Applied Water Science (2024) 14:211
https://doi.org/10.1007/s13201-024-02255-7

ORIGINAL ARTICLE

Enhanced biodegradation of lignin and lignocellulose constituents in the pulp and paper industry black liquor using integrated magnetite nanoparticles/bacterial assemblage

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Received: 20 December 2022 / Accepted: 28 July 2024

Abstract
The study was designed to explore the efficiency of magnetite nanoparticles (Fe_3O_4 NPs)/bacterial cell assembly to biodegrade lignin and lignocellulose, decontaminate pulp and paper-contaminated wastewater and optimize lignin adsorption by Fe_3O_4 NPs. Water samples were collected from three paper and carton manufacturing companies, **Alexandria** Governorate, **Egypt**. *Pseudomonas otitidis* MCC10330, the most active and promising strain among 10 previously screened indigenous and exogenous isolates, was selected and decorated with magnetic Fe_3O_4 Nanoparticles, that were prepared by the co-precipitation method, characterized and used to decontaminate paper and pulp effluent in a batch mode bioassay for 4 h. Fe_3O_4 NPs/bacterial cell assembly achieved the highest removals (64.1, 52.0, 54.3 and 66.6%) of TSS, COD, BOD, and Total Tannin and Lignin after 1, 4 and 4 h, reaching residual concentrations (RCs) of 322, 216, 112 and 7 mg/L, which are still slightly higher (5.35, 2.7 and 1.86-fold) than their maximum permissible limits (MPLs), respectively. RCs of pH, DO and TDS in the treated effluent are accepted for safe discharging. Maximum lignin adsorption and removal (82.14%) using Fe_3O_4 NPs was achieved at the optimized conditions (pH 6, Fe_3O_4 NPs dosage of 100 mg and 10 min contact time). Results confirmed that the proposed magnetite-coated *Pseudomonas otitidis* treatment system is highly efficient and recommended to treat the highly contaminated pulp and paper wastewater. Also, as far as we know, this integrated assemblage is the first time to be used as a novel, very promising, eco-friendly, renewable and economical biotechnological approach to minimize/eliminate the involved pollutants with the least running time.

Keywords Biodegradation · Lignin and lignocellulose · Immobilization · Magnetite nanoparticles · *Pseudomonas otitidis* · Pulp and paper effluent · Treatment

Introduction

Pulp and paper industry consume huge amounts of water during pulping process and manufacturing of paper as well as generating more quantities of polluted and colored wastewater (Ahmed et al. 2022; Kumar et al. 2021a, b), which considered severely polluted and possess dangerous environmental impacts (Ramos et al. 2022). Among others, paper and pulp manufacturing is major energy-consuming industry and constitutes one of the primary sources of industrial wastewater together with some other industries (chemical and petrochemical, food processing, tannery etc.) (Ahmed et al. 2022). Their effluents are contributing directly to the production of greenhouse gases as well as other pollutants including sulfur compounds, nitrogen oxides, chlorinated by-products with high load of lignin fragments, hemicellulose, chlorophenolics, resins, fatty acids and inorganic salts, all of which greatly damaging the water quality as reported by Kumar et al. (2020); Kumar et al.(2015) and Tripathy et al. (2022). They are also toxic for aquatic organisms and showed strong mutagenic effects. Pulp and paper effluent characterized by very high organic pollution load originated from the residues of lignin and polysaccharide degradation and reflected by high **BOD**, **COD** and **TDS**(Núñez et al.

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Published online: 05 September 2024

2022). Chemical composition and the dark coloration of the black liquor effluents from this industry pose negative environmental impact, especially in the receiving aquatic systems where they adversely affect aquatic fauna and flora (Singh et al. 2022). Such effluents are highly toxic and consisting of more than **250** chemicals of high molecular weight lignin (aromatic rigid polymer presents predominantly in woody plants) (Yao et al. 2021). Lignin is highly recalcitrant compound and very difficult to be degraded either chemically or biologically due to the presence of aryl ether bonds and carbon–carbon linkages (non-hydrolysable) (Le et al. 2022), therefore, degradation of lignin is utmost difficult task to reach the strict levels stated by the environmental regulations (Kumar et al. 2021b).

Abhishek et al. (2017), Hooda et al. (2018) and Khan et al. (2022) stated that efficient treatments are necessary before discharge into water bodies to minimize and avoid the hazardous environmental negative impacts caused by the paper industry by-products. Membrane separation, chemical coagulation, precipitation using metal salts and advanced oxidation processes are available among several traditional physicochemical treatment methods for pulping and paper production wastewater (Esmaeeli et al. 2023; Cyganowski 2021). Other traditional methods used for the treatment of pulp and paper effluents include incineration, photochemical **UV/TiO_2** oxidation, adsorption on activated carbon and polymer resin as well as chemical coagulation/flocculation by synthetic or natural coagulants (Nordin et al. 2020; Teodor et al. 2021; Moreira et al. 2022; Jagaba et al. 2023), all of which exhibited non-sufficient effectiveness for proper treatment for safe discharging. Selecting highly efficient, eco-friendly and cost-effective decontaminating method is of top priority demands in the field of wastewater treatment (Costa et al. 2017). Biological processes that actively used in bioremediation of pulp and paper industrial wastewater include activated sludge (Kumar et al. 2021a; Kumar et al. 2021b), aerated lagoons (Jansson 2022; Sonkar et al. 2020; Dagar et al. 2022), anaerobic treatment processes (Ribeiro et al. 2023; Goycoechea et al. 2023; Hovikorpi et al. 2020) and sequential anaerobic/aerobic treatment systems (Saski et al. 2020; Coimbra et al. 2021). Microbial fermentation of lignocellulose materials considered an added value approach for remediation of pulp and paper effluent. Previous study reported using wood rotting fungi in valorization and degradation of lignocellulose, since they are effectively producing lignin and polysaccharides-degrading enzymes (Kipping et al. 2024). However, the relatively slow growth of filamentous fungi and loss of polysaccharides during lignin degradation considered as limitations of this method as stated by Sharma and Arora (2015) and Podkościelna et al. (2022). In contrary, cellulose and lignocellulos degrading bacteria are highly active for this industrial effluent compared to other microbes since they produce functional enzymes at high concentration leading to efficient conversion of the secondary degradation metabolites. Many cellulose/lignocellulos degrading bacteria that are used for pulp and paper effluents treatment are either native (belong to the contaminated environments) or exogenous consortia (from outer source) and can be isolated from the contaminated effluent or the pulp mill sludge (Brown et al. 2021). Limitations include high cost, longer times, large spaces for aerobic processes, as well as experiencing difficulty in controlling the microbial populations, pH, temperature, nutrients and toxic or recalcitrant compounds harmful to biological degradation (Esmaeeli et al. 2023). Integration of physical, chemical or biological systems such as coagulation and wet oxidation, ozone and biofilms, chemical oxidation with ozone as well as chlorine with activated sludge sometimes are the optimal solution allowing to benefit from their unique features (Jansson 2022; Jagaba et al. 2023 and Murshid et al. 2023). Also, COD and NH^{4+}–N levels could be reduced to the permitted level using post-treatment electrolysis or ozonation (Saeed et al. 2022).

Nanomaterials-based applications have gained increased attention in water purification and wastewater treatment due to their unique physiochemical properties for elimination of organic and inorganic pollutants as well as toxic metals at minimum energy consumption, low cost and potential reuse of the treated effluents (Kiss 2020; Palani et al. 2021). There are different methods for synthesis of metals NPs to control their size and shape, including co-precipitation, hydrothermal, thermal decomposition, microemulsion, electrochemical deposition, laser pyrolysis, solvothermal, sonochemical, chemical vapor deposition, microwave-assisted method and aerosol pyrolysis. Among these methods, co-precipitation is one of the most used methods in the synthesis of aqueous phases because it is the simplest and efficient. Other methods have many disadvantages including long reaction time, temperature of reaction is high or extremely high, high pressure, poor yield, amounts of solvent required are large, relatively expensive and poor reproducibility (Sulistyaningsih et al. 2015).

Currently, **Fe_3O_4 NPs** are widely used in decontamination and purification processes due to their high specific surface area, simple preparation, easy modification and low toxicity (Malik et al. 2022; Ning et al. 2021; Nayl et al. 2022). In addition of being cost-effective, they are converted into hydroxides during treatment, which work as flocculants / solid sorbent, and help removing suspended and colloidal inorganic and organic contaminants (Pandey et al. 2022; Abo-Zeid et al. 2020). Upon the contact with **Fe** nanoparticles in wastewater treatment systems, reduction reactions help to efficiently eliminate mobile or immobile contaminants (Zhao et al. 2020; Priyadarshini et al. 2022).

Conventional bacterial immobilization possesses many advantages such as efficient, stabilized and continuous catalytic activity in addition to increasing microbial biomass, which allow biodegradation of hazardous pollutants (He et al. 2017; Ranmadugala et al. 2018; Malik et al. 2022). Integration (immobilization) of bacterial cells with Fe_3O_4 NPs overcome the drawbacks of the conventional bacterial immobilization including limited mass transfer in the inner matrix, matrix fragility, cell leakage from the supporting matrix (solid medium) as well as adverse effects on cell viability and catalytic activity (Yoshimoto et al. 2017). Fe_3O_4 NPs remarkably upgrade bacterial efficiency, reduces mass transfer problems, facilities separation of the dispersed coated cells and reuse of the biomass, minimizes microbial death and avoids blocking of continuous flow systems as early reported by Khan et al. (2022) and Jabbar et al. (2022). Moreover, microbial cell surfaces are coated with Fe_3O_4 NPs through strong physical adsorption due to the high specific surface area and high surface energy of **NPs**, where the smaller size of the **NPs** increases the surface area of the bacterial cell membrane, which enhances chemical activity and capacity for adsorbing many types of pollutants on their surfaces (Darabdhara et al. 2017). Integration between **NPs** and bacterial cells upgrades microbial activities and increase their reaction rates through overcoming technological limitations such as reduced solubility of hydrophobic substrate, reduced bioavailability to the microorganisms, kinetic limitations on degrading enzymes, un-healthy microorganisms, in addition to restoring magnetic **NPs** after complete treatment using external magnetic field for reuse (Ranmadugala et al. 2018; Jabbar et al. 2022). Thus, the current study was designed to develop a new assemblage of magnetite-coated bacteria, as an efficient and magnetically separable system, for bioremediation of pulp and paper industrial wastewater under optimum operation conditions using promising environmental bacterium. This assemblage was expected to have superior efficiency in removing the tested contaminants compared to its individual components since they gather different removal mechanisms (biodegradation, sorption, flocculation) that synergistically promote decontamination process at a remarkable level.

Materials and methods

Wastewater sampling

Wastewater was collected from the end pipes of **3** different pulp and paper manufacturing companies (RAKTA, ALAHLYA and ALMAMOORA), located in **Alexandria** Governorate, **Egypt**, during the course of the study. Pulp and paper manufacturing wastewaters are rich in cellulose fibers as well as other organic and inorganic contaminants. They were investigated before and after treatment with the proposed treatment.

Microorganisms

Based on the screening of **6** indigenous bacterial species isolated from pulp and paper-contaminated wastewater and **4** exogenous species (*Pseudomonas stutzeri*, *Bacillus licheniformis, Bacillus sphaericus* and *Pseudomonas otitidis* MCC10330) as well as their efficiency in the reduction of the investigated parameters, *P. otitidis* MCC10330 (ATCC BAA-1130) considered the most promising (Mohamed 2022). It was selected since it had the broad and highest degradation/accumulation activities for the contaminants in the pulp and paper waste effluent compared to the other tested bacteria. *Pseudomonas otitidis* MCC10330 was kindly provided, from the microbial collection at the **Institute of Graduate Studies & Research, Alexandria University** (**IGSR**). The selected bacterium was maintained on nutrient agar (**NA**) medium and prior to each experiment, the culture was reactivated overnight. It was selected, decorated with magnetite nanoparticles (**NPs**) and the efficiency of Fe_3O_4 NPs / *P. otitidis* assemblage for decontaminating pulp and paper waste effluent was investigated.

Synthesis and characterization of magnetite NPs

$\mathbf{Fe_3O_4}$ Nanoparticles ($\mathbf{Fe_3O_4}$ **NPs**) were synthesized using reverse co-precipitation method under $\mathbf{N_2}$ stream during the whole preparation steps (Sulistyaningsih et al. 2015; Mahmoud et al. 2013). A mixture of **5.2** g of $\mathbf{FeCl_3{\cdot}6H_2O}$ and **2.674** g of $\mathbf{FeSO_4{\cdot}7H_2O}$ were dissolved in **50** mL deionized water and stirred for **15** min under inert atmosphere at room temperature. Ammonia solution (**25**%) was added to the mixture till **pH ~ 10** under continuous stirring for another **30** min to achieve nucleation and formation of magnetite nanoparticles. Glycine (**1.2** g) was added to the magnetite **NPs** ($\mathbf{Fe_3O_4}$) as a surfactant to stabilize their dispersion, adjust particle size and surface functionalization. The black solid product was sonicated using ultrasonic bath for **20** min. $\mathbf{Fe_3O_4}$ NPs were isolated from the solution using an external magnet, washed three times with deoxygenated water and ethanol then dispersed and stored in ethanol followed by drying at **50** °C until complete dryness. Formation of $\mathbf{Fe_3O_4}$ NPs followed the equation:

$$\mathbf{2FeCl_3 + FeSO_4 + 4H_2O + 8NH_3 \rightarrow Fe_3O_4 + (NH_4)_2SO_4 + 6NH_4Cl}$$

Morphological, chemical and thermal stability properties of the $\mathbf{Fe_3O_4}$ **NPs** were characterized. Their particles size and morphology were determined using transmission electron microscope (**TEM** JEOL, JEM 100 CX, Japan). X-ray diffractometer (**XRD** BRUKER D8 Advance Cu target, Germany) was employed to examine their crystal phase. It was operating with CuKα radiation ($\lambda = 1.54$ A°) generated at 40 kV and 40 mA. Stability/degradation processes was estimated by thermal gravimetric analysis (**TGA**). Fourier Transform Infrared Spectroscopy (**FT-IR**, Model 8400 S, Shimadzu, Japan) was used to determine the **Fe_3O_4 NPs** functional groups.

Development and characterization of the Fe_3O_4 NPs-immobilized bacterial assemblage

Pseudomonas otitidis **MCC10330** (the most active and promising strain) was cultured for **24** h at **37** °C in nutrient broth (**NB**) medium till the microbial density reached ~**0.5** g [dry weight cells/L]. **Fe_3O_4**-(**1.5** g) dissolved in **100** mL solution (**50** mL DW + **50** mL absolute ethanol) in a conical flask and incubated for **10** min in a sonicator water bath. **Fe_3O_4** powder breakage was achieved by sonication shock waves that help breaking intermolecular interactions, thus, speed dissolution. Then, a fixed volume of the culture (**200** mL = **0.10** g bacterial weight) was mixed with the prepared **Fe_3O_4** NPs solution equivalent to cells: magnetite (g/g) ratio of **1:3** (that resulted in the highest coating efficiency according to a previous study) (El Bestawy et al. 2020) using shaking incubator (**NEW BRUNSWICK SCIENTIFIC, NEW BRUNSWICK, N.J., USA**) at **180** rpm and **37** °C. After one hour, the **Fe_3O_4** NPs/bacterial assemblage was collected and separated from the supernatant plus the free uncoated cells, which can then be harvested.

Characterization of the **Fe_3O_4 NPs-**immobilized bacterial assemblage was previously performed (El Bestawy et al. 2020) using Fourier transform infrared (**FT-IR**) to determine its functional groups while its morphological characteristics were determined using scanning electron microscope (**SEM**). Their results described bacteria in the micrograph as rod-shaped cells that immobilized by several layers of **Fe_3O_4 NPs** and attributing the strong coverage to the size of the **NPs** (typically about **2** orders of magnitude smaller than the bacterial cell), which allows the attachment of multiple **NPs** onto a bacterial cell.

Optimization of lignin removal by Fe_3O_4NPs

Removal of lignin by magnetite **NPs** under different conditions of **pH** (**6–8**), amount of nanosorbent (**10–150** mg) and contact time (**5–15** min) was investigated to determine the optimized conditions for maximum lignin removal from contaminated wastewater effluent.

Treatment bioassay using Fe_3O_4 NPs/immobilized bacterial assemblage

Schematic diagram of the treatment process of pulp and paper wastewater using the developed **Fe_3O_4** NPs/bacterial biomass assembly is illustrated in Fig. 1. ***Pseudomonas otitidis*** **MCC10330,** the selected bacterium was cultured in **200** mL **NB** and incubated for **24** h at **37** °C to obtain high cell density. El Bestawy et al. (2020) reported that magnetite–microbial cells assembly prepared at **1:3** ratio bacteria: **Fe_3O_4** w/w provides the highest bacterial growth and immobilization ability, which is supported by Yan et al. (2019). It was left for one hour at **37** °C under **180** rpm shaking speed (the perfect time of coating process). Then, the prepared magnetite–microbial cells assembly culture (**200** mL) was seeded into to **800** mL raw industrial wastewater (**1**L culture) after determination of the start-up bacterial density (CFU/mL). **Fe_3O_4 NPs/**immobilized bacterial assemblage in the wastewater sample was incubated for **4** h under shaking (**180** rpm) and treated samples were collected at **1** h interval. A control culture (**Fe_3O_4 NPs** free–*P. otitidis* culture) was subjected to the same investigation in parallel and under the same conditions for **4** h to decontaminate pulp and paper wastewater. Tested parameters were determined in the raw and treated wastewater to define the initial pollution load and their residues after treatment as well as the effectiveness of the remediation process.

Characterization of the raw and treated industrial effluent

Total tannin and lignin, the main targeted contaminant in the pulp and paper wastewater, as well as other quality parameters (pH, temperature, dissolved oxygen content, total suspended solids, total dissolved solids, biochemical oxygen demand, chemical oxygen demand and total viable count of bacteria) were determined in the raw and treated wastewater according to the standard techniques of Rice et al. (2017) to determine the removal efficiency at each exposure time.

Statistical analysis

Multifactorial analysis (ANOVA) was performed for the different contaminants in the industrial effluents by Pearson's correlation coefficient (r) at different confidence levels.

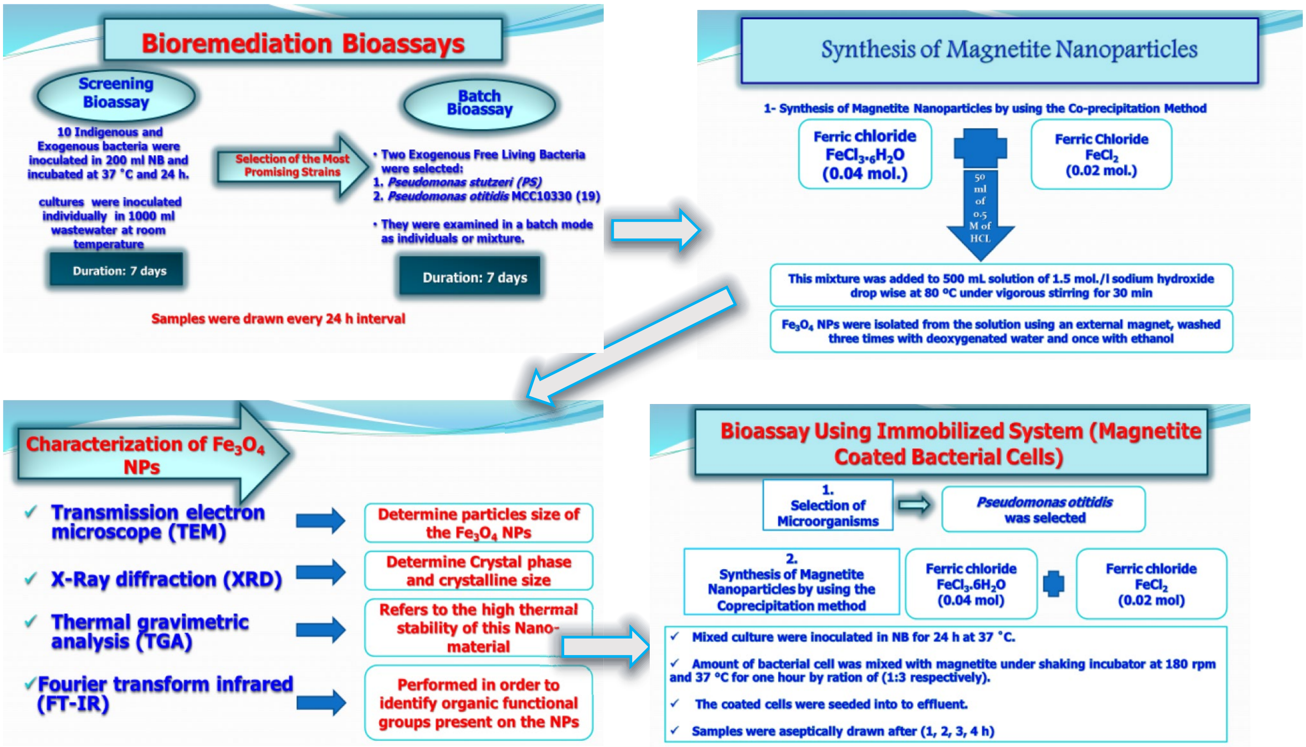


Fig. 1 Schematic Diagram of the Treatment Process of Pulp and Paper Wastewater Using Developed Fe_3O_4 NPs/Bacterial Biomass Assembly

Table 1 Physical properties of Fe_3O_4 nanoparticles

Se	Properties	
1	Appearance (Color)	Black
2	Appearance (Form)	Solid
3	Stability	Three months in solid state
4	Solubility	Dispersed in water / ethanol
5	Avg. size (TEM)	Around (8–14)nm

Results and discussion

As shown in the previous work by the authors (Mohamed 2022), ***P. otitidis*** proved being the most active to remove the involved pollutants. Therefore, it was selected to treat pulp and paper-contaminated wastewater as immobilized cells using magnetite nanoparticles.

Characterization of Fe_3O_4 NPs

Physical properties of magnetite nanoparticles (Table 1 and Fig. 2**A–D)** confirmed presence of nanoscale particles and aggregates with substantial interfacial contact areas per unit volume. Sonication was required to disrupt powder agglomerates and effectively break down the weaker Van der Waals forces.

1. **TEM** analysis illustrated spherical shape of **Fe_4O_3 NPs** with size ranging from **8.75** to **13.8** nm and partial aggregation (Fig. 2A).
2. **XRD** analysis indicated the **Fe_4O_3 NPs** crystalline structure with characteristic diffraction peaks at **$2\theta = 30°$, 35°, 43°, 50, 57.2°, 53.7°, 57, 63** and **73.8°** as shown in Fig. 2B**,** which are attributed to the cubic phase structure of **Fe_4O_3 NPs** (according to **JCPDS card, No. 01-089-0691**). Results confirmed that other types of iron oxides are nor present and also signposted that **Fe_4O_3 NPs** are well crystallized.
3. Two strong characteristic absorption peaks at **440** cm^{-1} and **570** cm^{-1} are shown in the **FT-IR** analysis of **Fe_4O_3 NPs**, which are related to the **Fe–O** bond (Fig. 2C). Intermolecular hydrogen bonding was illustrated by the absorption peak at ~ **3400** cm^{-1}as shown in all nanomaterials.
4. **Fe_4O_3 NPs** showed transition temperatures high thermal stability according to the **TGA** thermograms (Fig. 2D)**.**

Fe_3O_4 NPs are partially aggregated as shown in the **TEM** micrograph, attributed to their superparamagnetic behavior, which is supported by previous studies (Gnanamoorthy et al. 2020; Sallam et al. 2018; El Bestawy et al. 2020). Other characteristics of **Fe_3O_4 NPs** are consistent with those reported by other workers concerning **FT-IR,**

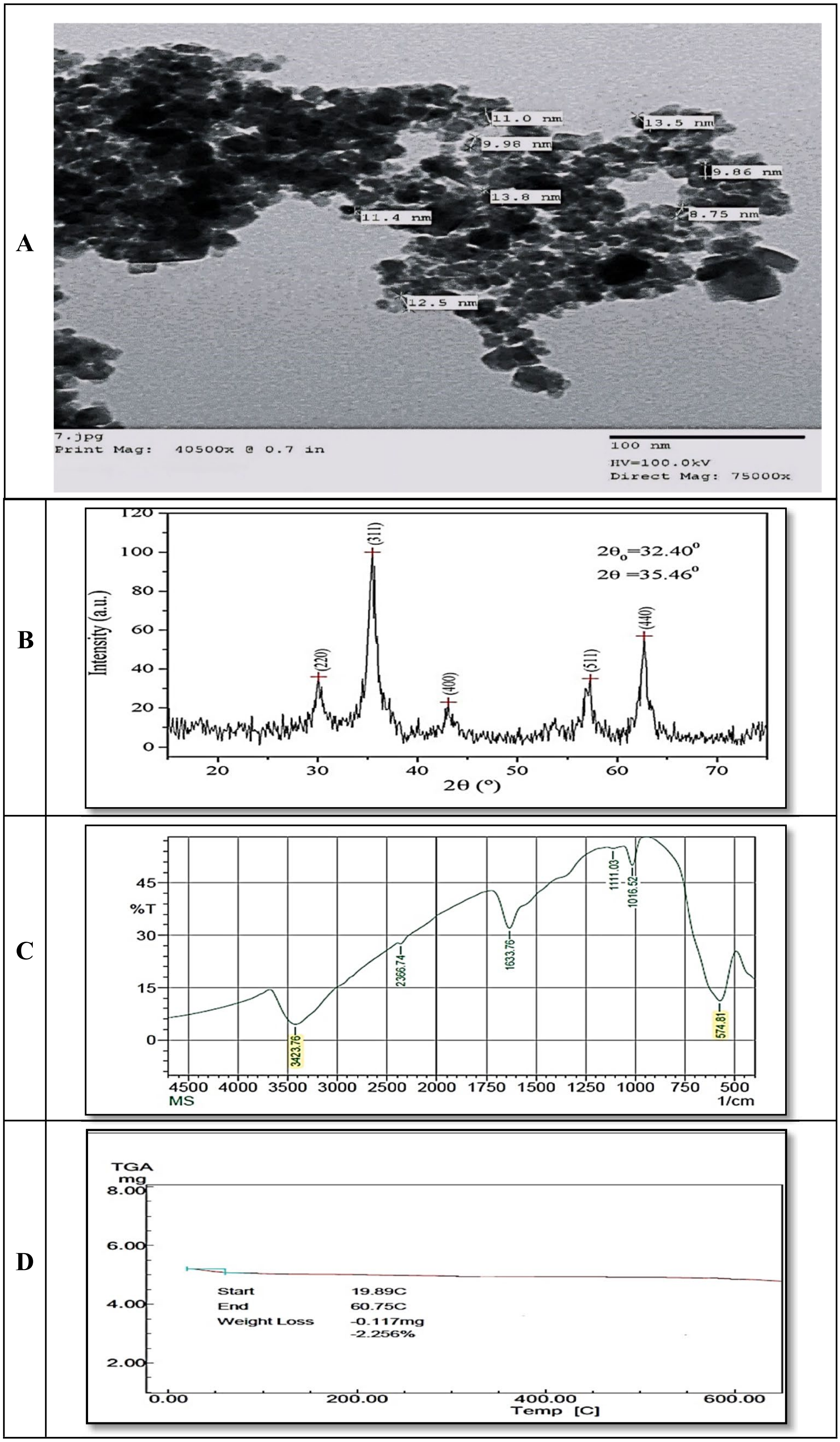


Fig. 2 Characterization of the synthesized Fe_3O_4 NPs; TEM micrographs (**A**), X-ray diffraction pattern (**B**), FT-IR spectrum (**C**) and TGA (**D**)

X-ray diffraction (Gonçalves et al. 2021) and **TGA**(Nayl et al. 2022).

Bioremediation of pulp and paper industrial effluent using Fe_3O_4/P. otitidis assemblage

Immobilization of bacterial cells with nanomaterial such as **Fe_3O_4 NPs** is a novel technique attracted much attention due to the remarkable enhancement in their activity and efficiency after acquiring the unique properties of **Fe_3O_4 NPs** such as super para-magnetism, biocompatibility, large surface area, high adsorption capacity of different pollutants and high surface energy that remarkably enhance adsorption, biodegradation and elimination of contaminants. Moreover, **Fe_3O_4 NPs** are easy to synthesize and can be regenerated and magnetically recyclable, which is very useful, sustainable and saves time in practical applications. The bacteria/ **Fe_3O_4 NPs** assemblage efficiency is controlled by physicochemical properties of **Fe_3O_4 NPs,** bacterial cells surface properties and environmental/culture conditions (Stylianou et al. 2021). In the present study, most of the **Fe_3O_4 NPs** have particle size more than **10** nm in diameter; therefore, they seem to be suitable for assembling with bacteria for remediation processes without the risk of their penetration into bacterial cell membrane, damaging the cells or creation of abnormal cell function at **10** nm or less (Banerjee et al. 2021).

Fe_3O_4 NPs/*P. otitidis* assemblage (**3:1**w/w) and **NPs**-free ***P. otitidis*** culture (control) were examined at **0, 1, 2, 3** and **4** h to decontaminate pulp and paper wastewater as shown in Table 2 and Figs. 3,4, and 5. Bioremediation assay concluded the following points:

1. Raw wastewater recorded **7.0**, **7.61**, **686**, **898**, **450**, **245** and **21** mg/L for **pH**, **DO**, **TDS**, **TSS**, **COD**, **BOD** and total tannin and lignin, respectively.
2. Magnetite/bacterial cells assembly recorded the highest removals (**64.1**, **52.0**, **54.3** and **66.6**%) of **TSS**, **COD**, **BOD** and total tannin and lignin after **1, 4, 4** and **3** h reaching **RCs** of **322**, **216**, **112** and **7** mg/L, respectively.
3. The highest removals of **TSS**, **COD, BOD** and total tannin and lignin achieved by the control (**Fe_3O_4 NPs**-free ***P. otitidis*** culture) recorded **82.8**, **52.7**, **50.0** and **57.1**% after **2, 4, 4** and **2** h, respectively, which is attributed mainly to microbial degradation process. Compared with **Fe_3O_4/*P. otitidis*** culture, it is clear that **Fe_3O_4 NPs** enhanced the removal of total tannin and lignin reaching **66.6**% instead to **57.1**% achieved by the control culture.
4. The **RC**s of **pH**, **DO** and **TDS** in the effluent treated with the proposed system are accepted for safe discharging, while **TSS, COD** and **BOD** levels are slightly higher (**5.35**, **2.7** and **1.86**-fold) than their **MPL**s (**60, 80** and **60** mg/L, respectively). Yet, there is no **MPL** stated for tannin and lignin in the **Egyptian** regulations.
5. Although a very short time (**4** h) was applied for this treatment bioassay, considerably very high reductions were achieved. Some of the tested pollutants are still higher than their **MPL**s for the safe discharge, which may be attributed to the short treatment time, small inoculum size or dose of the nanoparticles.

In nature, many species of fungi and bacteria can degrade lignin by secreting enzymes (Janusz et al. 2017). Metabolic pathway (mechanism) of lignin by the degrading microorganisms takes place through **2** steps (Zhao et al. 2022). De-polymerization of lignin is the first and most sophisticated step during lignin bioconversion into valuable products. Lignin de-polymerization involves two sequential processes; firstly, lignin is decomposed into oligomers or monomers (Xu et al. 2018), and secondly these products are completely degraded into carbon dioxide, water and minerals. Various specialized microbial enzymes that can depolymerize or mineralize lignin components have been isolated from specialized fungi and bacteria. They include class II heme-containing peroxidases, laccases and other auxiliary enzymes (Odwa

Table 2 Highest and lowest REs% of the different parameters in the raw and treated effluents during treatment using magnetite-microbial cells assembly

Parameter	Raw wastewater (mg/L)	Highest level/RE %	RC (mg/L)	Lowest RE %	MPL (mg/L)
pH	7.00	RL = 7.43, 2 h	7.43	7.04, 1 h	6–9
DO	7.61	RC = 7.86, 4 h	7.86	6.63, 1 h	≤4
TDS	686	34.4% SI, 4 h	922	30.9% SI, 2 h	2000
TSS	898	64.1%, 1 h	322	38.3, 1 h	60
COD	450	52.0%, 4 h	216	6.2, 1 h	80
BOD	245	54.3%, 4 h	112	10.2, 1 h	60
Total Tannin and Lignin	21	66.6%, 3 h	7.0	28.5% RE, 1 h	–

RL: residual level; RC: residual concentration; SI: salt increase

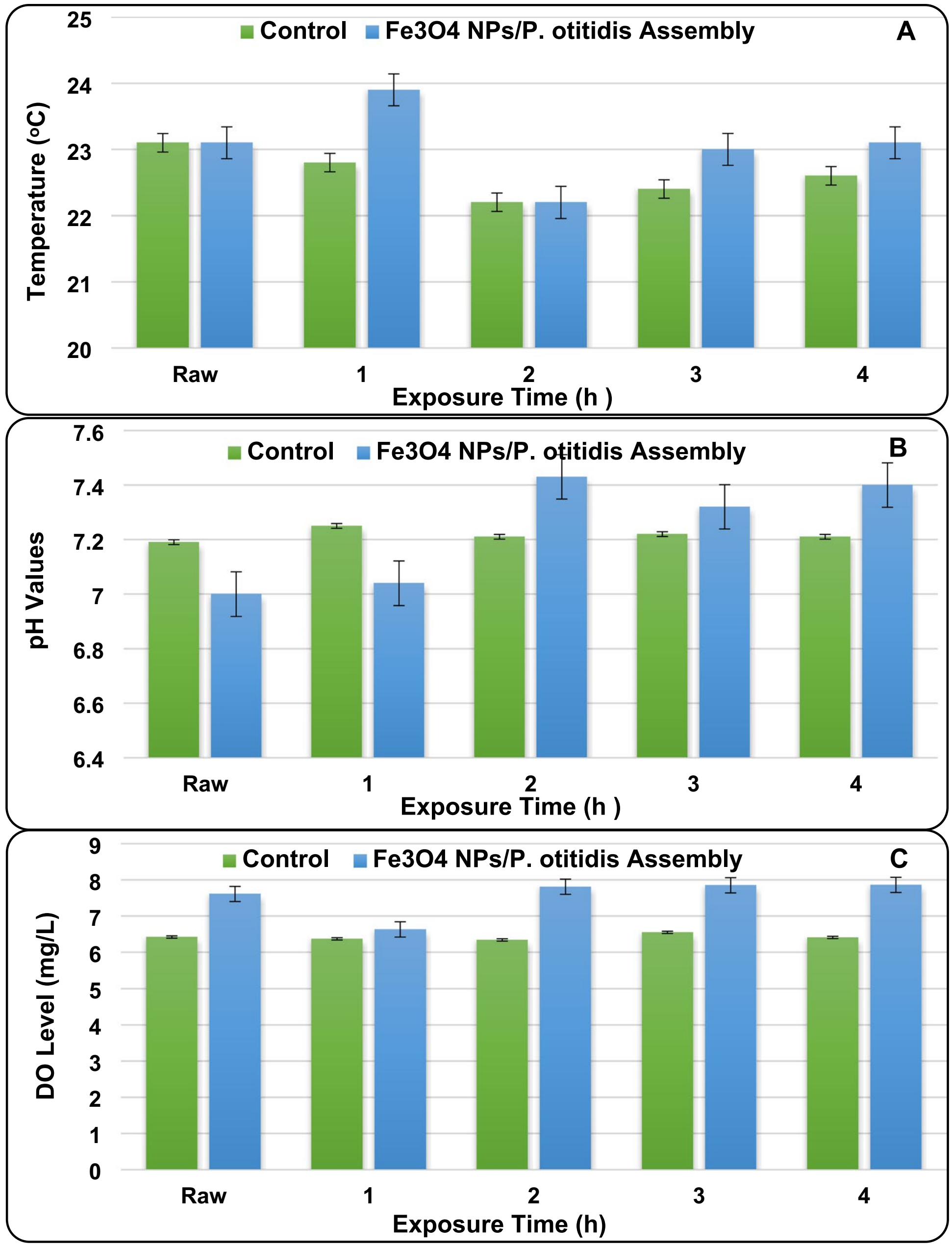


Fig. 3 Variations in the temperature (**A**), pH (**B**) and DO (**C**) Values in the raw and treated pulp and paper industrial effluent during the batch bioassay using Fe_3O_4/*P. otitidis* assemblage at different exposure times

et al. 2020). Minerals and low molecular weight phenolic compounds in the lignin as well as nutrient balance (C/N ratio) in the surrounding medium are stimulatory factors, which directly influence the production of ligninolytic enzymes (Raychaudhuri and Behera 2022). The second step of lignin biotransformation is microbial catabolism of aromatic derivatives (low molecular weight) of lignin, which is used by many microorganisms as carbon and energy sources for cell growth (Levy-Booth et al. 2022).

Bacteria/**Fe_3O_4 NPs** assemblage considered highly efficient system for wastewater treatment, where it characterized by cost-effective preparation, desirable electrical conductivity, paramagnetic and absorptive activity as well as being eco-friendly to the environment (Fang et al. 2022). In the present study, the proposed **Fe_3O_4 NPs/*P. otitidis*** assemblage could achieve considerably very high reductions of **TSS, COD, BOD** and total tannin and lignin within very short time (**4** h), confirming tremendous enhancement in the bacterial activity due to the presence of the nanoparticles, as reported also by El Bestawy et al. (2020) and Liu et al. (2020). Compared to other conventional treatment methods, the proposed technology can be considered as an efficient, simple, economical and flexible alternative to be used in wastewater bioremediation (Table 3).

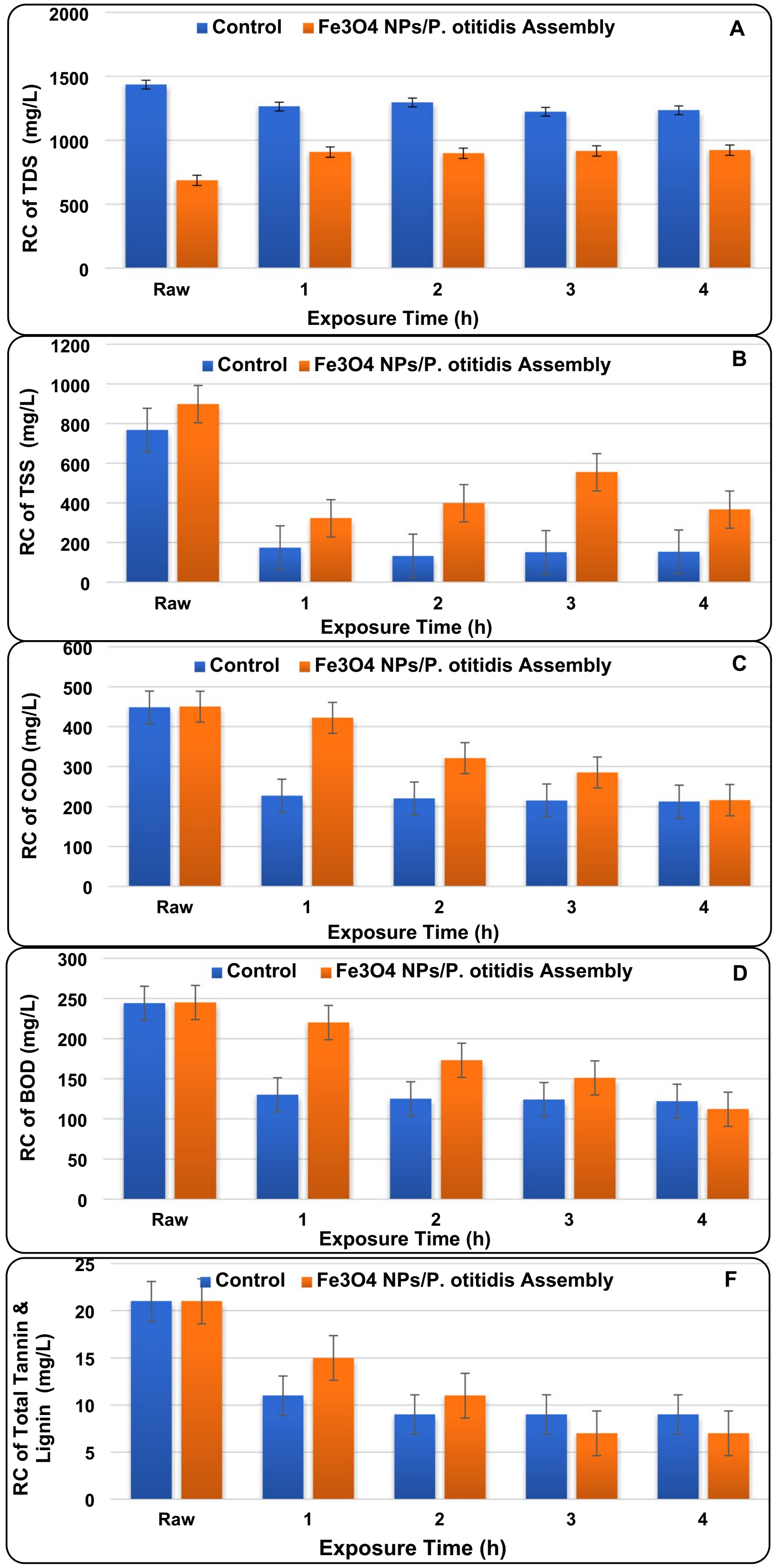
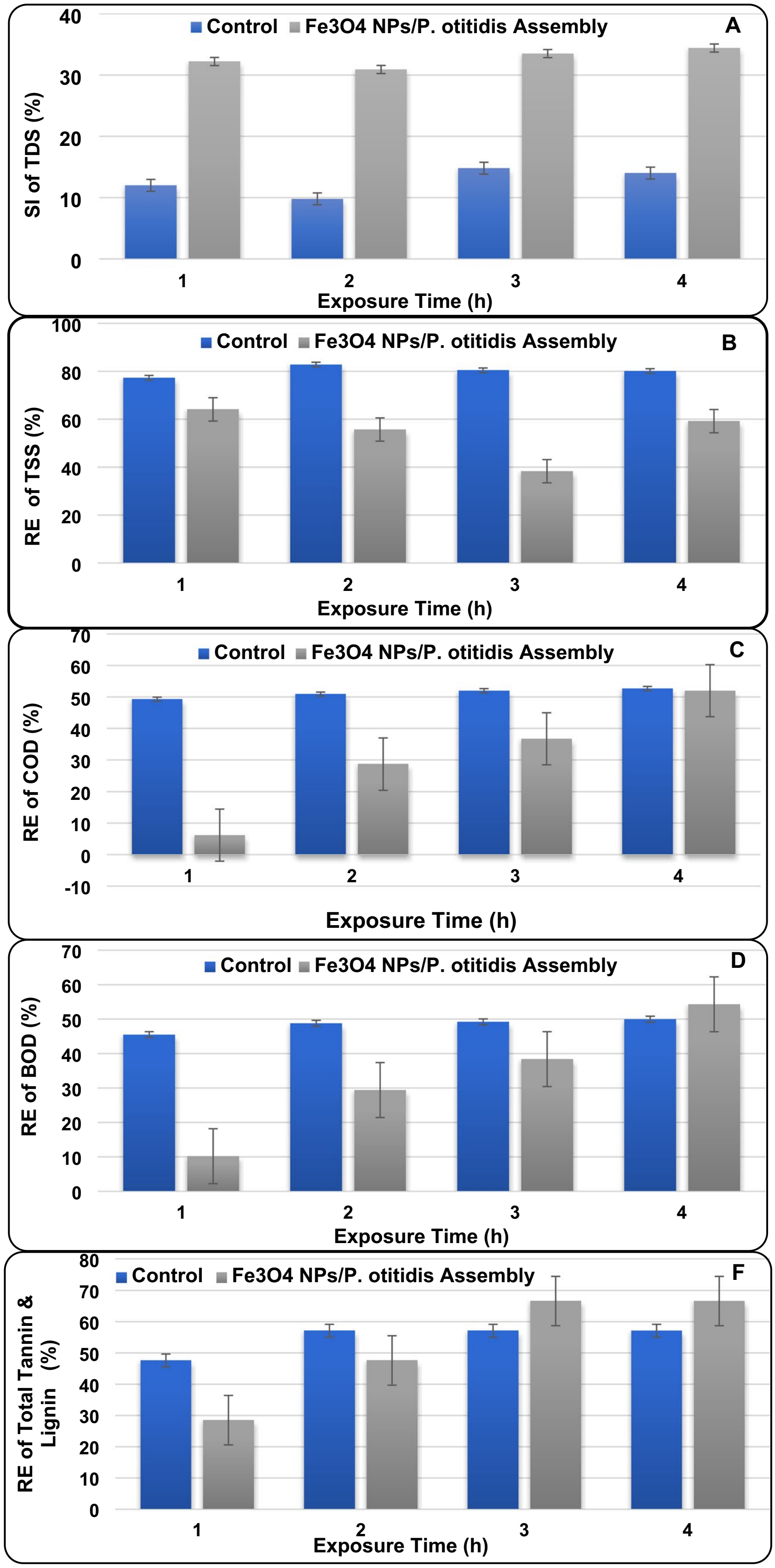


Fig. 4 Residual Concentration (RC) of TDS (**A**), TSS (**B**), COD (**C**), BOD (**D**) and Total Tannin and Lignin (**F**) in the raw and treated pulp and paper industrial effluent using Fe_3O_4/*P. otitidis* assemblage at different exposure times

Fig. 5 Removal Efficiency/Increase (RE/I%) of TDS (**A**), TSS (**B**), COD (**C**), BOD (**D**) and Total Tannin and Lignin (**F**) in the raw and treated pulp and paper industrial effluent using Fe_3O_4/*P. otitidis* assemblage at different exposure times



RCs of **pH, DO and TDS** in the effluent treated with the proposed system are accepted for safe discharging, while **TSS, COD** and **BOD** levels (**5.35, 2.7** and **1.86**-fold) were slightly higher than their **MPLs**, respectively. The main causes of having **RCs** higher than their **MPLs** (short treatment time, small inoculum size or dose of the nanoparticles) can be optimized to produce high quality and safe effluent. This is supported by other workers. Results indicated also high recyclability of $\mathbf{Fe_3O_4}$ NPs and recommend its use as a good adsorbent to remove lignin from aqueous solutions (Li et al. 2024).

Stimulatory effect of the pulp and paper industrial effluent on the growth of the tested bacterium

Effects of the tested wastewater on the stimulation or inhibition of the tested bacterium growth estimated as total viable count (**TVC**) were investigated at the starting and ending points of the treatment process (Fig. 6). ***P. otitidis***

Table 3 Comparison of the present achievements concerning treatment of pulp & paper effluents and those of previous works

Se	RE%						Refs.
	Lignin	COD	BOD	TSS	TOC	Conditions	Magnetite/bacterial cells assembly, present study
1	66.6	52.0	54.3	64.1	–	Within 1–4 h at room temperature, no agitation, no pH adjustment	Abhishek et al. (2017)
2	65.0	67.0	–	–	61.0	At 34 °C, pH 8.2 and 140 rpm by *Citrobacter freundii and Serratia marcescens* mixed Culture	Jansson (2022)
3	82.0	74.0	–	–	–	At 90 °C, pH 4 and 1 atm. after 2 h using Ce0.4Fe0.6O_2 mixed oxides	Kumar et al. (2015)
4	60.0	–	–	–	–	After 7 days *Pseudoxanthomonas* sp. R-28	Núñez et al. (2022)
5	29.4	–	–	–	–	At 55 °C using 30% trioctylamine in heptane (v/v)	Ribeiro et al. (2023)
6	–	≈15.0	–	–	–	Using granulated biomass fly ash (GBFA) with Fenton process	Teodor et al. (2021)
7	46.83	–	–	–	–	Using active carbon adsorption and TiO_2/UV decolorization (at 0.4 g/L TiO_2 concentration, 59.99 min irradiation time and 2.85 cm UV path length)	

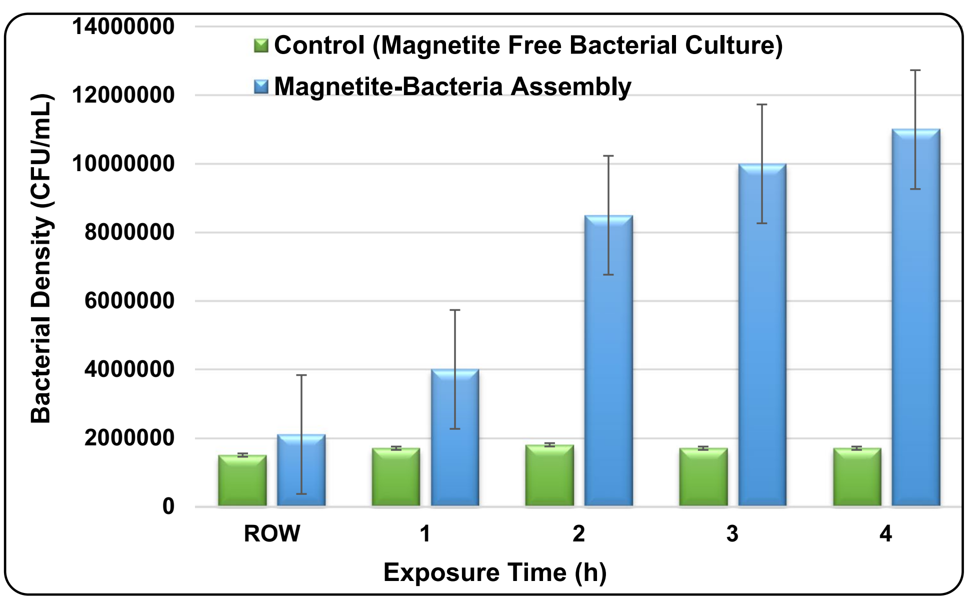


Fig. 6 Stimulatory and/or inhibitory effects of the tested wastewater on the growth (CFU/ml) of the tested bacterium during the treatment using Fe_3O_4/bacteria assembly

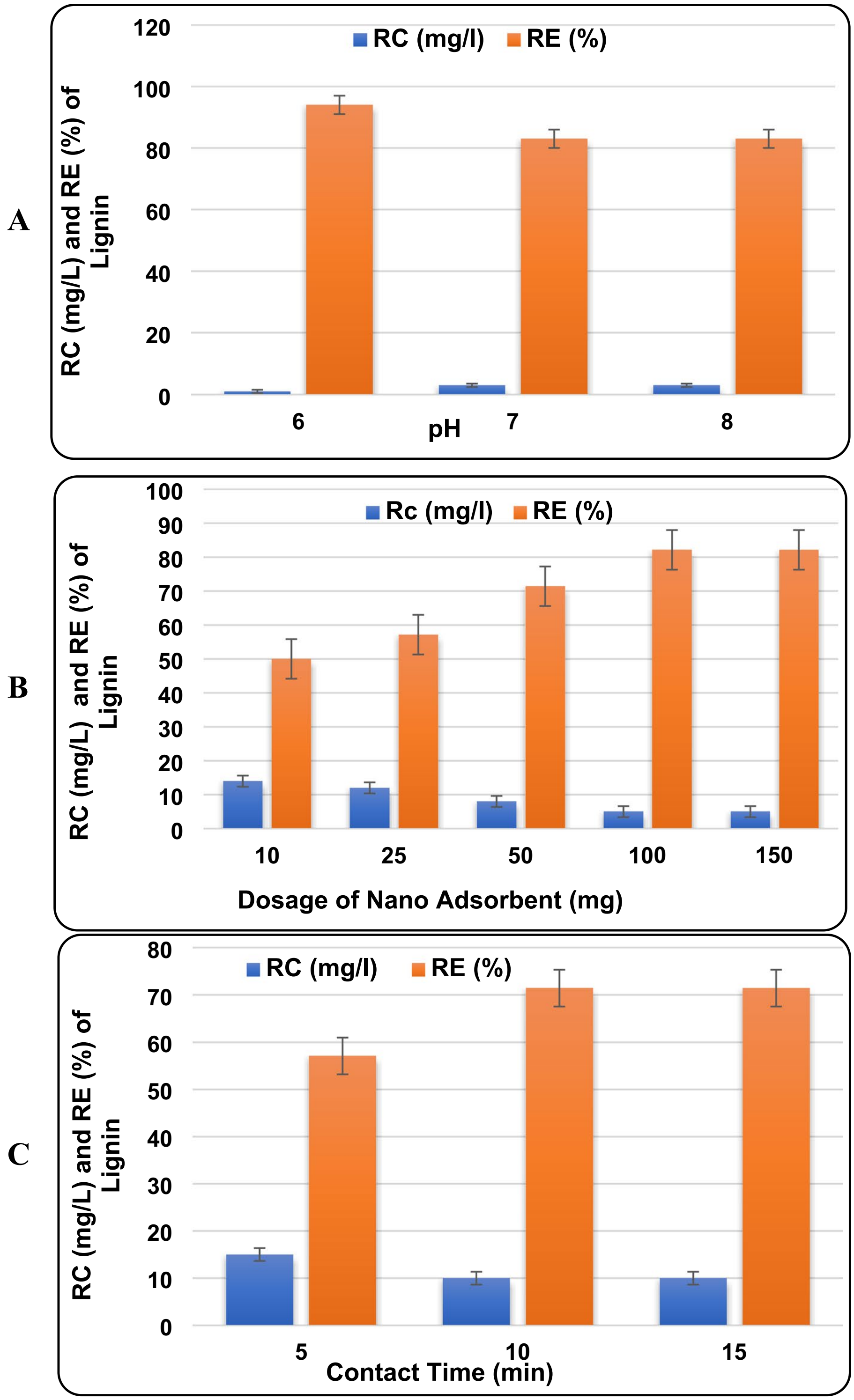


Fig. 7 Optimization of lignin adsorption on Fe_3O_4 NPs under different conditions; pH (**A**), nanoadsorbent dosage (mg) (**B**) and contact time. Such conditions are the key factors that are greatly controlling the efficiency of the remediation process as well as adjusting the shortest time for the best treatment, which is the main focus of the present study

decorated with **Fe_3O_4 NPs** at **3:1** ratio (magnetite NPs: bacteria) recorded the highest growth rate (**11.0×10^6** CFU/mL) after **4** h, which is equivalent to **7.4-**fold higher density than the initial inoculum at the starting point, indicating high metabolic activity. Moreover, **Fe_3O_4 NPs** coated bacteria showed remarkable increase in the growth rate compared to the control (**Fe_3O_4 NPs**-free culture) that reached the highest growth rate of only **1.8×10^6** CFU/mL after **2** h followed by slow decline till the end of the bioassay. This clearly confirmed the stimulation and protective effect of the **Fe_3O_4 NPs** on the growth of the associated bacteria as well as their low toxicity on living bacterial cells as stated by Konate et al. (2018) and El Bestawy et al. (2020).

Stimulation of bacterial growth rate with increase in magnetite concentration, especially at magnetite to bacterial cells ratio **3:1** (w/w), was supported by other workers who reported growth enhancement up to **500** ppm magnetite NPs (Konate et al. 2018). It was also proved that the **3:1** ratio resulted in the highest values of biomass (**7.4-**fold higher density than the initial inoculum) after only **4** h. **Fe_3O_4**-bacterial culture biomass showed continuous increase till the last exposure compared to the start-up density. Such results confirmed the regular ascending stimulation effects of the nanoparticles on the growth and multiplication of the tested bacteria till the end of the experiment. This stimulation resulted from the super paramagnetic behavior of **Fe_3O_4** NPs accompanied by their low toxicity on the living cells. Moreover, glycine modification of **Fe_3O_4** NPs with different surfactants has negligible toxicity on eukaryote cells compared to free **NPs** (Kafayati et al. 2013; El Bestawy et al. 2020).

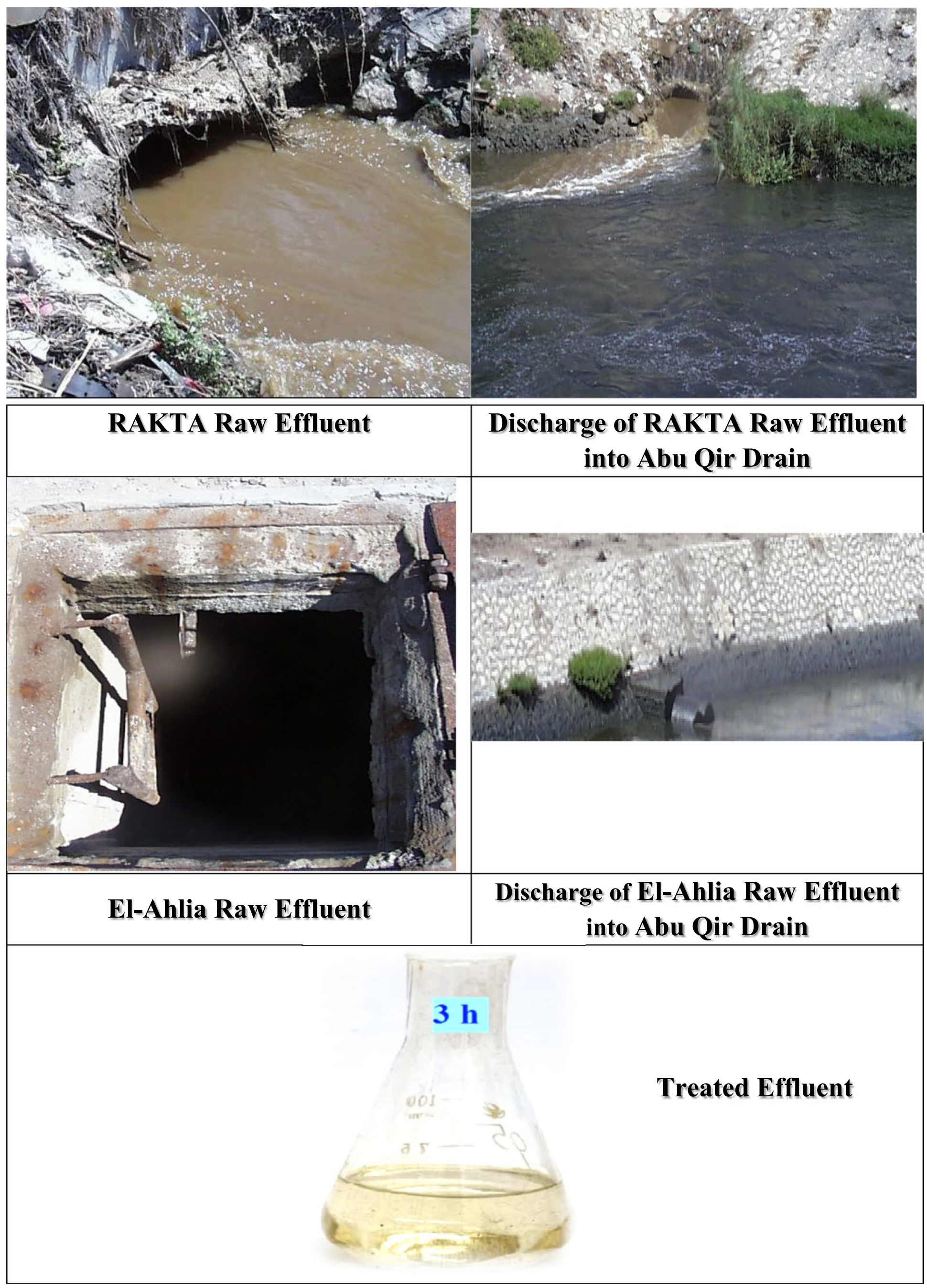


Fig. 8 RAKTA and El-Ahlia pulp and paper manufacturing companies raw and treated effluents using developed Fe_3O_4 NPs/bacterial biomass assembly

Table 4 Correlation Coefficients (Pearson's r) among the Different Parameters (Contaminants) During Bioassay Using Coated Bacteria

	TDS	TSS	BOD	COD	Total Tannin and Lignin
TDS	1	0.234	−0.689	−0.647	−0.671
TSS	0.234	1	−0.339	−0.364	−0.649
BOD	−0.689	−0.339	1	0.998**	0.935*
COD	−0.647	−0.364	0.998**	1	0.941*
Total Tannin and Lignin	−0.671	−0.649	0.935*	0.941*	1

*Correlation is significant at the 0.05 level (1-tailed)

**Correlation is significant at the 0.01 level (1-tailed)

Optimization of lignin adsorption using synthesized magnetite NPs

In order to reach the maximum lignin removal, optimization of lignin (**100** mg/L solution) adsorption on magnetite NPs under different **pH** values, nanoadsorbent dosage and contact times was investigated. Results revealed the following points:

1. Results showed decrease in lignin adsorption with the increase in **pH** value from **6.0** to **8.0** (Fig. 7A). Therefore, **6** considered the optimal **pH**.
2. There was a regular increase in the **RE** of lignin (**28** mg/L) with increase in Fe_3O_4 NPs dosage (**10, 25, 50, 100, 150** mg), reaching the highest removal of **82.14**% at **100** mg adsorbent and still constant at **150** mg (Fig. 7B). Therefore, Fe_3O_4 NPs dosage of **100** mg was considered the optimal dose.
3. Similarly, results indicated increase in the **RE** of lignin (initial concentration of **35** mg/L) with increase in contact time (**5, 10** and **15** min) reaching the highest removal (**71.4**%) after **10** min, which still constant up to the last contact time of **15** min (Fig. 7C). Therefore, **10** min was considered the optimal contact time.
4. It was then concluded that the optimized conditions for the maximum lignin adsorption and removal using Fe_3O_4 **NPs** were found to be achieved at **pH 6**, Fe_3O_4 NPs dosage of **100** mg and **10** min contact time.

The effect of solution **pH** on lignin absorption may be attributed to the influence on the surface properties of the adsorbent and the ionization/dissociation of lignin. Decrease of absorption was detected with **pH** increase. Similar findings reported that adsorption decreased with increase in **pH** in the range **2.0–9.0**. Moreover, the maximum lignin adsorption was achieved at **10** min contact time and increased with increasing Fe_3O_4 NPs dosage, as also supported by other workers (Li et al. 2024).

It is worth to clarify that only **0.3** g is required to prepare the Fe_3O_4 **NPs/*P. otitidis*** assemblage (as shown in the material and methods) that was used to remediate pulp and paper manufacturing wastewater with **21** mg/L total lignin. In case of using Fe_3O_4 **NPs** only, dosage of **100** mg Fe_3O_4 **NPs** is required for lignin (**28** mg/L) removal. Considering the fact that Fe_3O_4 NPs are easy to synthesize and can be regenerated and magnetically recyclable, as shown by Stylianou et al. (2021) and Sulistyaningsih et al. (2015), it is confirmed that the proposed technology is very useful, sustainable, fast and cost-effective in practical applications for treatment of real pulp and paper manufacturing wastewater on industrial scale**.** Moreover, no sludge was produced during the treatment with the proposed system; pollutants were digested (biologically decomposed), which is another valuable advantage. This is clearly shown in Fig. 8, which compares between pulp and paper effluent before and after treatment with the proposed technology. Moreover, comparison with similar previous works confirmed that the proposed technology present superior solution for the removal of lignin and other included contaminants from heavily polluted real pulp and paper manufacturing raw effluents. Not only the high removal efficiency, but economically, it is much cheaper than other technologies, very fast (within **4** h) and performed at the prevailed conditions with no agitation, temperature or **pH** adjustment are required. Therefore, the proposed treatment considered highly effective, environmentally friendly, renewable and strongly recommended for use for decontamination of pulp and paper industrial wastewater.

Statistical analysis

Multifactorial analysis (ANOVA) for the different contaminants in the industrial effluents was performed during the batch bioassay of Fe_3O_4/***P. otitidis*** assemblage by Pearson's correlation coefficient (r) at different confidence levels (**95** and **99**%) and presented in Table 4. Data in the table concluded the following points:

1. **BOD** showed highly significant positive correlations (at **0.01**) with only **COD,** while it was insignificantly correlated with other tested parameters.
2. **BOD** showed significant positive correlations (at **0.05**) with **total tannin and lignin**
3. **COD** showed significant positive correlations (at **0.05**) with **total tannin and lignin**
4. No other significant correlations were detected**.**
5. Statistical analysis confirmed that high and significant correlations were recorded among most of the tested parameters during the bioremediation process and are all affecting positively or negatively the remediation efficiency.

Conclusions

Results of the present study achieved the following conclusions.

1. Pulp and paper effluent contained extremely high levels of all the tested pollutants, especially **COD**, **BOD** and **TSS**, which creates many difficulties in its treatment and dangerously affecting the receiving environments.
2. ***P. otitidis*** **MCC10330 (ATCC BAA-1130)** proved being the most active when previously screened with other **9** bacterial species. Therefore, it was selected, immobilized using magnetite nanoparticles and used to treat pulp and paper-contaminated wastewater.
3. Using the proposed magnetite-coated bacterial assembly system is remarkably efficient since it enhanced the growth of *P otitidis* and achieved the highest removals of **TSS**, **COD, BOD,** total tannin and lignin, prevented sludge production and reduced treatment time during the treatment process. This is also confirmed by comparison with previous works (Table 3).
4. Although the very short time (**4** h) applied for this bioassay, considerably very high reductions were achieved. However, some of the tested pollutants (**TSS**, **COD, and BOD**) are still slightly higher than their **MPL**s for the safe discharge, which may be attributed to the short treatment time, small inoculum size or dose of the nanoparticles. To overcome pollutants **RCs** that are not compiling with the environmental laws, it is highly recommended to scale up a suitable unit for remediation, increase $\mathbf{Fe_3O_4}$ NPs dose and bacterial inoculum size as well as performing physical pre-treatment step (sedimentation) before treatment using the proposed $\mathbf{Fe_3O_4}$**/** bacteria assembly system.
5. Finally, the proposed assemblage can be directly augmented, at the optimized dose, into the existing treatment system (i.e., activated sludge) without the need to any infrastructures, or used as a very compacted tertiary biofilter unit after the secondary (biological) treatment in a continuous system, all of which confirmed the ease of application.

Funding Open access funding provided by The Science, Technology & Innovation Funding Authority (STDF) in cooperation with The Egyptian Knowledge Bank (EKB). The authors declare that no funds, grants or other support were received from any organization during the preparation of the submitted work. The authors have no relevant financial or nonfinancial interests to disclose.

Data availability Data obtained and analyzed in this study are presented in the main work. The manuscript was not submitted to a preprint server before submitting it to "Applied Water Science" and data are not shared with any other party.

Declarations

Conflict of interest The authors declare no conflict of interest or have also no competing interests to declare that are relevant to the content of this article.

Ethical approval The authors confirmed that all elements of the submission are in compliance with the journal publishing ethics policy and that we have not submitted the manuscript to any preprint server before submitting it to Applied Water Sciences.

Informed consent The authors declare that no human participants and/or animal studies were involved in the submitted research.

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