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MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF NOVOSPHINGOBIUM SPECIES AND THEIR OPTIMAL GROWTH KINETICS

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ABSTRACT

Novosphingobium species are Gram-negative, rod-shaped bacteria containing sphingoglycolipids. In this study, *Novosphingobium* species were isolated and characterized based on morphology and biochemical analysis. The research also investigated the impact of different culture media, temperature, time and pH on the growth of *Novosphingobium* species, aiming to determine its optimal growth kinetics. The culture media (LB, 0.1% glucose LB, NB, 0.1% glucose NB, and MM), pH (4, 5, 6, 7, 8, 9 and 10), and incubation temperatures (25, 30, 37, 45, and 55°C), were examined for representative *Novosphingobium* sp. growth, measured as optical density (OD). The results indicated that all the strains displayed yellow colony morphology with rounded raised edges and smooth surfaces. Strains were rod-shaped without spore production. All the strains assimilated maltose, sucrose, xylose and oxidized glucose. Representative strain of *Novosphingobium* species grown rapidly on 0.1% glucose LB medium and optimal temperature were identified 37°C. The growth of representative strain was significantly influenced by the initial pH of the culture media, but 7 pH was identified as optimal. This study provides valuable information regarding the characterization and optimal growth conditions of *Novosphingobium* species.

Keywords: Isolation; Characterization; Morphology; Biochemical; *Novosphingobium* species; Optimization of culture conditions.

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1. INTRODUCTION

Novosphingobium species are Gram-negative bacteria widely found in nature. These species do not produce spores and can utilize both oxygen and carbon dioxide from inorganic substrates. Moreover, they can also utilize glucose and other carbon sources during their growth. *Novosphingobium* species belong to *Sphingobacteriaceae* family (Huang et al. 2023; Gan 2013; Aylward 2013). *Novosphingobium* species are important environmental microorganisms with the ability to degrade a variety of aromatic compounds, such as toluene, aminobenzene, nitrobenzene polycyclic aromatic hydrocarbons (Tiirola et al. 2002: Yuan et al. 2009; Hashimoto et al. 2010). These species serve as effective agents for bioremediation in environments contaminated with aromatic hydrocarbons. Specifically, *Sphingobacterium multivorum* and *Sphingobacterium thalpophilum* have been identified for their ability to degrade petroleum. Notably, *S. multivorum* SWH-2 exhibits robust petroleum degradation activity. Through the optimization of its culture conditions, there are potential modifications in normal strain growth, enzyme secretion and activity. This optimization process can further enhance degradation rate of the strain (Sheng Wen et al. 2013). *Novosphingobium* species possess diverse metabolic pathways and ecological functions, providing significant application values in degradation of environmental pollutants, biotransformation, antioxidant activities, and interactions with plants (Chen et al. 2012; Mulla et al. 2016).

Novosphingobium species are known to be widely distributed in deserts, soils, toxin-contaminated soil, seawater, glacial meltwater, activated sludge, and plants (Teng et al. 2015; Huo et al. 2015; Chen et al. 2014; Niharika et al. 2013; Choi and Lee 2012). Many studies have reported the role of environmental bioremediation of *Novosphingobium* species, but few studies have been reported about the growth characteristics. In this experiment, we isolated several strains of *Novosphingobium* species from soil and water samples based on morphological and biochemical characterization. To proper understanding of *Novosphingobium* application, it is recommended to conduct further growth characterization of one of the more robust isolated strains of *Novosphingobium*. The objective of this study was isolation of *Novosphingobium* species based on morphological and biochemical

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characterization and optimize their superior growth conditions. This research is not only contributing to a comprehensive understanding of the optimal growth conditions for *Novosphingobium* species but will also serve as a foundational step in the application of *Novosphingobium* strains for the degradation of toxic substances.

2. MATERIALS AND METHODS

2.1. Materials and Reagents

2.1.1 Samples and Reagents: The collected samples included soil and water samples. The reagents used were glucose (Shanghai Macklin Biochemical Technology Co., Ltd), yeast powder (Beijing Land Bridge Technology Co., Ltd.), and Gram's stain (Guangdong Huan Kai Technology Co., Ltd.).

2.1.2 Primary Culture Mediums and their Composition:

(a) Luria-Bertani (LB) liquid medium: LB liquid medium is composed of 10g of peptone, 5g of yeast powder, 5g of sodium chloride (NaCl), and 1g of dextrose. Double distilled (DD) water is adjusted to 1L and after thorough mixing, the pH is set to 7. Subsequently, it was autoclaved at 121℃ for 15min, and stored at 4℃ for future use.

(b) 0.1% glucose LB liquid medium: 1g of glucose per liter was added to the LB liquid medium, with all other conditions remaining unchanged.

(c) Nutrient Broth (NB) medium: Consisting of 10g of peptone, 3g of beef paste powder, 5g of NaCl and DD water adjusted to 1L. The pH was set to 7.4 after sufficient mixing, followed by autoclaving at 121℃ for 15min. The medium was then stored at 4℃ for future use.

(d) 0.1% glucose NB medium: 1.0g of glucose per liter was added to the NB liquid medium, while keeping all other conditions unchanged.

(e) Minimal medium (MM): 2g $(NH_4)_2SO_4$, 0.01g $CaCl_2$, 0.2g $MgSO_4$, 0.002g $MnCl_2$, 0.005g $Fe_2(SO_4)_3$, 10.5g K_2HPO_4, and 4.5g KH_2PO_4 was mixed up to 1L of DD water. The pH was adjusted to 7.5. and filtration was performed with a sterile 0.22μm filter membrane.

2.2. Instruments and Equipment

The experiment utilized the following instruments and equipment: EX20 Digital Biomicroscope (Sunny Optical Co., Ltd.), Multi FC microplate reader (Thermo Fisher Scientific Co., Ltd.), pH Meter (Mettler Toledo Instrumentations CO., LTD.), and Constant Temperature Shock Incubator (Suzhou Jie Mei Electronics Co., Ltd.).

2.3 Experimental Methods

2.3.1. Sample Preparation: For each collected sample, 10g was measured, and 20mL of sterile water was added. The mixture was then vortexed and shaken thoroughly for proper mixing and allowed to stand for 30min at room temperature and collected the supernatant suspension.

2.3.2. Strain Isolation and Purification: For isolation and strain purification, 200μL sample suspension was taken and added it to 50mL of LB liquid medium and incubated it in a constant temperature shaking incubator at 37℃ and 180r/min for 24h. Following that, each bacterial suspension was prepared by mixing 1mL of the bacterial suspension with 9mL of sterile water. Subsequently, gradient dilutions, including 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}. were prepared. These gradient dilutions (200μL) were streaked on LB solid medium and incubated at 37℃ for 24h. The strains were initially isolated by observing the growth of the strains with naked eyes and picking single colonies based on colony morphology. The isolated strains were cultured for three times to make purified strain and preserved in 20% glycerol LB medium at -18℃.

2.3.3. Strain Morphology and Identification: The best-growing strains (n=10) were selected and placed on LB solid medium then incubated at 37°C for 24h. The colony color and morphology were observed. The morphological study was done by using EX20 Digital Biomicroscope (Sunny Optical Co., Ltd.). After Gram staining study, the strains were identified based on physiological and biochemical characterization and description were matched as a reference to Berger's Manual of Systematic Bacteriology. Based on identification, one representative strain was selected for further study.

2.3.4. Optimal Medium for Strain Growth: Single colony of representative strain was picked and inoculated with LB liquid medium following by incubated at 37℃ and 180r/min for 24h. The bacterial suspension (10^7 cfu/mL) was prepared. The bacterial suspension (100μL) of the representative strain was inoculated into LB, 0.1% glucose LB, NB, 0.1% glucose NB and MM medium for 24h. The OD value of the bacterial solution at 600nm was determined by using a microplate reader, and the strain's optimal growth medium was determined by parallelizing each sample three times with three replications.

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2.3.5. Growth Curve of the Strain: The bacterial suspension (100µL) of the representative strain was inoculated into the 0.1% glucose medium for 48h. The OD value of the bacterial solution at 600nm was determined every 4h. The growth curve was plotted with time as the horizontal coordinate and the OD value as the vertical coordinate to determine the optimal growth time of the strain.

2.3.6. Optimization of Growth Conditions for Strain: The bacterial suspension of the representative strain was inoculated into the optimal medium at a 1% inoculum volume. The inoculated samples were incubated at different temperatures (25, 30, 37, 45, and 55°C). The optimal temperature was then selected, and the pH of the optimal medium was adjusted to values ranging from 4.0 to 10.0 for the subsequent incubation. Furthermore, the optimal medium was incubated at different loading volumes (20, 40, 60, 80, and 100mL) to determine the optimal temperature for incubation. After 24 hours, the absorption values of the bacterial solution at 600nm were determined using a microplate reader. The samples were incubated in parallel three times, two times, and two times, respectively. Each sample underwent parallel runs three times with three replications to determine the temperature, medium pH, and loading volume at which the strain grows optimally.

2.4 Data Analysis

The obtained data were analyzed by using Microsoft Excel (ver. 2311) and SPSS (ver. 26.0) and plots were drawn by using Origin (ver. 2019b).

3. RESULTS

3.1 Morphological, Physiological and Biochemical Characterization of N*ovosphingobium* Species

The colonies of all strains (n=10) showed yellow color on LB solid medium after 24h. The colony morphology of all the strains were yellow, with rounded raised edges and smooth surfaces. The microscopic study showed that all the strains were rod-shaped and did not produce spores. (Fig. 1). All the strains were identified Gram-negative N*ovosphingobium* species based on morphology. For biochemical analysis, all the strains demonstrated the ability to assimilate and utilize maltose, sucrose, and xylose. Additionally, they oxidized glucose for acid production and could grow under Simon's citrate. However, they did not hydrolyze gelatin and peptone. Furthermore, the strains tested negative for ornithine decarboxylase, lysine decarboxylase, arginine decarboxylase, oxidase, nitrate reduction, and hydrogen sulfide tests. On the positive side, they exhibited positivity for β-galactose and heptapeptide tests, as detailed in Table 1. It was tentatively identified as a *Novosphingobium* species according to Berger's Manual of Systematic Bacteriology. Based on morphology and biochemical characterization all the strains (n=10) were identified *Novosphingobium* sp.

Table 1: Physiological and biochemical test of *Novosphingobium* species

Test	Result	Test	Result
Gelatin liquefaction	-	Nitrate Reduction	-
Glucose	+	Hydrogen Sulfide	-
Maltose	+	Simon's citrate	+
Sucrose	+	Peptone Water	-
Xylose	+	Heptoside Utilization	+
Ornithine decarboxylase	-	Urea	-
Lysine decarboxylase	-	β-Galactoside	+
Arginine decarboxylase	-	Oxidase reagent	-

Note: "+" for positive, "-" for negative

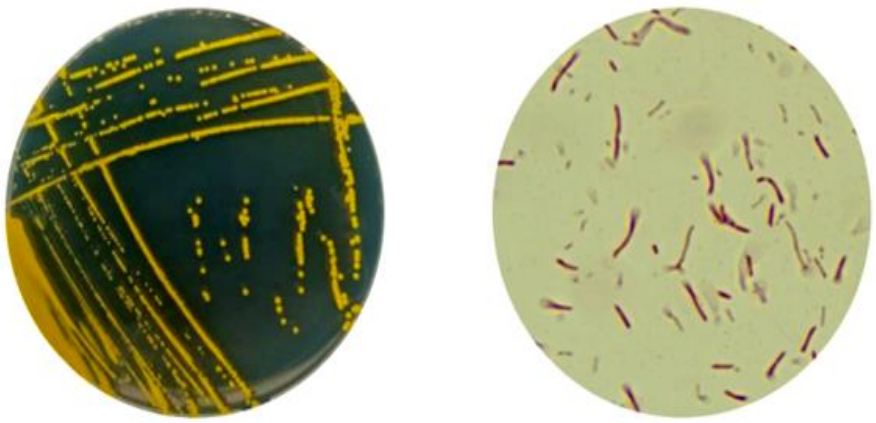

Fig. 1: Colony morphology and microscopic study (Scale bar = 100µm) of representative *Novosphingobium* sp. strain.

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3.2. Optimal Medium for Strain Growth

The representative strain was cultured in LB, 0.1% glucose LB, NB, 0.1% glucose NB and MM media for 24h. Its OD value was highest in LB medium, indicating that LB medium was more suitable for the growth of *Novosphingobium* species. When glucose was added to LB medium as carbon source, its OD value increased to 0.827, which was higher than that of LB medium without glucose. Representative strain could utilize glucose as carbon source, which was beneficial to its growth. Therefore, LB medium containing 0.1% glucose was chosen as the best medium for the subsequent experiments (Fig. 2).

3.3. Optimal Growth Time of the Strain

The results of the growth curve of the representative strain in Fig. 3 showed that the strain was in the delayed period at 0 to 4 h, the strain grew and developed rapidly into the logarithmic growth period at 4 to 20h, which was the best stage for inoculation, and the growth of the strain tended to level off at 20 to28 h, entering the stabilization period, in which the strain carried out the accumulation of metabolites, and the strain entered into the decline period when the incubation time reached 32h. The strain was in the decline period at 24h, which was the best stage for inoculation, and it was the best stage for inoculation. It can be seen that at about 24h the strain carried out a large number of amplification and reproduction, so choose to cultivate 24h of the bacterial fluid for subsequent experiments.

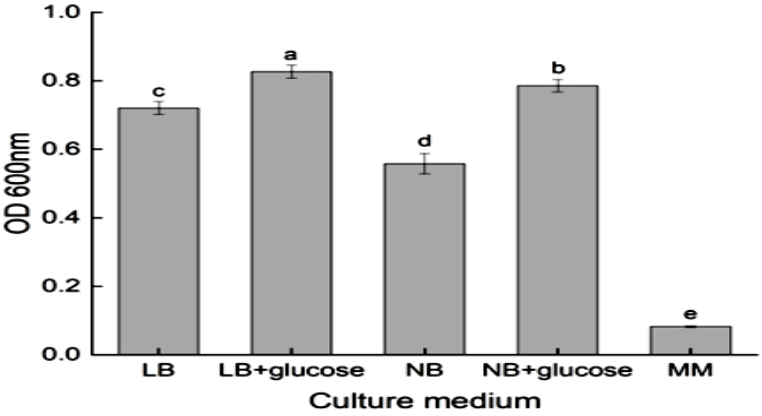

Fig. 2: Effect of different culture media on the growth of representative *Novosphingobium* sp. strain

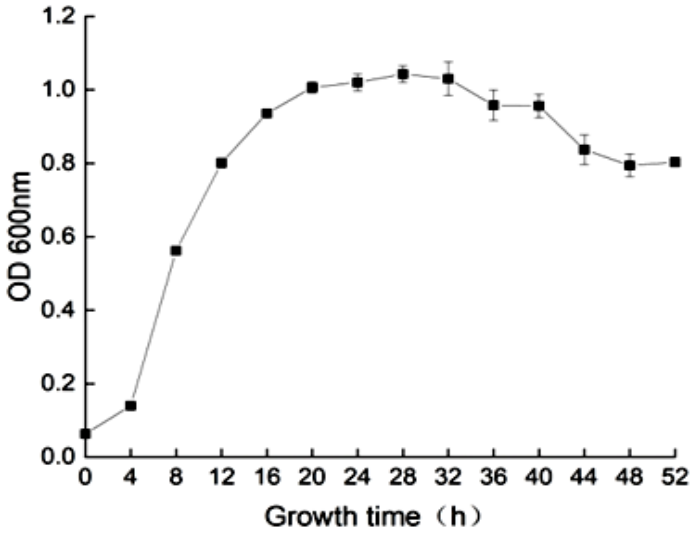

Fig. 3: Effect of time on the growth of representative *Novosphingobium* sp. strain.

3.4. Optimum Temperature for Strain Growth

The representative strain in the range of 25 to 55℃, the strain growth amount showed first growth and then decline. In the range of 25 to 37℃, the bacterial volume gradually increased with the increase of temperature; in the range of 37 to 45℃, the bacterial volume decreased abruptly with the increase of temperature; in the range of 45 to 55℃, the growth ability of the strain was retarded. The results showed that the strain was not resistant to high temperature, and the bacterial volume was maximum at 37℃, so 37℃ was selected as the optimal culture temperature for the strain (Fig. 4).

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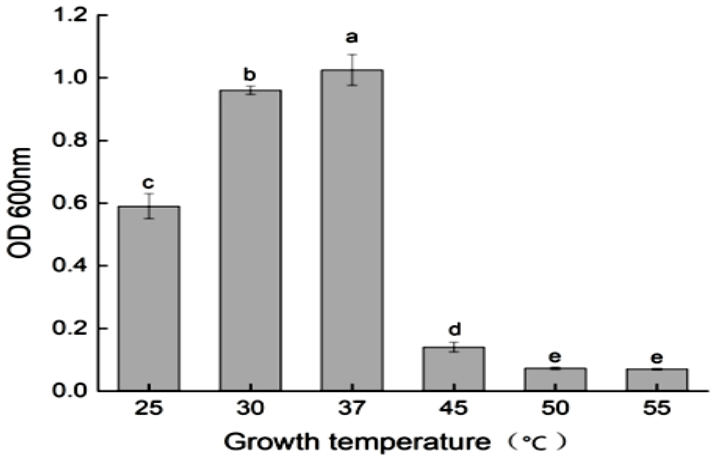

Fig. 4: Effect of incubation temperature on the growth of representative *Novosphingobium* sp. strain

3.5. Optimal pH for Strain Growth

The representative strain is suitable for growth in the range of pH 5.0~8.0, while it grows slowly under strong alkaline and acidic conditions, and the strain grows maximally when the pH is 7.0, and the OD600 can reach 0.919, so the optimal pH for the growth of strain SF09 is 7.0 (Fig. 5).

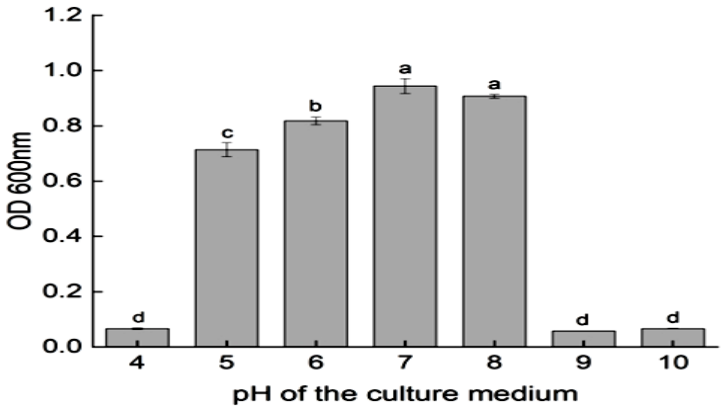

Fig. 5: Effect of different pH on the growth of representative Novosphingobium sp. strain.

3.6. Optimal Loading Volume for Strain Growth

The growth of the bacteria increased when the loading volume was increased within the range of 20 mL/150 mL to 60 mL/150 mL; the growth of the bacteria decreased when the loading volume was greater than 60 mL/150 mL. Since *Novosphingobium* species is an aerobic bacterium, the strain grows well when the ratio of nutrients to oxygen is optimized. Therefore, the optimal loading volume was determined to be 60 mL/150 mL (Fig. 6).

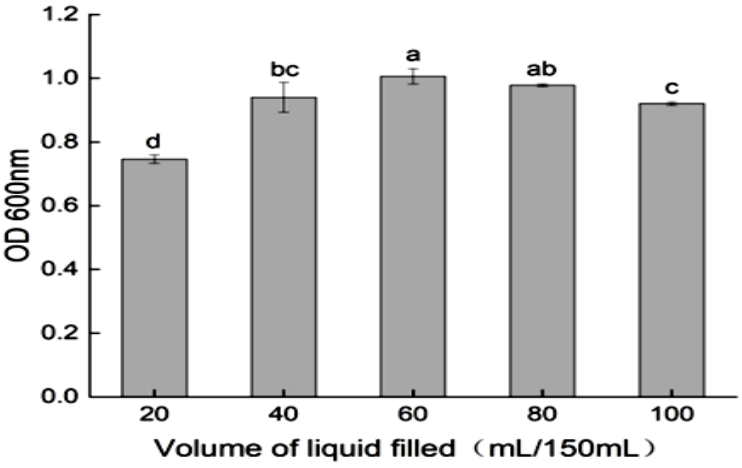

Fig. 6: Effect of loading volume on the growth of representative *Novosphingobium* sp. strain

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4. DISCUSSION

Novosphingobium sp. is a Gram-negative non-fermenting rod-shaped bacterium containing large amounts of cell membrane sphingolipids, which is a rich class of novel microbial resources (Tiirola et al. 2005; Notomista et al. 2011). *Novosphingobium* species capable of both aerobic and anaerobic metabolism, exhibiting motility, and showing positive results for catalase and oxidase activities, as well as starch hydrolysis (Belmok et al. 2023). Most studies in recent years have shown that polycyclic aromatic hydrocarbons have high hydrophobicity and very low water solubility, and *Novosphingobium* sp. can effectively sense and absorb it (Yan et al. 2007; Sohn et al. 2004), effectively repairing the environment. Studies have shown that the culture medium can provide a good growth environment for strains, and different medium components can affect the growth and metabolism of strains and even change the expression of certain genes (Xu et al. 2020). Among them, carbon and nitrogen sources are important parts of nutrients, which constitute the structural skeleton of bacterial cells, provide energy for the growth and metabolism of the bacteria, and are important conditions to ensure the optimal growth of microorganisms (Rabiya and Sen 2022). The pH has a great influence on the growth and metabolism of microorganisms, which can change the biological activity of enzymes, proteins, and other macromolecular materials in microorganisms, change the charge of the cell membrane of the bacterial cells, and change the ability of the bacterial cells to absorb nutrients from the environment. ability to absorb nutrients from the environment (Elyasi et al. 2020). *Sphingobacteriaceae* are widely distributed in a variety of complex extreme environments, mostly strictly aerobic or parthenogenetic anaerobic; nutritional requirements are broader, and metabolic types are complex and varied; they do not have much demand for ambient temperatures, and they can grow within the temperature range of 5 to 42℃ (Zhu et al. 2022).

This experiment showed that the strain grew better in LB medium containing 0.1% glucose. Numerous studies have shown that by improving and optimizing the culture conditions, the propagation ability and secretion of more metabolites can be improved, among which the culture temperature, medium pH and loading volume play a major role in the growth of the strain. The growth characterization of this strain showed that the bacterium could grow well under pH 5.0 to 8.0, 25 to 37℃ and 40 to 80mL/150mL loading volume, indicating that this strain could grow under weak acid and weak alkali, which is of research significance compared with the strain of *Novosphingobium* sp. Recent studies have shown that *Sphingobacteriaceae* has the ability to degrade zearalenone and vomitoxin (He, 2017). *Novosphingobium* species are renowned for their ability to adapt metabolically and their significant potential for effectively addressing pollution challenges (Liu et al. 2021; Chen et al. 2021). So it is hypothesized that *Novosphingobium* sp. is also effective in degrading fungal toxins, which will be followed up by the study on the degradation of fungal toxins by these strains.

5. CONCLUSION

In this study, *Novosphingobium* species were characterized based morphological, physiological, and biochemical. The growth conditions were optimized, revealing that representative *Novosphingobium* sp. strain exhibited enhanced growth under conditions 37°C and 7 pH. Specifically, the optimal growth was observed in 0.1% glucose LB medium with a pH of 7 at 37°C and a loading capacity of 60mL/150mL. These findings establish a foundation for further exploration and study of *Novosphingobium* species.

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