short rod shape and a size of 0.2-0.3μm × 0.6-1.2μm. The one with the best growth vigor was selected for further identification (Fig. 1b).

According to the Physiological and biochemical analysis, this strain was aescin, liquefied gelatin oxidase positive, but lysine decarboxylase, ornithine decarboxylase and arginine decarboxylase, Simon's citrate, hydrogen sulfide, ONPG, glycerol fermentation, peptone water, nitrate reduction negative It was able to utilize fructose, xylose, maltose, glucose, cellobiose, arabinose, but not inulin, sucrose, sorbose, sorbitol, mannitol, starch, urea.

The properties of morphology and physiology of this strain were studied. According to Bergey's Manual of Determinative Bacteriology, the strain was identified as a new strain of the genus *Pseudoxanthomonas* sp.

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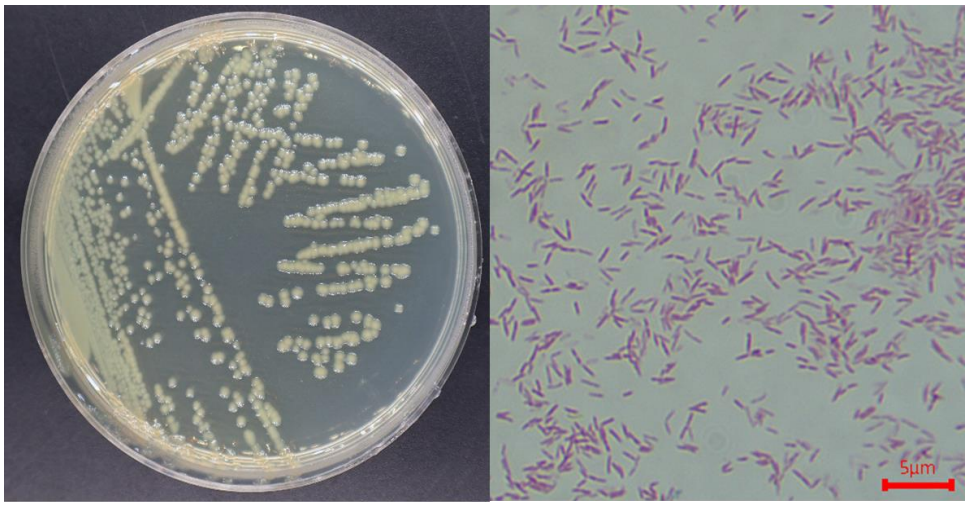

Fig. 1: Colony and cell morphology of *Pseudoxanthomonas* sp. (a) Colony morphology (grown at 37°C for 1 day); (b) Gram staining (magnification 1000 times).

3.2. Growth Curves of *Pseudoxanthomonas* sp.

The growth curve of *Pseudoxanthomonas* sp. was shown in Fig. 2. The strain was inoculated into LB medium, and the growth curve showed that 2-18h was an exponential phase, in which the growth was rapid in 2-10h, slowed down in 12-18h, and a stationary phase appeared after 20h.

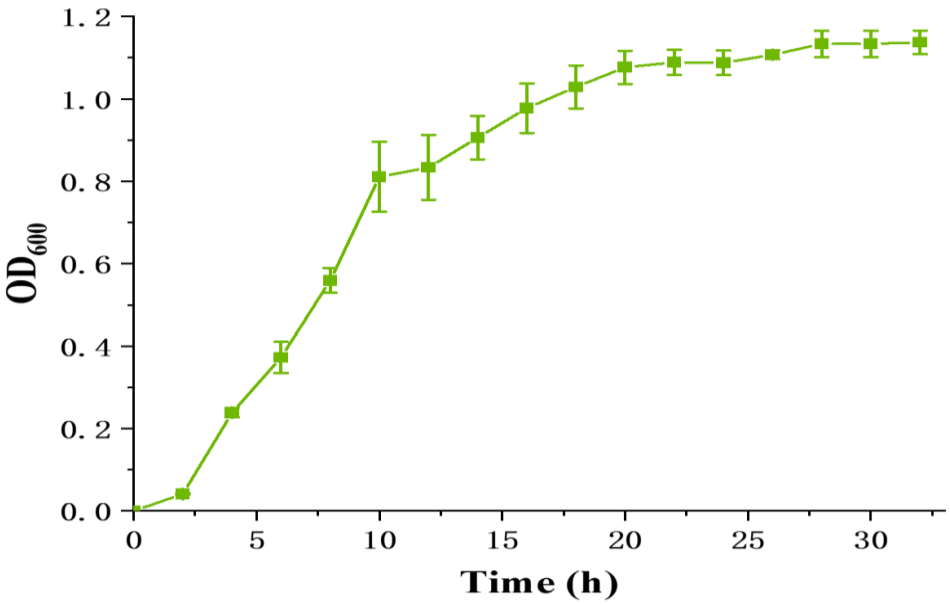

Fig. 2: The growth curve of *Pseudoxanthomonas* sp.

3.3. Selection of Optimal Carbon Source and its Concentration for *Pseudoxanthomonas* sp. Growth

The effect of different carbon sources on the growth of *Pseudoxanthomonas* sp. was shown in Fig. 3. The addition of glucose to the basal medium resulted in the highest OD_{600} value of 1.40 for the strain, which was significantly greater than the control OD_{600} value of 1.33. In contrast, the strains utilized fructose, lactose, sorbitol, and soluble starch less effectively as carbon sources compared to glucose, which resulted in lower OD_{600} values than the control. Therefore, glucose was identified as the most suitable carbon source for the strain's growth.

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To explore the optimal glucose concentration for strain growth, six concentrations ranging from 0.5 to 3% were added to the basal medium using glucose as a carbon source. The results shown in Fig. 4, indicate that the optimal glucose concentration for strain growth is 1%. Under this condition, the strain's OD_{600} value reached 1.45. The strain's OD_{600} value decreased to varying degrees when the glucose concentration in the medium was below 1% or above 1%. When the glucose concentration exceeded 2%, the strain's OD_{600} value significantly decreased. Overall, the strains exhibited the highest glucose utilization, and 1% glucose addition was the most suitable for their growth and metabolism.

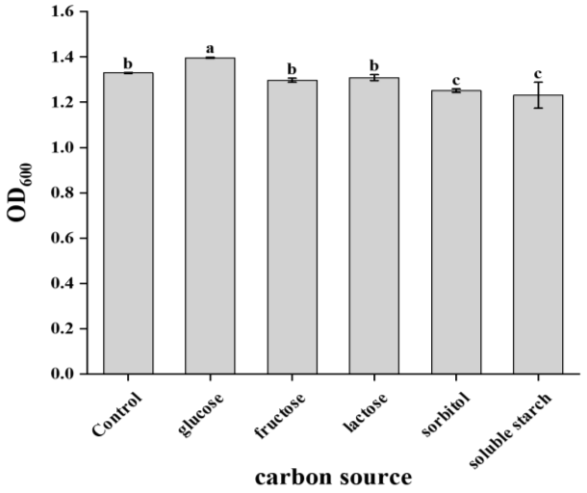

Fig. 3: The OD_{600} of *Pseudoxanthomonas* sp. with different carbon resources.

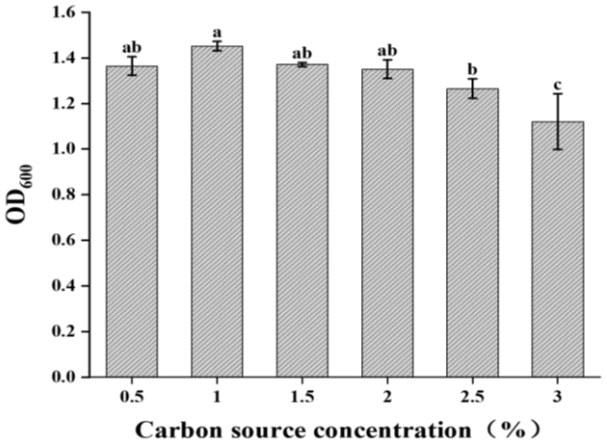

Fig. 4: The OD_{600} of *Pseudoxanthomonas* sp. with different concentration of glucose.

3.4. Selection of Optimal Nitrogen Source and its Concentration for *Pseudoxanthomonas* sp. Growth

The effect of different nitrogen sources on the growth of *Pseudoxanthomonas* sp. was shown in Fig. 5. Compared with the control group, adding yeast extract, soya peptone, tryptone and beef extract to the basic medium increased the OD600 value of the strain to varying degrees. The best nitrogen source was yeast extract, and the OD600 value of the strain was the highest, reaching 1.53, which was significantly higher than that of the group adding other kinds of nitrogen sources. On the contrary, the addition of urea as a nitrogen source would significantly inhibit the growth of the strain.

Yeast extract was used as a nitrogen source, and six concentrations of 0.5, 1, 1.5, 2, 2.5 and 3% were added to the basal medium to explore the yeast extract concentration that was most suitable for the growth of the strain. The result was shown in Fig. 6, the yeast extract additions in the range of 0.5-3%, the strains maintained high OD_{600} values, in which the most suitable yeast extract concentration for the growth of the strains was 2%, and under this condition, the strains had the highest OD_{600} value.

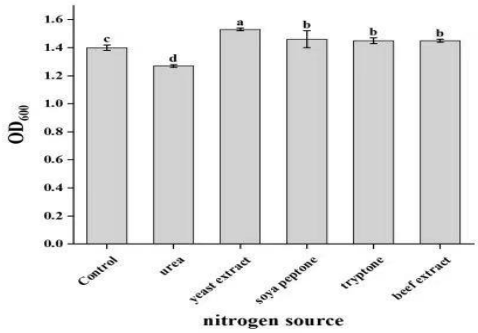

Fig. 5: The OD_{600} of *Pseudoxanthomonas* sp. with different nitrogen resources.

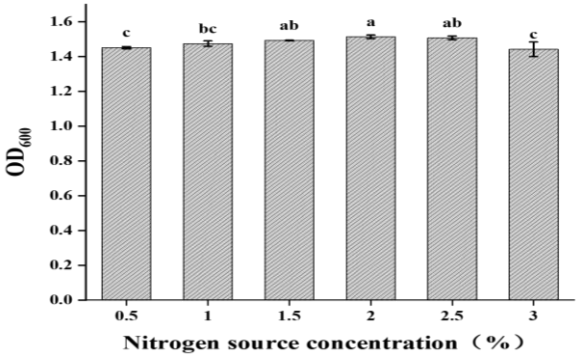

Fig. 6: The OD_{600} of *Pseudoxanthomonas* sp. with different concentration of yeast extract.

In conclusion, when the yeast extract was used as a nitrogen source and added at 2%, it was most suitable for the growth and metabolism of the strain.

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3.5. Selection of Optimal pH for *Pseudoxanthomonas* sp. Growth

The effect of pH on the growth of *Pseudoxanthomonas* sp. was shown in Fig. 7. The growth of the strains showed an increasing and then decreasing trend in the range of pH 5-8, the growth of the strains was inhibited in the acidic medium, and the good growth condition was maintained between pH 6-8, and the best growth was observed at pH 7 with an OD_{600} value of 1.46.

3.6. Selection of Optimal Temperature for *Pseudoxanthomonas* sp. Growth

The effect of temperature on the growth of the strains is shown in Fig. 8. Temperature is one of the important factors affecting the growth status of the strain, the strain in the culture temperature in the range of 16-51°C, the growth amount showed a trend of increasing and then decreasing, the temperature is too low or too high will significantly inhibit the growth of the strain, at 37°C, the maximum growth of the strain, OD_{600} value of 1.57.

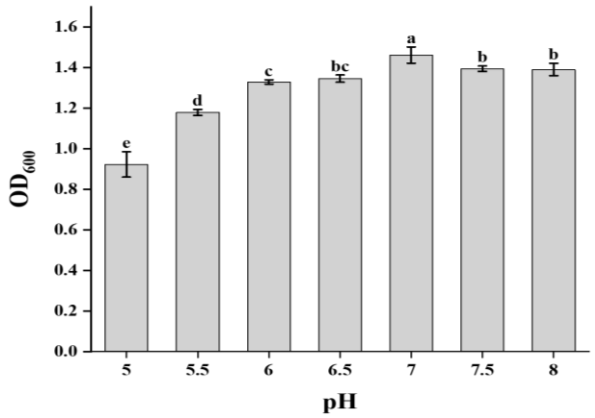

Fig. 7: The OD600 of *Pseudoxanthomonas* sp. with different pH value.

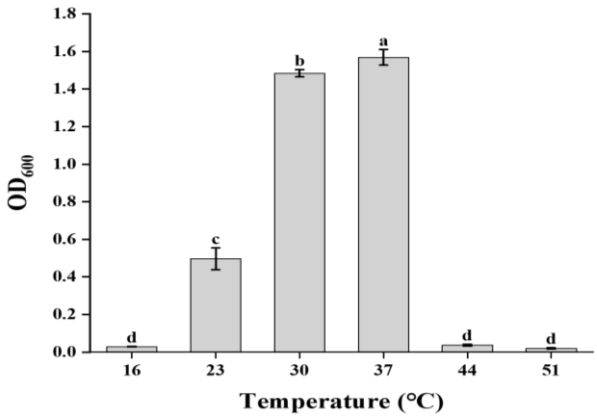

Fig. 8: The OD600 of *Pseudoxanthomonas* sp. with different temperature value.

3.7. The Result of Orthogonal Experiment

The dominant carbon source, nitrogen source, pH and temperature favoring the growth of the strain were determined by the single-factor experiment. Based on the results, a 4-factor, 3-level orthogonal experiment was designed, and the design scheme and result were shown in Table 2.

According to the analysis of polar deviation, the factors affecting the growth of the strain were in the following order: D＞A＞C＞B, i.e., temperature＞glucose addition＞pH＞yeast extract addition, and the optimal combination was D2A3C3B1 after the analysis of the mean value. There was no such option in the table of orthogonal experiments, and the group with the highest results in the table was $D_2A_3C_3B_1$.Therefore, further experiments were done to compare the result, and the result was as shown in Table 3. The result of the orthogonal experiments using The OD_{600} value of cultured bacteria using $D_2A_3C_3B_1$ medium was greater than that of $D_2A_3C_2B_1$ group (OD_{600} value: 1.45 > 1.41), therefore, $D_2A_3C_3B_1$ was determined to be the optimal formulation for culturing the strain.

Table 2: Design and result of orthogonal experiment

Experiment number	Glucose (%) A	Yeast extract (%) B	pH value C	Temperature (°C) D	OD_{600} value
1	1	1	1	1	0.83±0.12
2	1	2	3	2	1.29±0.07
3	1	3	2	3	0.03±0.00
4	2	1	3	3	0.03±0.00
5	2	2	2	1	0.69±0.10
6	2	3	1	2	0.33±0.09
7	3	1	2	2	1.31±0.02
8	3	2	1	3	0.05±0.00
9	3	3	3	1	1.04±0.09
k_1	0.72	0.72	0.40	0.85	
k_2	0.35	0.68	0.68	0.98	
k_3	0.80	0.47	0.79	0.04	
R	0.45	0.25	0.39	0.94	

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Table 3: Selection of optimal medium formula

Group	OD_{600} value
$D_2A_3C_3B_1$	1.45±0.06
$D_2A_3C_2B_1$	1.42±0.05

4. DISCUSSION

Determining the nutritional, energetic, and cultural environment for microorganisms is a crucial step in developing microbial preparations. The composition of the culture medium is a significant parameter, and the components and their concentrations affect the growth of microorganisms and the accumulation of metabolites. Therefore, to achieve cost-effectiveness, it is necessary to optimize the composition and concentration of the medium based on a standardized medium to obtain the most suitable conditions for microorganism growth (Batista and Fernandes 2015; Zhou et al. 2023).

In this study, we screened a strain of *Pseudoxanthomonas* sp. with the most vigorous growth from the environment through morphological and physiological and biochemical characterization, and after obtaining the growth curves of *Pseudoxanthomonas* sp., we investigated the effects of different culture conditions on the growth of *Pseudoxanthomonas* sp. In terms of carbon source, glucose was the most suitable carbon source for the growth of the strain compared to fructose, lactose, sorbitol and soluble starch. This may be due to the fact that glucose is more easily utilized by microorganisms as a monosaccharide compared to other carbon sources and is the preferred carbon source for most of the bacteria (Stülke and Hillen 2000; Galinier 2018). Among the nitrogen sources evaluated, *Pseudoxanthomonas* sp. grew best in yeast extract. This may be due to the fact that yeast extracts can be used as amino acids and peptides that better provide nitrogen (Podpora et al. 2016). In addition, pH and temperature are also important factors affecting the growth of the strain. pH has a significant effect on complex physiological phenomena such as membrane permeability and cellular morphology, and in this way, at the same time, temperature significantly affects the activity of enzymes that catalyze metabolic reactions. It has been reported that a temperature of 37°C and a pH close to neutrality are more suitable for the growth of *Pseudoxanthomonas* sp. In this study, *Pseudoxanthomonas* sp. grew best at a temperature of 37°C and pH 7.5, which is consistent with previous reports (Morales-Borrell et al. 2020; Azish et al. 2021; Morales-Borrell et al. 2021).

5. CONCLUSION

In this study, a vigorous bacterial strain, *Pseudoxanthomonas* sp., was screened from the environment through morphological and physiological and biochemical characterization, and the optimum culture conditions for the growth of the strain were designed through one-way and orthogonal experiments. The optimal culture conditions for the strain were as follows: using LB medium as the base medium, adding 1.5% glucose as the carbon source, 1.5% yeast extract as the nitrogen source, adjusting the pH of the medium to 7.5, and incubating at a temperature of 37°C. Based on this experiment, the development of the strain can be further processed to provide a guarantee for the sustainable development of agriculture.

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