

BIOCOMPATIBILITY ASSESSMENT OF A NUTRIENT-LOADED PICKERING EMULSION USING BENEFICIAL AGRICULTURAL MICROORGANISMS

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ABSTRACT

The biological compatibility of a nutrient-loaded Pickering emulsion was evaluated using three complementary biochemical assays: the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, the neutral red uptake (NRU) assay, and the lactate dehydrogenase (LDH) assay. The assessment involved agriculturally beneficial microorganisms, including *Rhizobium* and zinc-solubilizing bacteria (ZSB). The MTT assay showed that microbial viability remained above 94% in both *Rhizobium* and ZSB, even at a formulation concentration of 1600 ppm. Similarly, the NRU assay indicated high viability at 1200 ppm, with values of 95.6% in *Rhizobium* and 94.8% in ZSB. In contrast, LDH activity increased with formulation concentration, indicating a concentration-associated increase in membrane-related cellular stress. Nevertheless, the high viability observed in the MTT and NRU assays suggests that the formulation did not substantially reduce microbial viability under the tested conditions. These results provide preliminary evidence that the developed Pickering emulsion is compatible with agriculturally beneficial microorganisms within the tested concentration range. However, further long-term and soil-based studies are required to establish its environmental safety.

KEYWORDS: nanofertilizer, microbial compatibility and Pickering emulsion

1. INTRODUCTION

The development of advanced agricultural formulations using nano- and microstructured delivery systems has attracted considerable attention, as these systems can improve nutrient-use efficiency, enable controlled nutrient release, and support targeted delivery (Yadav et al., 2023; Ray et al., 2025). However, their environmental compatibility is an important consideration: agricultural nanomaterials may interact with soil microorganisms and alter their abundance, metabolic activity, and ecosystem functions (Atanda et al., 2025). Beneficial microorganisms are especially important because they contribute to nutrient cycling, nitrogen fixation, nutrient solubilization, and plant–microbe interactions (Wei et al., 2024; Hayat et al., 2010). Therefore, assessing microbial compatibility should be an important part of the safety evaluation of novel nano-enabled agricultural formulations (Feng et al., 2026). Pickering emulsions represent an important class of colloidal delivery systems in which solid particles, rather than conventional molecular surfactants, stabilize the interface between two immiscible liquids. The particles adsorb at the oil–water interface and form a protective interfacial layer that inhibits droplet coalescence. Particle wettability, size, morphology, surface charge and concentration strongly influence the resulting emulsion stability and functionality (de Carvalho-Guimarães et al., 2022; Yang et al., 2017). The distinctive interfacial structure of Pickering emulsions provides several advantages for agricultural delivery. Solid particles can provide enhanced physical stability, while the dispersed phase can serve as a reservoir for hydrophobic or poorly water-soluble active ingredients. Recent research has particularly investigated bio-based and lignin-derived particles as Pickering stabilizers because of their renewable origin and potential biodegradability. Lignin-stabilized Pickering emulsions have been proposed for controlled pesticide delivery and for improving formulation stability and persistence (Yu et al., 2024).

Nanoparticle–microorganism interactions depend on several physicochemical properties, including particle size, surface charge, chemical composition, concentration, and surface area (Shin et al., 2015; Suresh et al., 2012). Based on these properties, nanoparticles can have limited or substantial effects on microorganisms through mechanisms such as membrane interactions, oxidative stress, and the release of reactive or toxic species (Eker et al., 2024; Suresh et al., 2012; Manke et al., 2013). Microbial responses should therefore be evaluated experimentally, rather than inferred solely from a formulation's physicochemical characteristics.

The present study assessed the compatibility of the developed nutrient-loaded Pickering emulsion with two agriculturally relevant beneficial microorganisms: *Rhizobium* and zinc-solubilizing bacteria (ZSB). Three complementary assays were used. The MTT assay relies on the conversion of tetrazolium salt to formazan by metabolically active cells and is widely used to assess cellular metabolic activity and viability (Riss et al., 2016). The NRU assay measures the uptake and accumulation of neutral red by viable cells, providing an independent measure of cellular viability (Rodrigues et al., 2023). In contrast, the LDH assay measures extracellular LDH

released when cellular membrane integrity is lost and is commonly used to indicate cellular damage or cytotoxicity (Riss et al., 2019; Hiebl et al., 2017). Using these three endpoints together provides a more informative assessment because metabolic viability and membrane integrity represent different aspects of microbial response.

2. MATERIALS AND METHODS

2.1 Microbial Toxicity

The study aimed to visualise the toxicity in different microorganisms. Rhizobium bacteria, zinc-solubilising bacteria, and PPFM were subjected to different ppm of nanofertilizer

Media used for Microbial Toxicity

S.no.	Microorganism	Media
1	Rhizobium	Yeast Mannitol Agar with congo red
2	Zinc Solubilising Bacteria	Nutrient Agar + Zinc oxide

2.2 Lactose DeHydrogenase (LDH) assay

The lactate dehydrogenase assay determines the number of non-viable cells by detecting lactate dehydrogenase released by cells under adverse conditions. Lactate dehydrogenase converts the stored energy form (lactate) into the active energy form (pyruvate). During this conversion, NAD⁺ is reduced to NADH. The proton generated by the oxidation of NADH to NAD⁺ enables the exogenous diaphorase enzyme to transform the INT tetrazolium salt into a red-coloured formazan product. The red-coloured formazan product was measured at 490 nm. Absorbance increases with the number of non-viable cells and vice versa. The substance-mediated cytotoxicity assay on *Bacillus subtilis* and *Azotobacter chroococcum* bacteria was determined using the LDH assay (EZcount TM Lactose dehydrogenase cell assay kit).

The overnight-grown cultures of Rhizobium bacteria, zinc-solubilizing bacteria, and PPFM were inoculated into treatments individually and incubated in an orbital shaker at 37°C and 80 rpm for 5 hours. Untreated control (90 µl culture + 10 µl water) and maximum LDH (90 µl culture + 10 µl lysis buffer) were prepared and incubated at 37°C for 30 to 45 minutes. All the above samples were centrifuged, and the assay utilized the supernatant. Take 100 µl of samples in a 96-well plate and add LDH reagent mix to all treatments, including the untreated control and maximum LDH. Incubate at room temperature for 15 to 30 minutes under dark conditions, as the LDH reagent mix is light-sensitive. Later, add 50 µl of stop solution into samples and observe OD at 490 nm.

2.3 Neutral Red uptake assay

The neutral red uptake assay is a cell viability assay used to quantify xenobiotic-induced cytotoxicity in vitro. This assay depends on the incorporation and binding of neutral red, a weak cationic dye, within the lysosomes of live cells, driven by the pH gradient established after treatment. Overnight-grown cultures of Rhizobium bacteria, zinc-solubilising bacteria, and PPFM were inoculated into treatments separately and incubated on an orbital shaker at 37°C and 80 rpm for 5 hours.

Add 10 µl of 10% neutral red reagent to all treatments, which are then incubated in an orbital shaker at 37°C, 80 rpm for 2-4 hours. All the samples were centrifuged and the pellet was used for the assay. To the collected pellet, add 100 µl of neutral red solubilization solution and incubate the 96-well plate at room temperature for 10 minutes, then observe OD at 540 nm. The cell viability percentage was calculated using the formula after background correction.

$$\text{Cell viability \%} = \frac{\text{control OD} - \text{Test sample OD}}{\text{Control OD}} \times 100$$

2.4 MTT Assay

The MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay) is a colorimetric method widely used to evaluate cellular metabolic activity and viability following exposure to a test substance, such as a nano-fertilizer, nanoparticle, chemical compound, or extract. The assay is based on the ability of metabolically active cells to reduce the yellow MTT reagent to insoluble purple formazan crystals, primarily through cellular reductase activity.

Viable and metabolically active cells reduce MTT to purple formazan, whereas cells with severely impaired metabolic activity show reduced or negligible MTT reduction. After incubation, the formazan crystals are dissolved using an appropriate solubilizing solution, and absorbance is measured spectrophotometrically, commonly at 570 nm with a reference wavelength around 630–690 nm.

The percentage of cell viability can be calculated as:

$$\text{Cell viability \%} = \frac{\text{Absorbance of control cells}}{\text{Absorbance of treated cells}} \times 100$$

3. RESULTS AND DISCUSSION

3.1 Effect of Pickering emulsion on microbial viability assessed by MTT assay

The MTT assay demonstrated that exposure to the Pickering emulsion resulted in only a relatively small reduction in metabolic viability of *Rhizobium* and zinc-solubilizing bacteria over the tested concentration range (Table 1). The MTT assay is based on the reduction of tetrazolium salts by metabolically active cells, resulting in the

formation of a colored formazan product; consequently, the measured signal is associated with cellular metabolic activity (Riss et al., 2016; Janjic and Wollheim., 1992).

In *Rhizobium*, the viability was 99.4% at 200 ppm, 98.6% at 400 ppm and 98.9% at 600 ppm. With further increases in formulation concentration, viability decreased to 97.6%, 96.5%, 96.2%, 95.4% and 94.2% at 800, 1000, 1200, 1400 and 1600 ppm, respectively. Thus, even at the highest tested concentration of 1600 ppm, the reduction in MTT-derived viability relative to the control was approximately 5.8%.

A comparable response was observed in ZSB. Viability was 99.2% at 200 ppm and remained above 97% at 400 and 600 ppm. At 800, 1000, 1200, 1400 and 1600 ppm, viability values were 96.2%, 95.2%, 95.2%, 94.9% and 94.6%, respectively. The overall reduction at 1600 ppm was therefore approximately 5.4%.

Table 1. Effect of Pickering emulsion concentration on microbial viability determined by MTT assay

Pickering emulsion concentration	<i>Rhizobium</i> (%)	ZSB (%)
Control	100.0	100.0
200 ppm	99.4	99.2
400 ppm	98.6	97.3
600 ppm	98.9	97.5
800 ppm	97.6	96.2
1000 ppm	96.5	95.2
1200 ppm	96.2	95.2
1400 ppm	95.4	94.9
1600 ppm	94.2	94.6

The relatively high MTT response across all treatments indicates that the formulation did not substantially suppress microbial metabolic activity under the experimental conditions. The gradual decrease with increasing concentration may indicate a concentration-associated response; however, the magnitude of the reduction remained comparatively small.

This observation is particularly relevant for agricultural nanoformulations because previous studies have demonstrated that nanoparticle effects on soil and plant-associated microorganisms can vary substantially according to nanoparticle composition, concentration and physicochemical characteristics. Thus, the high viability observed in the present study provides experimental evidence of compatibility of the developed formulation with the tested beneficial bacteria.

It should be noted that MTT reduction represents metabolic activity rather than a direct enumeration of viable colony-forming units. Therefore, the results are most appropriately described as maintenance of metabolic viability/activity, rather than complete absence of microbial effects.

2.2 Effect of Pickering emulsion on microbial viability assessed by NRU assay

The NRU assay provided an independent assessment of microbial viability (Table 2). The assay is based on the ability of viable cells to take up and retain the supravital dye neutral red; reduced uptake is associated with impaired cellular viability or function (Repetto et al., 2008; Zhang et al., 1990).

In *Rhizobium*, the viability remained very high at lower concentrations, with values of 99.9%, 98.4% and 98.1% at 200, 400 and 600 ppm, respectively. At 800 ppm, viability was 97.9%, followed by 96.8% at 1000 ppm and 95.6% at 1200 ppm.

Similarly, ZSB showed viability values of 99.8%, 98.7% and 98.2% at 200, 400 and 600 ppm, respectively. At 800, 1000 and 1200 ppm, viability was 96.5%, 95.9% and 94.8%, respectively.

Table 2. Effect of Pickering emulsion concentration on microbial viability determined by NRU assay

Pickering emulsion concentration	<i>Rhizobium</i> (%)	ZSB (%)
Control	100.0	100.0
200 ppm	99.9	99.8
400 ppm	98.4	98.7
600 ppm	98.1	98.2
800 ppm	97.9	96.5
1000 ppm	96.8	95.9
1200 ppm	95.6	94.8

At 1200 ppm, the reduction relative to the control was only 4.4% in *Rhizobium* and 5.2% in ZSB. The close agreement between the MTT and NRU results strengthens the interpretation that the formulation did not cause substantial impairment of microbial viability under the conditions evaluated.

The use of complementary viability assays is particularly useful in nanoformulation studies because nanomaterials can interfere with individual assay components or influence cellular responses through different mechanisms. Therefore, interpretation based on multiple endpoints provides greater confidence than relying on a single biochemical assay.

2.3 Effect of Pickering emulsion on LDH activity

LDH activity was evaluated as an indicator of cellular membrane-associated damage (Table 3). LDH is an intracellular enzyme that can be released into the surrounding medium when membrane integrity is compromised. The LDH-release approach has consequently been widely used as an indicator of cellular cytotoxicity or membrane damage (Yuan et al., 2019; Hwang and Yen, 2008; Decker and Lohmann-Matthes, 1988).

The control LDH values were 0.4, 1.1 and 0.8 for *Rhizobium* and ZSB respectively. Following exposure to 200 ppm Pickering emulsion, LDH activity increased to 2.5 and 3.8, respectively. At 400 ppm, the corresponding values were 3.9 and 4.4.

At 600 ppm, LDH activity was 3.3 in *Rhizobium* and 4.3 in ZSB. A further increase was observed at higher concentrations, with values of 4.7 and 5.9 at 800 ppm and 5.6 and 6.1 at 1000 ppm.

At 1200 ppm, LDH values increased to 6.5 and 6.6 for *Rhizobium* and ZSB, respectively. At 1400 ppm, values were 6.1 and 7.8, while the highest values were recorded at 1600 ppm: 7.2 for *Rhizobium* and 9.5 for ZSB.

Table 3. Effect of Pickering emulsion concentration on LDH activity

Pickering emulsion concentration	<i>Rhizobium</i> (%)	ZSB (%)
Control	0.4	1.1
200 ppm	2.5	3.8
400 ppm	3.9	4.4
600 ppm	3.3	4.3
800 ppm	4.7	5.9
1000 ppm	5.6	6.1
1200 ppm	6.5	6.6
1400 ppm	6.1	7.8
1600 ppm	7.2	9.5

The increasing LDH activity at higher concentrations suggests that exposure to the formulation was associated with an increase in membrane-associated cellular stress. The strongest response at 1600 ppm was observed in ZSB, followed by PPFM and *Rhizobium*. However, the LDH results should not be interpreted independently as evidence of substantial loss of viability. LDH release reflects membrane integrity, whereas MTT and NRU provide information on metabolic and cellular viability, respectively. Consequently, the simultaneous maintenance of high MTT and NRU responses suggests that the increase in LDH activity did not correspond to extensive loss of microbial viability under the tested conditions.

3.4 Integrated assessment of microbial compatibility

The combined results from MTT, NRU and LDH assays provide a more comprehensive understanding of the interaction between the Pickering emulsion and beneficial microorganisms.

The MTT assay showed that *Rhizobium* and ZSB maintained viability values of 94.2% and 94.6%, respectively, even at 1600 ppm. Similarly, the NRU assay showed viability of 95.6% in *Rhizobium* and 94.8% in ZSB at 1200 ppm. These results indicate that the majority of the microbial population retained metabolic and cellular viability following exposure.

In contrast, LDH activity increased progressively with increasing formulation concentration, particularly above 1000 ppm. This pattern suggests that the formulation may induce some degree of concentration-dependent membrane stress without producing a corresponding substantial decline in overall viability. Such differential responses are important because nanoparticle–microorganism interactions can involve several simultaneous processes, including surface interaction, membrane perturbation, oxidative stress and changes in cellular metabolism.

The preservation of microbial viability is particularly relevant for agricultural formulations because beneficial microorganisms contribute substantially to nutrient cycling and plant growth. Previous reviews have emphasized that nano-enabled agricultural inputs may influence plant-associated and soil microbial communities, and that their effects depend on the nature, concentration and environmental behavior of the nanomaterial.

Therefore, the present findings are encouraging because the tested Pickering emulsion produced only a relatively small reduction in MTT- and NRU-derived viability. Nevertheless, these results should be regarded as preliminary laboratory evidence of microbial compatibility rather than definitive proof of environmental safety.

4. CONCLUSION

The developed nutrient-loaded Pickering emulsion exhibited a high degree of compatibility with the tested beneficial agricultural microorganisms based on MTT and NRU assays. *Rhizobium* and ZSB retained more than 94% viability at the highest concentration tested in the MTT assay, while NRU viability remained above 94% at 1200 ppm. The LDH assay showed a concentration-dependent increase in LDH activity, indicating increased membrane-associated cellular stress at higher formulation concentrations.

Taken together, the results indicate that the Pickering emulsion did not cause substantial loss of microbial viability under the tested experimental conditions, although the elevated LDH response at higher concentrations indicates the need for further investigation of membrane-level effects. Because the response of soil microorganisms to nano-

enabled agricultural formulations can vary with particle properties, concentration and environmental conditions, further studies involving microbial growth kinetics, colony-forming ability, longer exposure periods, soil microcosms and field-relevant application concentrations are recommended.

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