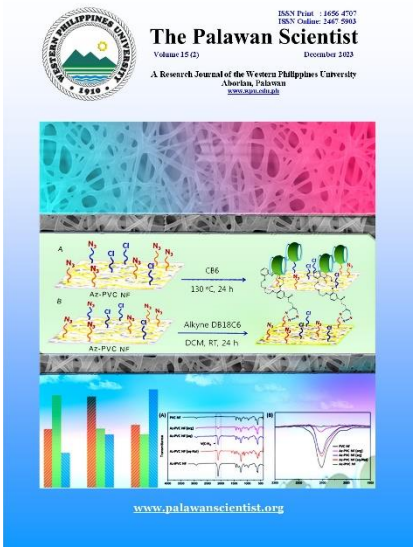

Selected physiological requirements of Purple non-sulfur bacteria isolated from Los Baños, Laguna for potential biohydrogen production

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ABSTRACT

Biohydrogen is gaining traction in energy research due to its high energy content and minimal carbon footprint. A typical method of producing biohydrogen is photofermentation using purple non-sulfur bacteria (PNSB). Exploring novel strains of PNSB and studying their versatile metabolism can aid in bioprospecting their potentially valuable by-products and applications, particularly in energy generation. This study investigated the physiological requirements of top biohydrogen-producing PNSB isolated from various Los Baños, Laguna, Philippines sites by measuring their biogas production and growth when subjected to different incubation conditions and macronutrient requirements. Results showed that the three local isolates grown anaerobically in mesophilic conditions without agitation preferred incandescent light. The high biogas yield and growth may be attributed to the light-harvesting pigments in PNSB, which are excited primarily by infrared and near-infrared wavelengths of incandescent light. Furthermore, the isolates can grow from various carbon sources, such as volatile fatty acids (malate, succinate, acetate, butyrate, and propionate) and sugars (glucose and starch). However, two of the isolates (MAY2 and PR2) did not produce biogas when supplied with acetate as a carbon source, which suggests a competing pathway that may have affected the photofermentation of the isolates. Also, the isolates prefer more complex organic sources such as yeast extract and peptone than inorganic sources such as ammonium and less complex organic sources such as urea. Finally, experiments on salt tolerance showed that 0.04% and 0.85% NaCl concentration favors biohydrogen production and growth, as exhibited by high biogas production, yield, and optical density. Results from this study can serve as a basis for future research on optimizing media composition and conditions for biohydrogen production from these isolates.

Keywords: biohydrogen, photofermentation, purple non-sulfur bacteria

INTRODUCTION

The threat of global warming and the world's energy dilemma have urged experts to find more sustainable energy sources. Along with other alternatives, biobased systems are being explored as replacements for hydrocarbon fuels. Recently, research on biohydrogen has been gaining traction, as it is an efficient energy carrier with around three times more energy than gasoline (EIA 2022) and has negligible greenhouse gas emissions (Kotay and Das 2008). Hydrogen (H_2) production can be achieved sustainably (Ahmed et al. 2021) through dark fermentation, direct and indirect biophotolysis, and photofermentation. Nevertheless, despite these various biological routes, H_2 technology is limited due to the low efficiency of the production process (Saratale et al. 2019; Tiang et al. 2020).

Photofermentation using purple non-sulfur bacteria (PNSB) is among the widely studied methods for biohydrogen production. Photofermentation can ultimately convert carbon and nitrogen sources to H_2 but is limited by various factors such as light and enzyme activity (Chandrasekhar 2015). In photofermentation, H_2 is generated as a by-product of energy generation via anoxygenic photosynthesis, where electrons from the tricarboxylic acid cycle are subsequently transferred to redox proteins embedded in the intracellular membrane, resulting in a proton gradient that powers the adenosine triphosphate cycle synthase. The nitrogenases and hydrogenases then convert the electrons and the protons to H_2. Thus, researchers are exploring ways to improve the photofermentation pathway by bio-prospecting novel species, improving strains, and identifying culture conditions that complement the growth requirements of PNSB. Among the growth requirements considered include macronutrients such as carbon and nitrogen sources, which constitute the significant biomolecules in living systems (Bonnet et al. 2020). It is already established that the amount and the type of carbon and nitrogen sources have considerable effects on the H_2 productivity of PNSB, making them primary candidates for optimization studies (Abdullah et al. 2020; Hakobyan et al. 2019). Furthermore, the H_2 photofermentative pathway requires light as an energy source, and light's spectrum and intensity have significant effects on H_2 productivity (Bosman et al. 2023; Rashid et al. 2022). Hence, the light requirement of the isolates is one essential consideration for optimization. On the other hand, there are known salt tolerant PNSBs studied mainly for their ability to utilize waste and wastewater as substrates (Chen et al. 2020; Hülsen et al. 2019). Plans for hybrid setups of wastewater treatment and photofermentation plants could benefit from salt tolerant PNSB isolates. There are limited local studies on the isolation of purple non-sulfur bacteria. One of which is by Montano et al. (2009), which isolated PNSB from rice paddies in Bulacan. Another local study is by Del Socorro et al. (2013), which isolated PNSB from rice paddies and aquatic sediments in Iligan City. These studies, however, have no emphasis on the biohydrogen production capability of the isolates and are, therefore, currently untapped in the Philippines.

This study characterized selected physiological requirements of the top biogas producers from the pool of PNSB isolates from various areas in Los Baños, Laguna. Specifically, this study investigated the effects of light (blue light-emitting diode (LED), red LED, fluorescent and incandescent), carbon (volatile fatty acids (VFAs), succinate, malate, glucose, and starch), nitrogen (ammonium, urea, glutamate, peptone, yeast extract), and NaCl concentrations (0.04%, 0.85%, 3.0%, 10.0%) on the biohydrogen production potential of the isolates. Studies suggest that the gas generated by PNSB photofermentation is composed primarily of H_2 (Craven 2019; Turon 2018; Ventura et al. 2019). Thus, biogas was measured to approximate biohydrogen production potential

METHODS

Purple Non-sulfur Bacteria Isolates

The top three biohydrogen-producing strains (MAY2, IRRI1, PR2) out of the 19 PNSB isolates obtained from water sediments in Los Banos, Laguna, Philippines (Ventura et al. 2021) were the focus of this study. MAY2 and PR2 isolates were identified under the genus *Rhodobacter*, and IRRI1 was verified to be under the genus *Rhodopseudomonas* using 16s rRNA sequencing.

Main Culture Preparation

The main cultures of these isolates were revived by multiple subsequent transfers in an acetate-yeast extract medium (AYE) (Montano et al. 2009). This medium has the following components (in g L^{-1}): K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.02; $Na_2S_2O_3$, 0.10; $NaCH_3COO$, 2.2; and yeast extract, 4.0. A loopful of each culture from stock was streak-plated in Acetate Yeast Extract (AYE) agar. Cultures were incubated under incandescent light using an anaerobic set-up previously described by Maiti et al. (2013). The isolate grown from this stage was then used for the succeeding experiments.

Seed Culture Preparation

Modified Biebl and Pfennig (MBP) medium was used for the seed culture preparation. The medium contains the following components (in g·L^{-1}): $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.4; KH_2PO_4, 0.5; $CaCl_2 \cdot 2H_2O$, 0.05; ferric citrate, 0.005; yeast extract, 0.3; vitamin solution (nicotinic acid, 0.0002; nicotinamide, 0.0002; thiamine HCl, 0.0004; and biotin, 0.008); and 1 ml trace element solution. Trace element solution

has the following composition (in mg·L^{-1}): $ZnCl_2$, 0.07; H_3BO_3, 0.06; $MnCl_2 \cdot 4\ H_2O$, 0.1 mg; $CoCl_2 \cdot 2\ H_2O$, 0.2; $CuCl_2 \cdot 2\ H_2O$, 0.02; $NiCl_2 \cdot 6H_2O$, 0.02; $(NH_4)2MoO_4 \cdot 2\ H_2O$, 0.04 and HCl, 0.025%v/v. The pH of MBP was adjusted to pH 6.5, particularly when supplied with volatile fatty acids as the carbon source.

Before the biogas production set-up, a pre-activated seed culture was prepared. A loopful of each isolate from the AYE medium was grown in a 40 ml MBP medium supplied with 7 mm malate and 10 mm glutamate in 150 ml glass vials. The set-ups were then purged with argon gas for 3 min and crimp sealed to simulate anaerobic conditions, vortex-mixed, and incubated under incandescent light (300 nm to 1400 nm) for 3-5 days at room temperature (20-25ºC). Growth from the pre-activated cells was then harvested via centrifugation (4000 x g for 5 min) and then transferred to a fresh batch of MBP medium supplied with 7.0 mm malate and 10 mm glutamate. Their optical density (OD) was adjusted to 0.5. Four milliliters of the adjusted culture were then transferred to 36 ml of fresh MBP medium; they were purged with argon and crimp-sealed, vortex-mixed, and then incubated for three days as previously described.

Biogas Production Set-up

Four milliliters from the adjusted culture was collected and centrifuged. The pellet was then placed in 150 ml serum bottles with 40 ml fresh MBP. The setup was purged with argon gas for 3 min and then crimp-sealed. The reactor bottles were incubated without agitation under anaerobic conditions at room temperature.

Effect of Different Light Source

The biogas production set-up used for the investigation of the effect of light source utilized MBP with mixed acids - 15 mm acetate, 7.5 mm butyrate, and 10 mm propionate (approximately 2.0 g·L^{-1} in total), and 2.0 mm glutamate as nitrogen source. The set-ups were incubated under different light types: red LED (660 nm), blue LED (460 nm), incandescent light (300-1400 nm), and fluorescent light (400-700 nm). The light intensity of each light source was also measured using a lux meter (see Table 1).

Effect of Carbon and Nitrogen Sources

Using the previously mentioned biogas production setup, the capability of the three isolates to utilize different carbon sources was also investigated. Instead of mixed acids, the set-ups used individual carbon sources- malate, succinate, acetate, butyrate, propionate, glucose, and soluble starch. The concentration of each carbon source was 2.0 g L^{-1} (Basak and Das 2007; Assawamongkholsiri 2019).

Similarly, the nitrogen requirements of the isolates were also investigated using the same biogas assay setup with modification on the nitrogen source. The nitrogen sources investigated were ammonium, urea, glutamate, yeast extract, and peptone. 2.0 mM of ammonium, glutamate, and urea were used for MBP (Ventura et al. 2021), while 2.0 g L^{-1} concentration was used for peptone and yeast extract (Hakobyan et al. 2012). The setups were processed and incubated as previously described.

Effect of Salt (NaCl) Concentration

The isolates were subjected to different salt (NaCl) concentrations by adjusting the amount present in the MBP medium. The variation in salt concentrations was as follows: 0.04% (recommended amount in standard MBP medium), 0.85% (physiological saline), 3.0% (marine saline concentration), and 10% (hypersaline) (Xiao et al. 2022; Irwin 2020). The MBP medium used in the investigation of the effect of NaCl concentration was similar to the light source investigation setup. The setups were processed and incubated as previously described.

Measurement of Biogas Production and Optical Density

The cumulative biogas was measured by recording the displaced gas using a sterile syringe every 24 hours, and the cumulative readings were plotted. Biogas yield was calculated according to the method of Wu et al. (2016; equation 1).

The sampling for biogas assay in the investigation of light source ran for 20 days; while that of carbon and nitrogen sources and salt tolerance ran for ten days.

The optical density in each setup was measured on the last day of observation to approximate cell growth. Two ml of the culture broth was placed in disposable cuvettes, and optical density was measured using a Shimadzu® 1800 UV-Vis spectrophotometer set at 660 nm.

$$\text{Biogas yield ml g}^{-1} = \frac{Total\ biogas\ generated\ per\ set{-}up\ (ml)}{Volume\ of\ media\ \ (ml) \times substrate\ concentration\ (g \cdot ml^{-1})} \qquad \text{equation (1)}$$

Table 1. Light sources used in the experiment.

Light type	Brand/Model	Wattage (W)	Illuminance (lux)	Wavelength (nm)	Intensity (lux)
Incandescent	Firefly® FINS60F	60	1000	300-1400	~700
Fluorescent	Firefly® 3S32	32	1000	400-700	~3,200
Red LED	Perfin® PFLG02	10	200	660 nm	~200
Blue LED	Perfin® PFLG02	10	300	460	~200

RESULTS

Effect of Different Light Source

The isolates MAY2, PR2, and IRRI1 prefer incandescent light for biogas production. As shown in Figures 1A and 1B, the highest biogas production (in ml) of the three isolates was recorded in setups incubated under incandescent light. MAY2, IRRI1, and PR2 produced 63.6 ml (795 ml·g^{-1} substrate), 59.6 ml (745 ml·g^{-1} substrate), and 47.4 ml (592.5 ml·g^{-1} substrate) biogas, respectively. Incubation under different light sources produced minimal to almost negligible amounts of biogas. Setups incubated under red LED light did not produce biogas, while those exposed to blue LED and fluorescent light produced minimal amounts only (< 5.0 ml). Optical density was found to have the highest biomass in set-ups exposed to fluorescent and incandescent light (Table 2). As listed in Table 2, the highest OD recorded for setups under incandescent light were 1.667 and 1.574, respectively, for PR2 and MAY2, while IRRI1 gave the highest OD (1.446) for the reactors incubated under fluorescent light. The highest recorded OD for IRRI was 1.446 in fluorescent light setups, while the lowest OD values (< 0.5) were recorded in setups exposed to red LED.

Carbon and Nitrogen Requirements

The isolates were observed to variedly utilize different carbon sources (Figures 2A and 2B). For MAY2 and PR2, the highest biogas production was recorded in butyrate at 67.6 ml (845 ml·g^{-1} substrate) and 55.0 ml (687 ml·g^{-1} substrate) of biogas, respectively. On the other hand, IRRI1 supplied with acetate produced 62.7 ml of biogas (781.25 ml·g^{-1}substrate). Interestingly, PR2 and MAY2, which both belong to the genus *Rhodobacter*, did not produce biogas in acetate. These two isolates were also able to produce gas when supplied with glucose, while IRRI1 did not. All the isolates did not produce biogas when supplied with soluble starch. In terms of OD, low OD was observed in setups supplied with succinate, as shown in Table 3. The highest recorded OD was in IRRI1 supplied with butyrate at 2.091. The highest OD for MAY2 and PR2 were also recorded in setups supplied with butyrate at 1.817 and 1.419, respectively.

While there was an observed variation in the utilization of carbon sources for the three isolates, a similarity was observed in the preference for nitrogen sources (Figures 3A and 3B). The three isolates produced biogas when supplied with glutamate, yeast extract, and peptone. However, they did not yield gas in ammonium (< 0.5) and urea setups. The three isolates exhibited the highest biogas production in setups supplied with yeast extract: MAY2, PR2, and IRRI1 produced 132 ml (1650 ml·g^{-1}substrate), 107.3 ml (1446.25 ml·g^{-1} substrate), and 88.5 ml (1106.25 ml·g^{-1} substrate) of biogas, respectively. The highest OD values were also observed in yeast extract set-ups of MAY2 and PR2 (Table 4). The highest OD value for IRRI1 was recorded in set-ups supplied with peptone.

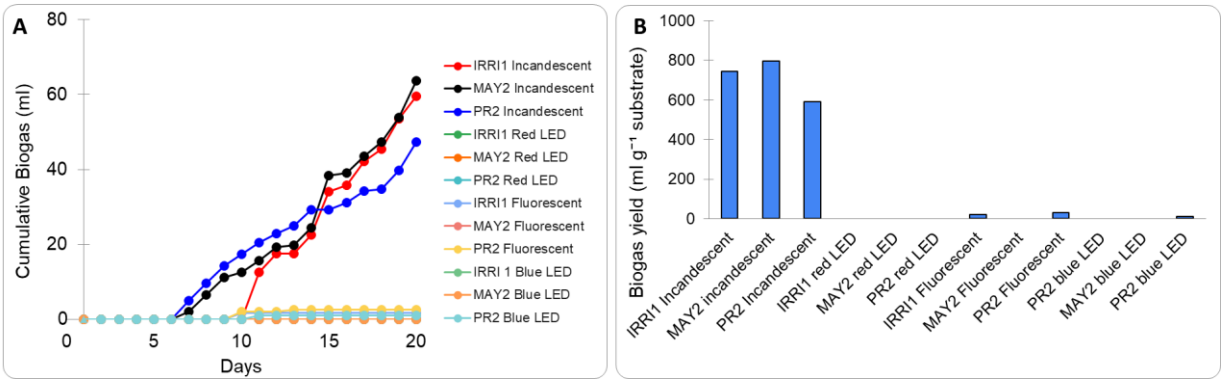

Figure 1. (A) Cumulative biogas production and (B) biogas yield of the purple-non-sulfur bacteria isolates incubated under different light conditions.

Table 2. Optical density at 660 nm (OD_{660}) measurement of purple non-sulfur bacteria (PNSB) isolates set-ups incubated under different light types.

Isolate	Light Type			
	Incandescent	Fluorescent	Red LED	Blue LED
IRRI1	1.011	1.446	0.294	1.183
MAY2	1.574	1.002	0.613	0.977
PR2	1.667	1.651	0.363	0.965

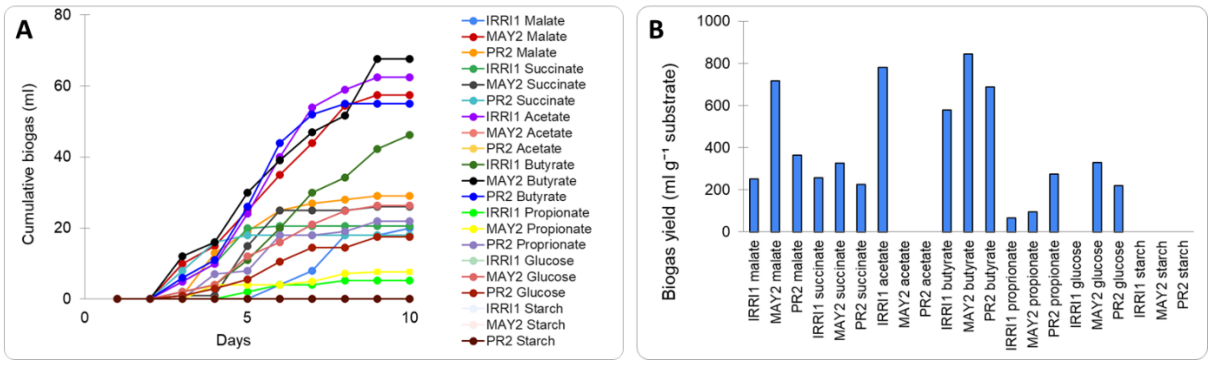

Figure 2. (A) Cumulative biogas production and (B) biogas yield of the purple non sulfur bacteria isolates under different carbon sources.

Table 3. Optical density at 660 nm (OD_{660}) measurement of the purple non-sulfur bacteria (PNSB) isolates set-ups incubated across different carbon sources.

Isolate	Carbon source						
	Malate	Succinate	Acetate	Butyrate	Propionate	Glucose	Starch
IRRI1	0.604	0.736	1.329	2.091	1.569	0.459	1.693
MAY2	1.323	0.717	1.417	1.817	0.736	1.645	1.485
PR2	0.814	0.712	1.408	1.419	0.514	1.225	1.114

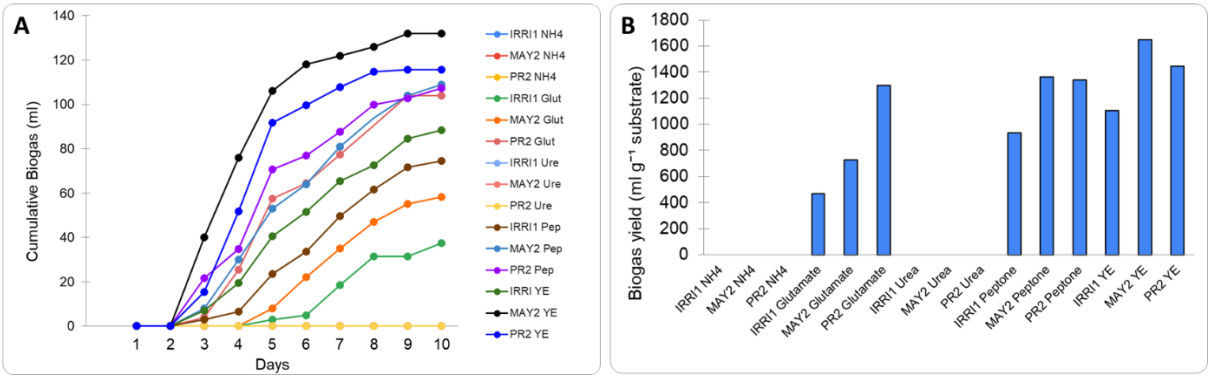

Figure 3. (A) Cumulative biogas production and (B) biogas yield of the purple non sulfur bacteria isolates under different nitrogen sources.

Table 4. Optical density at 660 nm (OD_{660}) measurement of the purple non-sulfur bacteria (PNSB) isolates set-ups incubated across different nitrogen sources.

Isolate	Nitrogen Source				
	NH_4	Glutamate	Peptone	Yeast Extract	Urea
IRRI1	0.534	0.598	0.765	0.614	0.398
MAY2	0.170	0.561	0.449	1.208	0.973
PR2	0.205	0.940	0.431	1.133	0.823

Effect of Salt (NaCl) Concentration

Thus, the salt tolerance of the isolates was also investigated. The results in Figures 4A and 4B showed that the isolates produced the highest amount of biogas in set-ups supplemented only with 0.04% NaCl. This concentration is the standard NaCl concentration of the MBP medium. MAY2, PR2, and IRRI1 produced 139.6 ml (1745 ml·g^{-1}substrate), 64 ml (800 ml·g^{-1} substrate), and 117 ml (1462.5 ml·g^{-1} substrate) of biogas, respectively. Adjusting the salt concentration to 0.85% (physiological concentration) decreased the production of biogas. Moreover, set-ups supplemented with 3.0% and 10% NaCl did not produce biogas. As shown in Table 5, cell growth is also affected by the salt concentration, as OD also decreased with the increase in NaCl. A 0.85 salt concentration exhibited the highest OD for MAY2 (1.129), followed by PR2 (0.936). IRRI1, on the other hand, produced the highest OD (0.949) at 3.0% NaCl concentration. The 10% salt concentration barely supported the cell growth of the isolates, as evidenced by an OD below 0.5.

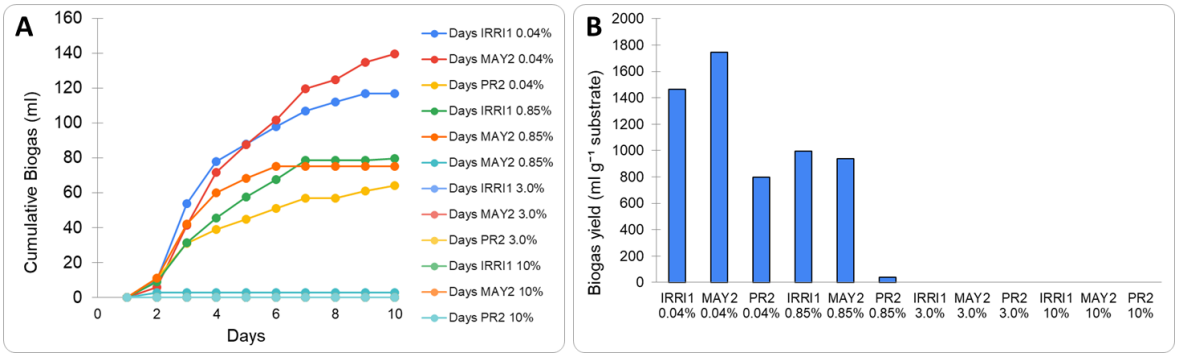

Figure 4. (A) Cumulative biogas production and (B) biogas yield of the purple non-sulfur bacteria isolates at varying salt concentrations.

Table 5. OD_{660} measurement of the purple non-sulfur bacteria isolates set-ups incubated under different salt concentration.

Isolate	Salt Concentration			
	0.04%	**0.85%**	**3.0%**	**10%**
IRRI1	0.798	0.872	0.949	0.223
MAY2	1.020	1.129	0.836	0.373
PR2	0.877	0.936	0.690	0.141

DISCUSSION

Biogas Production and Optical Density of the PNSB Isolates under Different Light Sources

Purple non-sulfur bacteria produce light-harvesting complexes (LHC) such as LH-I and LHII, which contain pigments that absorb far-red and near-infrared spectrum (Law et al. 2004). These pigments are mostly carotenoids and bacteriochlorophylls, which typically absorb light at 500 nm and above 800 nm, respectively. The absorbed light provides energy for photofermentation, which is the main pathway that generates H_2 (Deo et al. 2012). Incandescent lamps emitting an infrared spectrum (800 nm – 500 µm) were found to be the best option for H_2 production for *Rhodobacter capsulatus* (Monroy et al. 2013; Fox 2020). Furthermore, H_2 productivity is improved by the presence of both visible (400 nm – 750 nm) and infrared spectra provided by incandescent lamps (Turon et al. 2018; Halabe 2013). Therefore, the broad wavelength spectra of incandescent light provide the wavelength that complements the light absorption requirements of LHCs present in most PNSB. Observations from previous studies support the observed preference of the PNSB isolates in this study. For example, a study on *Rhodobacter capsulatus* found that replacing incandescent lamps with LED that only emit near-infrared light reduced H_2 production by 50% (Turon et al. 2018). In another study by Hu et al. (2018), the cell growth rate and H_2 production of four different species of PNSB were significantly higher when exposed to incandescent light systems compared to fluorescent. In contrast, LEDs and fluorescent are known to emit limited colored spectra (Abdel-Rahman et al., 2017), which may not provide the infrared and full visible spectra needed for optimized photofermentation, possibly limiting H_2 production. It is important to note, however, that light requirements for biohydrogen production may vary between species and can be affected by factors such as intensity and exposure time (Nath and Das 2009; Androga 2012). Additionally, the biogas production setups in the study took a more prolonged time (6 days) to produce biogas than what was usually observed (3-4 days). So, the 10-day observation period was extended to 20 days.

Based on the optical density results listed in Table 2, all the light source types used in the experiment can support the growth of the three isolates, but the cell growth of each isolate varies according to the type of source. For MAY2, incandescent light is favorable for biomass production, as evidenced by the high OD (1.574). For IRRI1, fluorescent light (1.446), while for PR2, both fluorescent and incandescent lights support high cell growth (1.667 and 1.651, respectively). Overall, many studies suggested that incandescent light best suits PNSB cultivation (Yu et al. 2021; Yu et al. 2022). For example, a study on *Rhodobacter sphaeroides* found that incandescent light generated the most biomass (5.66 g DCW L^{-1}) and protein (4.43 g L^{-1}) after 7-day cultivation compared to halogen lamp, infrared light, and variously colored LEDs. Full-spectrum incandescent light was also found to produce 3.2 times more biomass than spectral bands incandescent light (Yu et al. 2022). However, some studies focused on using other light types depending on the intended output or product. For example, Hülsen et al. (2019) utilized infrared light to selectively enrich PNSB in a non-sterile-rich medium for COD, NH_4-N, and PO_4 removal. In a study by Kuo et al. (2012), LED blue

light produced the highest cell and carotenoid concentration in cultures of *Rhodopseudomonas palustris* compared with incandescent, fluorescent, and other colors of LED lights. In a different study by Zhuo et al. (2014), red LED light was found to support higher biomass in production and COD removal of locally isolated *Rhodopseudomonas* compared with other sources of light. Moreover, the highest production of carotenoids was observed under yellow LED light. These studies suggest that blue and red light generally supports cell growth, although the optimum effect on productivity can be species-specific (Rashid et al. 2022).

These results suggest that higher biomass does not automatically mean higher biogas or H_2 production, and different light sources can support biomass growth but may not favor photofermentative biohydrogen production.

Biogas Production and Optical Density of the PNSB Isolates across Different Carbon and Nitrogen Sources.

The metabolic flexibility of PNSB allows them to assimilate different carbon sources. They are mainly studied for their capability to use volatile fatty acids as carbon sources for H_2 production. Most species of *Rhodobacter* prefer malate, succinate, butyrate, propionate, lactate, and pyruvic acid for H_2 production (Androga et al. 2012). This trend reported in the literature was also observed in the high H_2 productivity of MAY2 and PR2, both members of *Rhodobacter*. This preference can be due to the direct assimilation of the short-chain organic acids to the TCA cycle, which primarily provides electrons and protons to the photofermentation pathway. As the electrons are transferred in the pathway, a proton gradient is created, which drives the ATP synthase to generate energy, after which the protons and electrons are captured by nitrogenase to produce H_2 (Gabrielyan et al. 2015). Nonetheless, these isolates did not produce gas in acetate, which could be attributed to other competing pathways, such as the polyhydroxybutyrate (PHB) synthesis. In some *Rhodobacter* species, acetate is preferentially utilized for PHB synthesis, a pathway that competes with photofermentation for electrons obtained from the oxidation of VFAs. This relationship between the pathways puts a limit on how *Rhodobacter* can utilize acetate. *Rhodopseudomonas* members can utilize various VFAs with efficient acetate utilization (Oh et al. 2004; Touloupakis et al. 2021).

Although gas production was observed in glucose for MAY2 and PR2, the amount of gas produced was relatively lower compared to the VFA setups; starch, likewise, did not fare better. For PNSB to utilize glucose, it must be converted first to VFAs, resulting in inadvertent lowering of pH and a longer time in assimilating the substrate (Jeong et al. 2008). Starch also requires acid or enzymatic hydrolysis for it to be utilized by photosynthetic bacteria for photofermentation (Vendruscolo 2015).

The observed high biogas productivity of the isolates in setups supplied with yeast extract as a nitrogen source can be attributed to other micronutrients present in yeast extracts, such as amino acids, vitamins, minerals, and growth-stimulating compounds (Gabrielyan et al. 2015; Hay et al. 2013). These micronutrients may serve as co-enzymes and co-factors to the enzymes found in the photosynthetic electron transfer chain of the photofermentation pathway, as well as cofactors to the nitrogenases and hydrogenases. A study by (Hakobyan et al. (2012) compared the H_2 production of *Rhodobacter sphaeroides* supplied with yeast extract and glutamate as a nitrogen source. Yeast extract setups produced 6 times more H_2 than glutamate set-ups, which is attributed to the presence of growth-stimulating compounds, vitamins, and a variety of amino acids present in yeast extract. In another study by Liu et al. (2015), a novel strain of *Rhodopseudomonas* was able to grow and produce H_2 in setups supplied with peptone, beef extract, and glutamate but not in urea and ammonium. These results are similar to the obtained results of this study. The negligible production of biogas in setups supplied with ammonium can potentially be due to the inhibitory effect on ammonium ions to the nitrogenase enzyme, which either repressed the enzyme via feedback inhibition or by repression of nitrogenase-related genes (Androga et al. 2012). Similarly, urea can be broken down into carbamate and ammonium ions, possibly rendering the same effect (Alexandrova and Jorgensen 2007).

Salt (NaCl) Tolerance of the PNSB Isolates

Some PNSB members were found to withstand high salt concentrations even in seawater and hypersaline environments (Sakarika et al. 2019). Based on the results, high salt concentration halted the H_2 production of the isolates, although they were able to exhibit cell growth at 3.0% salt concentration. Many PNSB members were able to tolerate saline conditions, including species of *Rhodobacter* and *Rhodopseudomonas* (Asif et al. 2021). A study on one marine PNSB, *Rhodovulum sulfidophilum*, observed that biohydrogen production was still attained even at 3.0% salt concentration, and the optimum production is at 2.0% concentration (Cai and Wang 2012). However, for the isolates in this study, low NaCl concentration is still preferred for biogas production. These isolates were not obtained in saline or hypersaline environments and may not have the necessary adaptations for such conditions. Moreover, studies point out that increasing salinity can decrease the activity of the nitrogenase enzyme, which is the primary enzyme for photobiological hydrogen production (Severin et al. 2012).

Recommendations and Future Directions of the Study

Results from this study can serve as a baseline for future optimization experiments, as these results can narrow down the criterion and parameters of interest. Parameters such as carbon-to-nitrogen ratio and light intensity can also be explored. Furthermore, these can also be used for proper characterization and identification of the isolates.

The study relied only on crude measurement of biogas via displacement, that is why it is recommended to measure biohydrogen content and volatile fatty acid content via chromatography in future studies. Additionally, it is recommended to upscale the reaction and validate if it is comparable to the results from this study or other studies.

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ETHICAL CONSIDERATIONS

There were no animal or human subjects involved in the study.

DECLARATION OF COMPETING INTEREST

The authors declare that there are no competing interests of any authors.

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