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Influence of iron oxide nanoparticles on growth and activity of native Lignocellulosic bacteria

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Abstract
The influence of iron oxide (FeO) nanoparticles (NPs) on the growth and activity of native Lignocellulosic bacteria was investigated through series of laboratory experiments. FeO NPs at concentrations ranging from 5 to 100 ppm were tested to study their effect on growth of native bio decomposer bacterial strains at different time intervals through plate assay. No any adverse effect was recorded of different concentrations of FeO NPs on the growth of the bio decomposers. During the liquid assay experiment, the optimum growth of each bio decomposer bacteria was recorded at a concentration of 60 ppm of FeO NPs. Comparatively adverse effect of FeO NPs concentration were observed from 70 ppm onwards on growth of bio decomposers. During compatibility study, all four native bio decomposers were found compatible with each other. As far as cellulolytic and lignolytic activity is concerned, their efficiency on solid media revealed that FeO NPs at the concentration of 60 ppm was found to enhance cellulolytic and ligninolytic activity of the bio decomposer bacteria in comparison to other concentrations of FeO NPs.

Keywords: Bio decomposer, iron oxide, nanoparticles, cellulolytic, lignolytic

Introduction
Nanoparticles have applications in agriculture, including: (1) nano formulations of agrochemicals for applying pesticides and fertilizers for crop improvement, (2) the use of nanosensors in crop protection for detecting diseases and agrochemical residues, (3) nanodevices for plant genetic engineering, (4) plant disease diagnostics, (5) animal health, animal breeding, poultry production and (6) postharvest management are some of the topics covered. The growth of biomass-to-fuel generation could be improved by nanotechnology (Ghidan *et al*., 2020) [20]. Various studies showed that Fe O nanoparticles might considerably reduce the electrolyte leakage (EL), malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentration, while increase the activities of antioxidants in plants under cadmium stress (Manzoor *et al*., 2021) [8].

Frenk *et al*. (2013) [3] studied the effect of metal oxide nanoparticles on microbial community structure and function in two different soil types and revealed a growth in the number of *Bacilli* class members in CuO 1% and Fe_3O_4 0.1% treated soils, as well as an increase in the abundance of Betaproteo bacteria. Liu *et al*. (2013) [7] studied effects of Fe nanoparticles on bacterial growth and bio surfactant production and found that, after 72 h time growth of bacteria is higher with Fe nanoparticles than without Fe nanoparticles. Diao and Yao (2009) [2] reported that *Bacillus subtilis* survival rates were somewhat greater when pre-oxidized NZVI particles were treated with oxygen, *i.e*., oxidized to FeO (OH), were needle-shaped and strongly aggregated, and their size was significantly increased. The inactivation effects of oxidized NZVI particles on *B. subtilis* were not seen. They also found that in comparison to *B. subtilis*, *Pseudomonas fluorescens* is more sensitive to environmental stress. *P. fluorescens* survival rates after treatment with various iron suspensions used for *B. subtilis*.

Asha *et al*. (2012) [1] found that maximum cellulolytic enzyme activity (87.25+0.73) recorded when Fe was incorporated in the medium in comparison to control (20.75+0.60). They have also concluded that metal ion Fe^{2+} showed maximum stimulatory activity, and K^+, Mg^{2+}, and Zn^{2+} did not have any effect on the activity of the enzyme. The effects of a magnetic Fe_3O_4 (0.5%) for biogas production during anaerobic digestion of pig manure and they revealed that when compared to anaerobic digestion without Fe_3O_4, the use of 0.5% Fe_3O_4 had the most positive influence on biogas generation, with total biogas and methane content increasing by 13.81% and 35.13%, respectively (Liu *et al*., 2019) [6].

Looking to the environmental issues pertaining to different Lignocellulosic agro-waste and the applicability of FeO nanoparticles to enhance the biodegradation potential of Lignocellulosic waste, the current study was designed to study the effect of FeO nanoparticles on the growth and Lignocellulosic activity of native bio decomposer bacterial strains *in vitro*.

Material and methods

Bacterial strains used Native cellulose and lignin degrading bacterial strains i.e. *Pseudomonas stutzeri* AAU BDCT 1, *Bacillus velezensis* AAU BDCT 2, *Streptomyces rochei* AAUBD M 10 and *Streptomyces chartreusis* ss AAUBD M 16 available at the Department of Agricultural Microbiology culture collection were used for the study.

Plate assay the effect of FeO nanoparticles on the growth of bio decomposers was determined following a plate assay performed on Nutrient Agar (NA) medium supplemented with different concentrations of FeO nanoparticles *viz*. 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 ppm. Nutrient Agar supplemented with fertilizer grade $FeSO_4$ (@ 50 kg/ha of soil) has served as a treated check and NA medium without FeO nanoparticles has served as a control. Inoculated plates were incubated at 32 ºC and observations for enhancement or inhibition of the organism's growth were recorded at every 24, 48, 72 and 96 hrs of incubation (Liu *et al*., 2013) [7].

Liquid assay The effect of FeO nanoparticles on the growth of bio decomposers was determined following a liquid assay performed using 50 ml Nutrient Broth (NB) medium supplemented with different concentrations of FeO nanoparticles *viz*., 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 ppm. NB media supplemented with fertilizer grade $FeSO_4$ (@ 50 kg/ha of soil) has served as a treated check and NB medium without FeO nanoparticles has served as a control. After inoculation, flasks were incubated on a shaker at 32 ºC and 100 rpm for 96 hrs. Observations for enhancement or inhibition of the organism's growth were recorded in terms of O.D. (Optical Density) using spectrophotometer at 600 nm after 24, 48, 72 and 96 hours of incubation (Liu *et al*., 2013) [7]. *In vitro* compatibility for consortium preparation *In vitro* plate bioassay was carried out to determine the compatibility of selected isolates with each other on nutrient agar plates. The respective bacterial cultures were cross-streaked on Nutrient agar plates and incubated at 30±2 ºC for 5 days and observed every 24 hrs for interaction or inhibition of any of the test cultures at intercept on a plate (Prajapati *et al*., 2017) [9]. S Plate assay for cellulolytic activity to determine the effect of FeO nanoparticles on cellulolytic activity, a plate assay was carried out following methodology described by Liu *et al*. (2013) [7]. Car boxy methyl cellulose (CMC) agar plates supplemented with 5 to 100 ppm of FeO nanoparticles ware inoculated with native bio decomposers. CMC Agar supplemented with fertilizer grade $FeSO_4$ (@ 50 kg/ha of soil) has served as a treated check and CMC agar medium without FeO nanoparticles has served as a control. Inoculated plates were incubated at 32 ºC for 72 hrs. Observations were recorded at every 24 hrs up to 72 hrs. Cellulase activity was defined as the formation of a colorless zone around microbial colonies. At that point, the ratio of halo to colony diameters was recorded to work out Solubilization Index (SI). The FeO nanoparticles concentration, at which the highest SI was recorded, has been considered as the threshold concentration (In ppm) for that particular isolate.

$$\text{Solublization Index (SI)} = \frac{\text{Colony Diameter (mm)} + \text{Halo Zone Diameter (mm)}}{\text{Colony Diameter (mm)}}$$

Plate assay for lignolytic activity To determine the effect of FeO nanoparticles on lignolytic activity, a plate assay was carried out following methodology described by Rahman *et al.* (2013) [10]. Minimal Salt Medium agar plates incorporated with 1% alkaline lignin (as a sole source of carbon and energy) and supplemented with 5 to 100 ppm of FeO nanoparticles ware inoculated with bio decomposers. MSM-L agar supplemented with fertilizer grade $FeSO_4$ (@ 50 kg/ha of soil) has served as a treated check and MSM-L agar medium without FeO nanoparticles has served as a control. Inoculated plates were incubated at 32 ºC for 72 hrs. Observations were recorded at every 24 hrs up to 72 hrs. Lignolytic activity was defined as the increase or decrease in growth intensity of the organisms on the MSM-L agar plate. The FeO nanoparticles concentration, at which the highest growth was recorded, has been considered as the threshold concentration (in ppm) for that particular isolate.

Results and Discussion

Plate assay all bio decomposer bacterial strains showed mild growth at 24 h (Table 1). Moderate growth was recorded in bio decomposer cultures for FeO NPs concentrations at 5, 10, 20, 30, 80, 90 and 100 ppm and $FeSO_4$, while luxurious growth was observed for FeO NPs concentrations at 40, 50, 60 and 70 ppm at 48 h (Table 1). Similarly, at 72 and 96 hrs all bio decomposer cultures showed luxurious growth (Table 1). From the results presented in table 1, it can be concluded that there was no any inhibitory effect observed of FeO nanoparticles on the growth of all native bio decomposer bacterial strain on nutrient agar plates at different time intervals. The results of this experiment were further confirmed by evaluating the effect in liquid media. The results were aligned with, Diao and Yao (2009) [2], they have reported, *B. subtilis* survival rates were somewhat greater when pre-oxidized NZVI particles were treated with oxygen, *i.e.*, oxidized to FeO (OH), were needle-shaped and strongly aggregated, and their size was significantly increased.

Liquid assay after inoculation, flasks were incubated on a shaker at 32 ºC with 100 rpm. Observations for enhancement or inhibition of the organism's growth were recorded in terms of optical density using a spectrophotometer at 600 nm. The highest growth enhancement in terms of optical density 0.293, 0.323, 0.150 and 0.170 was recorded for *P. stutzeri* BDCT-1, *B. velezensis* BDCT-2, *S. rochei* AAUBD M-10 and *S. chartreusis* AAUBD M-16, respectively at 60 ppm FeO NPs concentration after 24 hrs of inoculation (Table 2). The highest growth enhancement in terms of optical density 0.538, 0.452, 0.176 and 0.204 was recorded for all four native bio decomposer microbial cultures at 60 ppm FeO NPs concentration after 48 hrs of inoculation (Table 2). The similar trend was recorded for all bio decomposer cultures at 72 and 96 hrs in NB media supplemented with 60 ppm (Table 2). The data presented in table 2 showed that the growth of all native bio decomposers was decreased from 70 ppm to higher concentration of FeO NPs at different time intervals. In comparison to results obtained from plate assay experiment, the results of liquid assay shown some different growth pattern, when FeO NPs concentration was increased from 70 ppm and onwards. The possible reason may, in liquid media the contact and exposure of the microorganisms and the NPs

Table 1: Effect of FeO nanoparticles on the growth of bio decomposer bacterial strains on nutrient agar plates

Q	Growth of bio decomposer bacterial cultures at 24 hrs				Growth of bio decomposer bacterial cultures at 48 hrs				Growth of bio decomposer bacterial cultures at 72 hrs				Growth of bio decomposer bacterial cultures at 96 hrs			
	***P. stutzeri* BDCT-1**	***B. velezensis* BDCT-2**	***S. rochei* AAUBD-M10**	***S. chartreusis* AAUBD-M16**	***P. stutzeri* BDCT-1**	***B. velezensis* BDCT-2**	***S. rochei* AAUBD-M10**	***S. chartreusis* AAUBD-M16**	***P. stutzeri* BDCT-1**	***B. velezensis* BDCT-2**	***S. rochei* AAUBD-M10**	***S. chartreusis* AAUBD-M16**	***P. stutzeri* BDCT-1**	***B. velezensis* BDCT-2**	***S. rochei* AAUBD-M10**	***S. chartreusis* AAUBD-M16**
FeO NPs (5 ppm)	+	+	+	+	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
FeO NPs (10 ppm)	+	+	+	+	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
FeO NPs (20 ppm)	+	+	+	+	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
FeO NPs (30 ppm)	+	+	+	+	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
FeO NPs (40 ppm)	+	+	+	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
FeO NPs (50 ppm)	+	+	+	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
FeO NPs (60 ppm)	+	+	+	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
FeO NPs (70 ppm)	+	+	+	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
FeO NPs (80 ppm)	+	+	+	+	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
FeO NPs (90 ppm)	+	+	+	+	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
FeO NPs (100 ppm)	+	+	+	+	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
$FeSO_4$ (22.32 ppm) (*i.e.* @ 50 Kg/ha)	+	+	+	+	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++

Solubilization

Table 2: Effect of FeO nanoparticles on the growth of bio decomposer bacterial strains in broth

Concentration	Optical Density at 600 nm at 24 hrs				Optical Density at 600 nm at 48 hrs				Optical Density at 600 nm at 72 hrs				Optical Density at 600 nm at 96 hrs			
	***P. stutzeri* BDCT-1**	***B. velezensis* BDCT-2**	***S. rochei* AAUBD-M10**	***S. chartreusis* AAUBD-M16**	***P. stutzeri* BDCT-1**	***B. velezensis* BDCT-2**	***S. rochei* AAUBD-M10**	***S. chartreusis* AAUBD-M16**	***P. stutzeri* BDCT-1**	***B. velezensis* BDCT-2**	***S. rochei* AAUBD-M10**	***S. chartreusis* AAUBD-M16**	***P. stutzeri* BDCT-1**	***B. velezensis* BDCT-2**	***S. rochei* AAUBD-M10**	***S. chartreusis* AAUBD-M16**
Control	0.050	0.035	0.030	0.040	0.106	0.184	0.063	0.065	0.112	0.337	0.09	0.078	0.126	0.423	0.125	0.11
FeO NPs (5 ppm)	0.103	0.158	0.066	0.070	0.164	0.200	0.107	0.101	0.086	0.323	0.095	0.102	0.080	0.262	0.084	0.095
FeO NPs (10 ppm)	0.123	0.166	0.075	0.090	0.174	0.211	0.113	0.112	0.088	0.326	0.108	0.104	0.085	0.296	0.090	0.096
FeO NPs (20 ppm)	0.126	0.206	0.075	0.092	0.176	0.210	0.120	0.124	0.097	0.396	0.112	0.112	0.095	0.383	0.108	0.105
FeO NPs (30 ppm)	0.130	0.207	0.110	0.125	0.220	0.268	0.160	0.150	0.128	0.410	0.114	0.143	0.125	0.433	0.110	0.137
FeO NPs (40 ppm)	0.175	0.235	0.117	0.155	0.230	0.363	0.165	0.175	0.155	0.487	0.130	0.204	0.134	0.475	0.126	0.171
FeO NPs (50 ppm)	0.280	0.278	0.126	0.165	0.233	0.452	0.176	0.204	0.158	0.562	0.156	0.225	0.145	0.556	0.138	0.184
FeO NPs (60 ppm)	0.293	0.323	0.150	0.170	0.538	0.517	0.208	0.210	0.185	0.607	0.208	0.357	0.180	0.595	0.186	0.226
FeO NPs (70 ppm)	0.160	0.230	0.095	0.100	0.213	0.346	0.148	0.128	0.122	0.366	0.120	0.139	0.109	0.353	0.116	0.129
FeO NPs (80 ppm)	0.101	0.154	0.090	0.067	0.177	0.295	0.125	0.126	0.095	0.334	0.078	0.116	0.090	0.329	0.064	0.115
FeO NPs (90 ppm)	0.093	0.117	0.045	0.065	0.128	0.195	0.087	0.096	0.083	0.318	0.068	0.096	0.080	0.312	0.054	0.073
FeO NPs (100 ppm)	0.060	0.095	0.035	0.060	0.118	0.191	0.076	0.045	0.066	0.249	0.063	0.080	0.060	0.210	0.013	0.067
$FeSO_4$ (22.32 ppm) (*i.e.* @ 50 Kg/ha)	0.058	0.040	0.032	0.048	0.110	0.187	0.069	0.040	0.064	0.246	0.057	0.030	0.053	0.195	0.007	0.019

Table 3: Effect of FeO nanoparticles on cellulolytic activity on CMC agar plate and on lignolytic activity MSM-Lignin agar

Concentration	Solubilization Index at 72 hrs after incubation				Growth of bio decomposer bacterial cultures 96 hrs			
	***P. stutzeri* BDCT-1**	***B. velezensis* BDCT-2**	***S. rochei* AAUBD-M10**	***S. chartreusis* AAUBD-M16**	***P. stutzeri* BDCT-1**	***B. velezensis* BDCT-2**	***S. rochei* AAUBD-M10**	***S. chartreusis*AAUBD-M16**
FeO NPs (5 ppm)	8.0	6.0	4.3	4.5	++	++	++	++
FeO NPs (10 ppm)	7.7	5.7	7.2	4.3	++	++	++	++
FeO NPs (20 ppm)	8.4	4.5	3.8	5.7	++	+++	++	++
FeO NPs (30 ppm)	5.8	7.2	6.0	3.6	++	+++	++	++
FeO NPs (40 ppm)	7.3	4.8	4.5	4.8	++	+++	+++	+++
FeO NPs (50 ppm)	5.5	4.3	5.3	4.3	+++	+++	+++	+++
FeO NPs (60 ppm)	11.0	9.0	8.5	6.3	+++	+++	+++	+++
FeO NPs (70 ppm)	5.5	3.6	6.3	5.7	++	-	++	++
FeO NPs (80 ppm)	8.0	9.0	6.0	5.4	++	-	++	++
FeO NPs (90 ppm)	5.8	3.6	5.7	6.0	++	-	++	++
FeO NPs (100 ppm)	7.3	6.0	5.7	4.8	++	-	++	++
$FeSO_4$ (22.32 ppm) (*i.e.* @ 50 Kg/ha)	8.0	5.0	4.0	4.3	+	+++	++	++

Note: +++ luxurious, ++ moderate, + mild and – no growth

is direct and constant. So, higher level of NPs can affect adversely on microbial growth in liquid media in comparison to solid agar medium at same concentration of NPs. A similar type of findings was reported by Zhang *et al*. (2022) [12], Tran *et al*. (2010) [11] and Gabrielyan *et al*. (2019) [4].

In vitro Compatibility for Consortium Preparation the compatibility of co-inoculated microorganisms is a key requirement for the effective establishment of microbial culture mixtures in the consortium. The compatibility of the four isolates was tested *in vitro* on the Nutrient agar plate, and they were all determined to be compatible. Microbial compatibility among members of mixed cultures has been found by several researchers around the world. Similarly, Prajapati *et al.* (2017) [9] were tested three native phylllospheric methylotrophic bacterial cultures (*S. saprophyticus*, *B. subtilis* and *B. methylotrophicus*) for compatibility on Nutrient agar plate *in vitro* and reported that, all the bacterial cultures were found compatible with each other. Plate assay for cellulolytic activity Measurements of halo zone of CMC degradation on agar plate were recorded and the efficiency of the bio decomposer bacteria was worked out in the form of Solubilization Index (SI). Highest SI 11.0, 9.0, 8.5 and 6.3 were recorded at 72 hrs for all native bio decomposers, respectively at 60 ppm concentration of FeO nanoparticles (Table 3). With increase in NPs concentration from 70 ppm onwards the SI was found to decrease gradually. Moreover, in the treatment $FeSO_4$ (@ 50 kg/ha; corresponding Fe concentration of 22.32 ppm), shown similar SI that was recorded for 20 ppm FeO NPs. Interestingly, with increase in FeO nanoparticle concentration from 5 to 60 ppm, SI found to increase for all four native bio decomposers.

Plate assay for ligninolytic activity to study the effect of FeO nanoparticles on microbial ligninolytic activity, an agar plate assay was performed using an MSM-L medium supplemented with 1% alkaline lignin as a sole source of carbon and energy along with different concentrations (5 to 100 ppm) of FeO nanoparticles. A treated check was also kept consisting 1% alkaline lignin and fertilizer grade $FeSO_4$ (@ 50 kg/ha of soil). The results of the plate assay are shown in table 3. From the data presented in the table it was observed that, all the native bio decomposer bacteria strains produced luxurious growth on MSM-L agar plate in presence of 60 ppm nanoparticles at 96 hrs. Of inoculation, indicating towards their strong lignolytic activity. Interestingly, from 5 ppm to 30 ppm FeO concentration, all the tested microbes shown moderate growth. But From 40 ppm onwards luxurious growth was observed for all tested isolates means, FeO nanoparticles from 40 to 60 ppm promoted lignolytic activity. Moreover, 70 ppm onwards the growth shown downward movement for all isolates, particularly isolates *B. velezensis* BDCT-2 shown complete growth inhibition from 70 ppm FeO NPs concentration onwards. Moreover, for $FeSO_4$ moderate growth was observed. Initial increase in growth of bio decomposer bacteria might be due to lower concentration of FeO NPs (up to 60 ppm) supports/increase lignin degradation activity. While at higher concentration of FeO nanoparticles (from 70 ppm onwards) exerted inhibitory effect on lignolytic activity.

Conclusion

Overall results indicated that, iron oxide nanoparticles significantly influenced growth and activity of native biodecomposer bacterial strains. FeO nanoparticles at 60 ppm found to enhance growth of native biodecomposer bacterial strains, while higher concentration shown bacteriostatic effect in terms of restriction in growth. Moreover, cellulolytic and lignolytic activity were also enhanced by FeO NPs at 60 ppm concentration.

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