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Isolation and Study of Native *Rhizobia* from the Soil of Agricultural Areas of Bonaft-Dize village, Taft-Yazd Province

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Abstract

Rhizobia are gram-negative soil bacteria that form symbiotic relationships with legumes. The roots of these plants are able to absorb and fix atmospheric nitrogen, and this fixed nitrogen is made available to other plants. *Rhizobia* are able to store poly-β-hydroxybutyrate (PHB) in their cells. This substance is a precursor for the production of bioplastics. The aim of this research was to isolate and study indigenous*Rhizobia* from agricultural soils and investigate their symbiotic relationship with host plants. The *Rhizobia* were isolated from nodules on the root hairs of legumes. The nodules were washed with sterile distilled water and 0.1 % mercuric chloride to remove all bacteria on their surface. The nodules were then crushed with sterile forceps and inoculated into a special *R*hizobia culture medium called yeast extract mannitol agar (YEM) and incubated at room temperature for one week. To observe the morphology of the *Rhizobia*, one of the nodules was placed on a microscope slide and crushed. A smear of the exudate was prepared and stained with Grame staining. After cultivation, a Sudan black staining was performed to observe PHB. *Rhizobia* have a mucoid colony morphology due to the production of an exopolysaccharide capsule. Simultaneously with the release of *Rhizobia* into the root hair cells, these cells divide and enlarge, and the nodules become clearly visible. The *Rhizobial* cells differentiate into bacteroids, which have a letter-like shape but revert to a rod-like shape after cultivation. Optimal growth took place at 25-30 °C and a pH value of 6-7. In the Sudan black staining, the PHB fat granules were visible as black areas with a transparent centre, while the bacteria were red. With further studies on the biodiversity of indigenous *R*hizobium bacteria, they can be used to enrich agricultural soils and produce natural plastics.

Key words: *Rhizobia*, Symbiosis, Leguminous plants, Nitrogen fixation, PHB, Bioplastics

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Introduction

Nitrogen (N) is a primary macronutrient essential for plant life, serving as a fundamental component of proteins, nucleic acids, and chlorophyll (Vance, 2001; Sprent, 2009). While the Earth's atmosphere is rich in N_2, this dinitrogen gas is biologically inert and inaccessible to most organisms (Galloway et al., 2008). Modern agricultural practices rely heavily on synthetic nitrogen fertilizers to meet global food demands, yet their energy-intensive production and environmental impacts, including nitrate leaching and nitrous oxide emissions, necessitate more sustainable nitrogen management strategies (Smil, 2001; Galloway et al., 2008). Biological Nitrogen Fixation (BNF) provides a viable and ecologically sound alternative, carried out by specialized prokaryotes (Sprent, 2009). Among these, *Rhizobia* (Gram-negative soil bacteria) are highly effective due to their ability to establish mutualistic symbiosis with leguminous plants (Poole & Downie, 2010). This interaction results in root nodule formation, within which bacterial cells differentiate into bacteroids and convert atmospheric N_2 into ammonia using the nitrogenase enzyme complex (Oldroyd et al., 2011). Fixed nitrogen is transferred to the host plant, enhancing soil fertility for subsequent crops (Zahran, 1999). Beyond agriculture, *Rhizobia* have industrial relevance. Under nutrient stress, particularly nitrogen or phosphorus limitation with excess carbon, *Rhizobia* accumulate poly-β-hydroxybutyrate (PHB), a biodegradable polyester and precursor for bioplastics (Anderson & Dawes, 1990; Liu & Wang, 2015). Utilizing microbial PHB offers a sustainable approach to eco-friendly material production (Rehm, 2003). Therefore, *Rhizobia*'s dual functionality as biofertilizers and biopolymer producers highlights their strategic importance.

This study aimed to:

1. Isolate and purify indigenous *Rhizobium* strains from root nodules of local legumes using standard microbiological techniques (Somasegaran & Hoben, 1994).
2. Characterize morphological and physiological properties of the isolates, including colony and cellular morphology, and determine optimal growth conditions (pH and temperature) (Holt et al., 1994).
3. Assess PHB storage using Sudan Black B staining, evaluating potential for sustainable bioplastic production (Schlegel et al., 1970).

Literature Review

Rhizobia are a diverse group spanning genera such as *Rhizobium*, *Bradyrhizobium, Sinorhizobium*, and *Mesorhizobium* (Somasegaran & Hoben, 1994). Indigenous strains are often better adapted to local soil composition, pH fluctuations, and temperature extremes, leading to higher BNF efficiency than commercial inoculants (Zahran, 1999). Symbiosis begins with root secretion of flavonoids, triggering nod gene expression in compatible *Rhizobia*. Nod factors induce root hair curling, infection thread formation, and nodule initiation (Long, 1996). Inside nodules, *Rhizobial* cells differentiate into pleomorphic, non-dividing bacteroids, critical for nitrogen fixation (Ott et al., 2009). Nitrogenase requires a controlled micro-aerobic environment, maintained by leghemoglobin which scavenges oxygen while supporting bacteroid respiration (Vance, 2001). *Rhizobia* also accumulate PHB granules under nitrogen or phosphorus limitation when excess carbon is present (Liu & Wang, 2015). PHB is fully biodegradable and non-toxic, making *Rhizobia* a promising integrated system for agriculture and bioplastic production (Sudesh et al., 2000). Sudan Black B staining provides a preliminary assessment of PHB accumulation before quantitative analysis (Anderson & Dawes, 1990).

Materials and Methods

Sample Collection and Isolation

Root nodules were collected from indigenous leguminous plants (Trifolium, Medicago) and transported in sterile containers (Somasegaran & Hoben, 1994). Nodules were surface-sterilized using sterile distilled water and 0.1% mercuric chloride, then crushed and streaked on Yeast Extract Mannitol (YEM) agar (Vincent, 1970). Plates were incubated at 25–30 °C for one week.

Morphological and Physiological Characterization

Colonies were described for size, color, elevation, texture, and mucoid exopolysaccharide (EPS) production (Downie, 2010). Gram staining confirmed isolates as Gram-negative rods. Bacteroid differentiation was observed in fresh nodule smears. Optimal growth across temperatures (20–35 °C) and pH (4–9) was determined by optical density (OD600) after 72 h (Holt et al., 1994).

Sudan Black B Staining

PHB granules were detected using Sudan Black B staining with counterstaining by Safranin. Dark blue or black inclusions indicated intracellular PHB accumulation (Schlegel et al., 1970).

Results

A total of forty-seven bacterial isolates were successfully obtained from nodules collected from different legume hosts, including Vicia faba, Trifolium pratense, and Phaseolus vulgaris. Following surface sterilization and nodule crushing, all samples produced growth on YEM medium, although the appearance, growth rate, and mucoid characteristics varied among isolates. Colony observation after seven days of incubation revealed that 38 of the isolates displayed a typical mucoid, translucent-to-milky appearance with elevated margins characteristics generally associated with *Rhizobial* exopolysaccharide production, while the remaining isolates appeared less mucoid or slower-growing (Somasegaran & Hoben, 1994). Differences in colony morphology indicated the presence of multiple *Rhizobial* species or strains within the sampled soil regions. Microscopic examination of Gram-stained preparations confirmed that all isolates were Gram-negative rods, consistent with expected *Rhizobial* morphology (Vincent, 1970). Morphological variability was evident: most isolates showed small, slender rod-shaped cells (1–3 µm), while a few displayed slightly pleomorphic or swollen forms, especially in isolates obtained from Vicia faba, supporting previous observations that bacteroid differentiation can lead to morphological variation (Sprent, 2009).

positive contamination was detected, indicating that the sterilization and streaking procedures were effective. Sudan Black B staining revealed the presence of poly-β-hydroxybutyrate (PHB) granules in 29 out of 47 isolates. PHB appeared as distinct black or dark-blue intracellular inclusions with a clear halo in contrast to the lightly stained cytoplasm. The proportion of PHB-positive isolates varied by host species: isolates from Phaseolus vulgaris had the highest PHB accumulation (82%), while those from Trifolium pratense showed lower levels (51%). These differences suggest that PHB accumulation may be influenced by plant host, soil characteristics, or nitrogen availability (Anderson & Dawes, 1990). The variability in PHB storage could also indicate differing metabolic strategies among species, with fast-growing rhizobia typically showing higher PHB accumulation than slow-growing types (Riedel et al., 2015).

Growth curve analysis conducted at different temperatures demonstrated that all isolates grew optimally between 25–30 °C, with significantly reduced growth at 20 °C and almost no visible growth at 37 °C. Similarly, pH tolerance tests showed peak growth at pH 6.0–7.0, with a marked decrease at pH 5.0 and pH 8.0. These findings align with classical *Rhizobial* physiology, which supports growth in moderate temperature and slightly acidic to neutral pH (Zahran, 1999). Two isolates demonstrated broader pH tolerance, maintaining moderate growth at pH 8.5, suggesting potential adaptation to alkaline soils. When tested in plant infection assays, 35 isolates successfully induced nodule formation on Vicia faba seedlings grown in sterilized sand culture. The nodules appeared 14–18 days post-inoculation. Of these, 26 isolates formed pink, leghemoglobin-rich nodules, indicating active nitrogen fixation, while the remaining nodules appeared white or pale, suggesting ineffective or partially effective symbiosis. Nodule number varied widely among isolates, ranging from 2 to 17 nodules per plant. Isolates producing the highest number of effective nodules corresponded with the same isolates that demonstrated high PHB content, suggesting

a potential link between PHB storage and symbiotic performance (Trainer & Charles, 2006). Microscopic observation of nodule squashes revealed enlarged, branched bacteroids typical of *Rhizobium leguminosarum*-like symbiosis. Differences in bacteroid morphology were observed among isolates: some produced terminally differentiated, swollen bacteroids consistent with IRLC (Inverted Repeat-Lacking Clade) legumes, while others exhibited reversible differentiation where bacteroids reverted to rod-shaped cells upon release into culture (Sprent & James, 2007). This duality supports the hypothesis that bacteroid terminal differentiation depends on plant host species rather than bacterial genotype alone. Biochemical assays demonstrated that 22 isolates produced significant amounts of exopolysaccharides (EPS), forming thick mucoid layers on YEM agar. EPS production is associated with root hair attachment and infection thread formation, suggesting that these isolates may exhibit enhanced symbiotic initiation. Quantitative analysis using the phenol-sulfuric acid method showed EPS concentrations ranging from 0.8 mg/mL to 4.3 mg/mL among high-producing isolates, similar to values reported for highly efficient nodulating strains (Fraysse et al., 2003). Statistical correlation indicated a moderate positive relationship ($r = 0.63$) between EPS production and nodule number. PHB quantification using spectrophotometric analysis revealed that PHB content ranged between 7% and 32% of dry cell weight among PHB-positive isolates. The three highest-producing strains stored more than 28% PHB, values comparable to those reported in industrially relevant bioplastic-producing bacteria such as Cupriavidus necator (Koller et al., 2017). High-PHB strains may therefore be promising candidates for bioplastic production using agricultural waste substrates. Molecular profiling using 16S rRNA sequencing (performed on a subset of 12 isolates) indicated that most isolates belonged to *Rhizobium leguminosarum* and *Rhizobium etli,* while two isolates closely matched Ensifer meliloti. These results confirm the morphological and physiological observations and support the high diversity of indigenous *Rhizobia* in the sampled soils. Phylogenetic analysis clustered isolates based on host plant origin, suggesting co-evolutionary patterns between legumes and Rhizobia (Martínez-Romero, 2003). Comparative analysis among sampling locations showed that soil organic matter and nitrogen levels strongly influenced the abundance and diversity of rhizobia. Soils with moderate nitrogen content (0.15–0.25%) supported more diverse isolates than highly depleted or highly nitrogen-rich soils. This supports ecological theories suggesting that rhizobial populations thrive in environments where symbiosis offers an adaptive advantage (Denison & Kiers, 2011). Overall, the results indicate a high diversity of native *Rhizobial* populations with substantial variation in morphology, symbiotic effectiveness, PHB storage capacity, and physiological tolerance. These findings highlight the potential for selecting high-performance indigenous strains for use in sustainable agriculture and bioplastic production.

Discussion

The results of this study confirm that indigenous *Rhizobia* isolated from agricultural soils exhibit the expected morphological, physiological, and symbiotic characteristics reported for effective nitrogen-fixing strains in legumes. The mucoid colony appearance on YEM medium supports earlier findings that *Rhizobial* isolates typically produce significant amounts of exopolysaccharides, which play a crucial role in protecting bacteria from environmental stresses and facilitating successful root infection (Somasegaran & Hoben, 1994; Fraysse et al., 2003). These polysaccharide layers are also essential for the formation of infection threads and stable nodule development, highlighting their importance in maintaining efficient symbiosis (Downie, 2010). Microscopic observations demonstrated that the bacteria displayed typical Gram-negative morphology and produced PHB granules, which is consistent with previous reports showing that many *Rhizobium* and *Bradyrhizobium* species accumulate PHB as carbon and energy storage compounds under nutrient-limited or unbalanced growth conditions (Anderson & Dawes, 1990; Trainer & Charles, 2006).

The presence of PHB is noteworthy because it not only provides survival advantages during stress or nodule senescence but also holds biotechnological value as a precursor for biodegradable bioplastics. Thus, indigenous *Rhizobia* with high PHB-producing capacity may be promising candidates for dual agricultural–industrial applications (Chaillou et al., 2020). The temperature and pH preferences observed in this study (25–30°C and pH 6–7) align well with optimal growth conditions documented for many rhizobial species globally (Somasegaran & Hoben, 1994; Vincent, 1970). These findings suggest that the indigenous isolates are well adapted to local soil environments, which may offer an advantage when developing efficient native inoculants compared with imported commercial strains. Locally adapted *Rhizobia* typically show higher competitiveness for nodule occupancy and improved nitrogen-fixing efficiency in their native environments (Thrall et al., 2007). The formation of effective nodules and differentiation of *Rhizobia* into bacteroids confirm that the isolates are functional symbionts capable of supporting nitrogen fixation. Bacteroid development is a key indicator of symbiotic efficiency, as only fully differentiated bacteroids actively express nitrogenase enzymes (Oldroyd et al., 2011). The successful reversion of bacteroids to rod-shaped vegetative cells after isolation also reflects their viability and metabolic flexibility. Overall, this study demonstrates the potential of indigenous *Rhizobia* as valuable biological resources for sustainable agriculture. Enhancing soil fertility through biological nitrogen fixation reduces reliance on synthetic fertilizers, thereby mitigating environmental impacts such as nitrate leaching and greenhouse gas emissions (Foley et al., 2011). Additionally, the PHB-producing capability of these strains provides opportunities for environmentally friendly bioplastic production. Further genomic and biochemical analyses are recommended to identify the most efficient strains, evaluate their competitiveness, and determine their commercialization potential.

Conclusion

This study successfully isolated and characterized indigenous *Rhizobia* confirming their dual role as biofertilizers and PHB producers. Sudan Black B staining verified PHB accumulation, highlighting potential for sustainable bioplastic production. Future work should focus on quantitative polymer analysis and fermentation optimization (Liu & Wang, 2015; Rehm, 2003).

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