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RESEARCH ARTICLE

Surfactin production in the bioreactor: Emphasis on magnetic nanoparticles application

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Abstract

Surfactin is one of the main lipopeptide biosurfactants produced by different species of *Bacillus subtilis*. This study aims to analyze the effect of starch-coated Fe^0 and Fe^{3+} nanoparticles on the biomass and biosurfactant production of *Bacillus subtilis*. Out of 70 soil samples, 20 *Bacillus* were isolated and genome sequenced by biochemical methods and 16S rRNA gene. Quantitative and qualitative screening methods were used to isolate and detect biosurfactant production. For the aim of this study, 61 and 63 (*Bacillus subtilis subsp. Inaquosorum*) were selected. Then, hemolytic activity, biomass amount, surfactant production, and reduction of surface tension in Minimal Salt Medium containing Fe^0 and Fe^{3+} nanoparticles were examined after 48, 72, and 96 h of culture. Strain 61 was the best bacterium and Fe^{3+} was the best nanoparticle. The results were compared with the results of non-nanoparticle bioreactor. The results showed the amount of biomass, surfactin, and surface tension decrease, 72 h after growth in 61 strain containing Fe^{3+} reached the highest values. Surfactin from strain 61 culture in the Fe^{3+}nanoparticle bioreactor after 72 h of growth showed higher production than the same strain culture after 72 h without Fe^{3+}, if continuing the research, this strain can be commercialized in the future.

KEYWORDS

Bacillus subtilis, biosurfactant, Fe^0 and Fe^{3+} nanoparticles, surface tension, surfactin

1 | INTRODUCTION

Biosurfactants are unique amphiphilic molecules and are widely used in different industries due to their surface activities such as purification, emulsification, and dispersing. Also are used in the removal of organic and metal environmental contaminants [1]. Biosurfactants are also used in industries such as the petroleum, food, pharmaceutical, and cosmetics industries. Some of the characteristics of biosurfactants that make them superior to chemical surfactants are reduced surface tension and interfacial tension, low toxicity, high biodegradability, emulsification, production from low-cost raw materials, antimicrobial properties, easier production, and greater diversity, as well as resistance to extreme conditions such as high temperatures and high salt concentrations and pH changes [2–5]. Biosurfactants also increase the biodegradation of petroleum hydrocarbons by enhancing the

solubility and emulsification [6]. Surfactin, a molecule with a molecular weight of 1036.3 g/mol produced by different species of *Bacillus subtilis* is one of the most effective biosurfactants with surface tension up to 25 mN/m. Surfactin has a lipopeptide structure consists of seven amino acids attached to the carboxyl and hydroxyl groups of a 14-carbon fatty acid [7,8]. Kinetic studies have shown that the production of surfactin is dependent on bacterial growth [1]. Today, extensive research is conducted on the use nanoparticles such as iron, gold, silver, and iron oxide for increasing the amount of production and improving the effects of biosurfactants [9–11]. It is reported that some nanoparticles at certain concentrations not only do not have antimicrobial effects but also increase the level of bacterial contact with compounds present in the medium and enhance the exchange of nutrients and thereby increase the rate of biosurfactant production. The results of these studies vary depending on the type of bacteria, the type of nanoparticles, the concentration of the nanoparticles, and the interaction time of the nanoparticles with the bacteria, but various studies have shown that the nanoparticles increase the growth of some bacteria and have no toxic effects on them [9]. In recent years, Fe nanoparticles have shown successful applications due to their excellent magnetic properties and high compatibility. Extensive researches have studies the effects of Fe nanoparticles and their derivatives on various bacterial processes such as bacterial growth rates and acceptable results have been observed [12].

Rangarajan et al. investigated the time-dependent dosing effect of Fe^{2+} on the production of surfactant from *Bacillus*. In this study, a single-dose Fe^{2+} supplementation (0.48 mmol/L) increased lipopeptide concentration to 3.3 ± 0.1 g/L after 8 h of fermentation. The results showed that lipopeptide production increased by 4.25 + 0.15 g/L by adopting a multidose Fe^{2+} feeding strategy. Finally, the concentration of biosurfactant produced by single-dose Fe^{2+} and multiple doses Fe^{2+} feeds was 4.7 and 5.8 times higher compared to the non-nanoparticle biosurfactant production condition [13]. Rashedi et al. showed that biofunctional nanoparticles improve the biodegradation of normal decane and hexadecane compounds by 91 and 89%, respectively. The reason for this increase is the effect of nanoparticles on increasing biosurfactant production in the test environment [14].

Fe nanoparticles also have considerable potential for functionalization because of their reactive surfaces. Zerovalent iron nanoparticles tend to condense quickly that leads to lack of their reactivity [15, 16]. The surface coating of nanoparticles with ionic compounds increases the repulsive force between the nanoparticles close together and prevents their agglomeration and aggregation [17]. Recently, several studies have used different types of polymers as lightweight matrices for coating and surface modification of nanoparticles [18]. The polymer coating preserves the core and its magnetic properties and makes the nanoparticles pragmatic. In addition, it prevents the agglomeration of nanoparticles, increases their stability and shelf life, and reduces their toxicity [19]. Polyacrylic, polyethylene glycol, and polyvinyl alcohol are among the synthetic polymers that are considered for coating of nanoparticles. The most common natural polymers are polysaccharides (a group of biopolymers). Polysaccharides are biocompatible, nontoxic, and renewable, and the presence of specific chemical groups in their structure makes them more bioactive [20] the most common polysaccharides for coating of nanoparticles are: agarose [20], chitosan [21], dextran [22], and alginate [23]. Among the polysaccharides, starch is the cheapest and most accessible biopolymer, which has been used to stabilize and coat some nanoparticles due to its unique characteristics such as biocompatibility, biodegradability, nontoxicity, and having OH functional groups [20]. Coating of Fe nanoparticles is often performed to reduce the oxidation rate of these nanoparticles [24, 25]. Rezazadeh et al. examined the biosurfactant production by *Pseudomonas aeruginosa* using Fe/starch nanoparticles. In this study, Fe/starch demonstrated no bacterial toxicity at 1 mg/mL and enhanced the growth rate and biosurfactant production up to 23.91 and 20.62%, respectively [8].

The aim of this study was to evaluate the biomass and surfactin production by *B. subtilis* and the reduction of surface tension of strains isolated from native soil of Iran in a stirred bioreactor without nanoparticles, compared to biomass and surfactin production and surface tension reduction in the presence of starch-coated Fe^0 and Fe^{3+} nanoparticles at 48, 72, and 96 h postgrowth.

2 | MATERIALS AND METHODS

2.1 | Isolation and purification of *Bacillus*

A total of 70 soil samples were collected from different parts of Iran at a depth of 7-10 cm and transferred to sterile glass containers at 4°C [26]. To isolate and purify *Bacillus* from other bacteria, spores retention and removal of vegetative cells, heat treatment was first used and the bacteria were incubated for 10 min at 80°C [27]. To perform Pour Plate culture, after preparation of dilution series, 1 mL of each dilution with 15 mL of the nutrient agar medium at 45°C was poured into the plate and incubated in an incubator at 37°C for 24 to 48 h [28]. After purification steps, the isolated strains were examined by Gram staining and catalase test and only Gram positive and catalase positive strains were selected. For biochemical detection

of *Bacillus* strains, Starch agar, Methyl red (MR)/Voges–Proskauer (VP), sulfide indol motility (SIM), Simmon citrate medium, nitrate recovery, and lecithin were used (Merck Company, Germany) [27].

2.2 | Selection of best surfactin-producing *Bacillus* strains

To isolate the better surfactin-producing *Bacillus* strains, red blood cell hemolysis, drop collapse, emulsification, and surface tension reduction tests were used. Surface tension hemolysis test was performed using Blood Agar culture medium [29]. While for Drop Collapse, Emulsification and Surface Tension tests, the nutrient agar medium was used. To do so, the strains were incubated at 37°C for 48 h and then were transferred to Minimal Salt Medium (MSM) containing glucose 40 g, KH_2PO_4 1.36 g, $CaCl_2$ 0.0007 g, $MgSO_4 \cdot H_2O$ 0.63 g, $MnSO_4$ 0.001 g, Na_2HPO_4 5/68 g ,Na-EDTA 0.0014 g, $FeSO_4 \cdot 7H_2O$ 0.0022 g (Merck Company) [30]. After transferring to MSM medium, the samples were stored in a shaking incubator at 37°C and 150 rpm for 72 h.

2.3 | Red blood cell hemolysis test

In this method, all purified strains were cultured on blood agar plates for 48 h at 37°C. Beta zone positive hemolysis strains (presence of transparent region around bacterial colonies) were selected to continue screening for surfactin-producing strains [31, 32].

2.4 | Drop collapsing test

Drop Collapses test is based on the collapse of drops of surfactant-producing bacterial suspensions on an oil-coated surface. In this condition, drops of bacterial suspensions which produce no or very low amount of biosurfactants remain stable. As biosurfactant concentration increases, the stability of drops decreases which is correlated with surface tension but not with emulsifying activity [33]. In this method, the strains were first incubated in the newest agar medium for 30 h at 30°C and incubated in MSM medium containing 20 g/L glucose 0.7 g/L KH_2PO_4, 0.4 g/L $MgSO_4$, 0.01 g/L $CaCl_2$, and 0.001 g/L $FeSO_4$ transferred. A total of 2 mL of freshly inoculated agar medium incubated for 48 h with 50 mL of MSM medium inoculated, and placed in a ShikranCubator for 150 min for 14 h, then centrifuged for 15 min at 6000 rpm and cell microfilter. Bacterial strips were separated, 20 mL of the suspension was placed on a mineral oil-coated surface, the strain remained unaffected but the strain with the surfactin-producing strain was destroyed. The standard lyophilized strain producing surfactin 21332 ATCC was used following trypticase-agar (TSA) assay plus 5% sheep blood. This test was done in three repetitions, and the average of the replicates is listed.

2.5 | Emulsification index test

Broderick Cooney method was used to select the most suitable strains [34]. Within four tubes of identical diameter, 4 mL of 24-h bacterial culture medium, including MSM medium containing 3% glucose and 0.03% yeast and 5 mL white oil, was poured. Each sample was subjected to severe vortexing for 1 min. The specimens were then kept in a static condition for 24 h. After this time, the residual emulsion height and total liquid height in the test tube were measured and the stability of the emulsion formed was calculated by the following formula [35–38]. This test was done in three repetitions and the average of the replicates is listed.

$$\mathrm{EC} = \frac{\text{Height of emulsion layer}}{\text{Height of total liquid}} \times 100. \quad (1)$$

2.6 | Surface tension measurement

In order to determine the main surfactant-producing strains, surface tension test was performed by tensiometer (FTM-TN-556, Toos Nano). The surface tension was measured by Du Nouy Ring Method. In this method, the device ring, made of platinum alloy, is immersed in the liquid, the force applied to the immersed ring is adjusted to zero and the ring is slowly pulled out of the fluid. The maximum force recorded by the device to remove the ring from the liquid surface is considered as the surface tension of the liquid. A total of 25 mL of 48-h culture of each sample was poured into the apparatus. The sample temperature was set to 25°C before measuring the surface tension [39]. This test was done in three repetitions and the average of the replicates is listed.

2.7 | Molecular detection of selected strains by PCR

PCR was used for molecular detection of selected strains. In order to isolate DNA from hemolytic bacteria containing the gene required for surfactin production, 2 mL of Luria–Bertani medium (LB) and a colony of each inoculated sample was incubated at 37°C for one night. The mediums containing bacteria were centrifuged at 6000 *g* for 20 min,

and the supernatant was removed from the biomass. In order to extract DNA from the sediment, DNA extraction kit (BAC) made in Shahid Beheshti University Medical Genetics Department was used. For the accuracy of DNA extraction, the resulting product was transferred to 1% agarose gel. The complementary DNA (cDNA) extracted as template DNA was used for PCR. The primer used for PCR was Rd1 (5′AGAGAGGTGATCCAGCC3 F) Fd1 (5′AGTTTGATCCTGGCTCAG3′) and 'Gene Amp PCR System 2700 (Applied Bio systems, USA). After amplification, the PCR product was sequenced with the ABI PRISMTM 3100 Genetic Analyzer (Applied Bio systems) software and the results were analyzed by Chrome plus software using the NCBI database [40–42]. The selected strains were cultured in the main medium for production of surfactin and the produced surfactant was extracted after PCR. The purified strains with beta hemolysis were cultured in 1 L MSM medium then acidified with 3 mM HCl and centrifuged at 6000 *g* for 20 min. The resulting precipitate was placed in a desiccator for 10 min and dried in oven at 100°C and biomass weight was measured using a digital balance [43]. Dichloromethane was added to the supernatant (containing crude surfactin) and placed on the shaker at 180 rpm for 14 h. Watman filter paper was used to remove impurities. Surfactin dissolved in dichloromethane was washed twice with equal volume of distilled water and shaken for 20 min. To separate the two liquid phases, it remained standing for 3 h. The white to yellow precipitate was separated by centrifugation at 15 000 *g* at 10°C for 25 min and dried in a desiccator for 24 h and held overnight at 4°C. This precipitate is surfactin [44–46].

2.8 | Preparation of Fe^0 and Fe^{3+} nanoparticles and surface modification of nanoparticle with starch

The iron nanoparticles were synthesized via liquid phase reduction method, which involves adding a strong reducing agent to a solution containing iron ions and reducing them to metal nanoparticles [47]. Starch coated iron nanoparticles were prepared as follows: first, 6 g of anhydrous iron chloride and 4.8 g of $FeCl_2{\cdot}6H_2O$ were poured into in a three-necked balloon equipped with a sparger, condenser, and separator funnel. Then, 100 mL of distilled water and 30 mL of ethanol were added to balloon. The mixture was stirred by magnetic stirrer at ambient temperature at 250 g and a uniform orange color solution was obtained. To remove the dissolved oxygen, a weak stream of nitrogen through the sparger was poured into the solution. Then, 2 g starch solution was added to the medium. After 30 min, potassium borohydride solution (5.4 g in 20 mL water) and 30 mL of ethanol were added drop wise to the medium with the rate of 1-2 drops per second through a separating funnel. With the addition of potassium borohydride droplets, black particles of iron nanoparticles were formed. After the oxidation, the mixture which was completely black was further stirred for 1 h at ambient temperature. Then, the mixture was filtered and washed twice with distilled water, once with ethanol, and finally with acetone for quick drying and dried overnight at 80°C (Merck Company) [48, 49]. TEM micrographs of synthesized magnetic nanoparticles are shown in Figure 2A,B.

2.9 | Evaluating the effect of starch-coated Fe^0 and Fe^{3+} nanoparticles in surfactin production, emulsification rate, and surface tension reduction

A total of 4500 mL of MSM medium was prepared and autoclaved under 1.5 atmospheric pressure and at 121°C. It was then divided into 250 mL portions. New culture was prepared from both bacterial strains in Nutrient Broth and inoculated into 250 mL of MSM medium and incubated in an incubator at 30°C and 150 rpm. Optimal concentration 1 mg/L of starch-coated Fe^0 and Fe^{3+} nanoparticles was added to culture media. At 48, 72, 96 h after growth, samples were taken from the culture medium, and beta hemolysis, biomass, surfactant production, and surface tension reduction tests were performed to evaluate surfactin production [50]. From each sample, three repetitions were done and the average of the three replicates is listed.

2.10 | Biosurfactant production and surface tension reduction of selected strain 61 with and without Fe^{3+} nanoparticles in bioreactor

The stored bacterial stock was cultured on a slant medium and incubated at 30°C for 48 h. Then, it was transferred to Luria–Bertani (LB) medium from the grown slant medium, and incubated overnight in a shaker incubator at 30°C and 150 rpm until reaching the optical absorption equivalent to 0.5 McFarland (1.5×10^8) (UV-Vis Spectrophotometer-4000, Agilent Company). It was then transferred to a 5 L stirred bioreactor of Saba Company (National Institute of Genetic Engineering) containing 3.5 L MSM medium. Two 5 L fermenters were used concurrently, one containing 61 strain with Fe^{3+}nanoparticles (1 g/L) and the other containing only 61 strain. Samples were taken from both bioreactors after 48, 72, and 96 h. From each sample, three repetitions were done and the average of the three replicates is listed.

FIGURE 1 Rapid beta hemolysis of sheep erythrocytes by surfactant produced

3 | RESULTS

3.1 | Quantitative and qualitative screening methods

To isolate the better surfactin-producing *Bacillus* strains, from quantitative and qualitative screening methods, such as red blood cell hemolysis, drop collapse, emulsification, and surface tension reduction tests in MSM containing Fe^0 and Fe^{3+} nanoparticles, were used. Hemolytic activity of the bacteria was considered to be the first qualitative evidence for biosurfactant production [51]. Anandaraj also used this method to investigate the production of biosurfactants by their isolated bacteria [52]. The bacteria in this research, were able to break down red blood cells and create a clear hemolysis halo around the colonies. In the study, 70 bacterial samples were isolated from soil and 36 bacterial samples were isolated by beta hemolysis test (Figure 1). Of these bacteria, 20 species had strong beta-hemolysis which were used for screening the best surfactant-producing strains (Figure 3).

The results of the Drop Collapses test in Table 1 (average of the three replicates) showed that out of the 20 strong positive hemolysis samples compared to ATCC 21332, four became positive fast (less than 5 min, strains 2, 61, 63, and 66) and were able to destroy and disperse the oil drop, which confirms the production of biosurfactants.These strains were used to isolate biosurfactants.

Emulsification Index Test, is to check the stability and emulsification of the bacterial soup after 24 h [38]. This test for all samples were done in three repetitions, and the average of the replicates is listed, and the results were calculated according to Equation 1. The results are shown in Table 2 (average of the three replicates). In this study, emulsifying activity in 10 strains, including strains 13, 21, 25, 53, 58, 61, 63, 64, 66, and 70, had the same results (88.88%) with standard strain ATCC 21332 and compared to ATCC 21332 which is a strong surface tension reducer and used as a positive control, exhibiting good performance [53].

The results of surface tension reduction test of 20 strong hemolysis samples compared to water surface tension (71.5 mN/m) and culture medium without bacteria (50.2 mN/m) (as control) showed 13 samples had the surface tension less than 40 mN/m (Table 3, average of the three replicates). Therefore, the biosurfactant produced by the strains studied has an acceptable ability to reduce surface tension. Makkar et al. produced lipopeptide biosurfactant from *B. subtilis* using a molasses carbon source at thermophile condition. The use of molasses by *Bacillus* strains to produce biosurfactant and growth at 45°C resulted in the production of biosurfactant and reduced surface tension of the medium to 29 and 31 Dyne/cm [54]. Also, the study of Sahebnazar et al. aims to promote the reduction of oily sludge viscosity and consequently oil recovery using biosurfactants. For this purpose, the effect of rhamnolipid purification on the oil recovery was investigated. Rhamnolipid was purified using Fe^0 nanoparticles. The measurement of surface tension and critical micelle concentration showed an increase in rhamnolipid purity from 47.61 to 83.33% at optimum conditions. The results of rhamnolipid application for oily sludge treatment showed that the purified biosurfactant decrease the interfacial tension of *n*-decane/water (1/1) from 27 to 1.2 mN/m while the unpurified rhamnolipid decrease the interfacial tension from 27 to 6.7 mN/m. Also, the reduction of oily sludge viscosity by purified biosurfactant is 27.2% more than that by unpurified biosurfactant [55].

Finally in this research, based on quantitative and qualitative tests, four strains in drop elimination test, 10 strains in emulsifying activity and 13 strains in surface

TABLE 1 Results of the average of the three replicates of Drop Collapses test

Samples/ Drop Collapses test results																			
2	3	7	11	13	15	21	22	25	26	31	53	56	58	61	62	63	64	66	70
++	+	+	+	+	+	+	+	+	+	+	+	+−	+	++	+*	++	−	++	+

++ Fast (less than 5 min), +* (15 min), +− (20 min), + (after 20 min).
ATCC Sample: ++ Fast (less than 5 min).

TABLE 2 Results of the average of the three replicates of the Emulsification test

Samples/Results										
Witness	**2**	**3**	**7**	**11**	**13**	**15**	**21**	**22**	**25**	**26**
88/88	81/25	86/66	85/10	84/44	88/88	82/60	88/88	85/10	88/88	84/44
	31	53	56	58	61	62	63	64	66	70
	86/66	88/88	85/10	88/88	88/88	88/88	88/84	88/88	88/88	88/88

TABLE 3 Results of the average of the three replicates of surface tension of purified strains using the duo ring method

No.	Sample	Surface tension
1	Distilled water	71.5
2	Culture medium	50.2
3	70	32.3
4	61	32.3
5	15	48.6
6	3	48
7	63	32.2
8	21	40.5
9	2	34.1
10	13	55.8
11	ATCC	47.3
12	26	33.6
13	66	33.2
14	22	37.8
15	58	34.9
16	25	35
17	53	32.9
18	64	34.1
19	37	36
20	56	32.4
21	7	48.6
22	11	46.3
23	62	46.2

tension reduction test were selected as biosorbent generating strains for subsequent screening.

3.2 | Phylogenetic identification of selected strains

The sequencing results of strains 70, 63, and 61 corresponded to the *B. subtilis subspecies*, *Inaquosorum*, and *Bervibacterium frigurtiolrance* strains, respectively with 99.9% homology. Since, the aim was to isolate the surfactin-producing *B. subtilis* from soil, so strain 70 identified as *B. frigurtiolrance* was removed from further investigation. According to all the tests, ultimately both 61 and 63 strains which were *B. subtilis subspecies, Inaquosorum* were selected to identify the best surfactant-producing strain among these.

3.3 | Evaluation of hemolysis, biomass, biosurfactant, emulsifier, and surface tension reduction of selected strains with and without Fe^0 and Fe^{3+} nanoparticles

After analyzing 18 samples containing 250 mL medium of strains 61 and 63 with and without nanoparticles at 96, 72, and 48 h after growth, the results of hemolysis, biomass, surfactant production, emulsification rate, and surface tension reduction were determined (Table 4, average of the three replicates). The results showed that strain 61 is the best strain, and the best time to increase biomass and the amount of surfactin produced is 72 h after growth and the best nanoparticle is Fe^{3+}nanoparticle that doubles the amount of surfactant produced and the surface tension of reduced 43.27 Nm/m to15.4 Nm/m. The morphological characteristics of nanoparticles surface were determined by Transmission Electron Microscopy (TEM, Hitachi, H-7500, 120 kV) (Figure 2A,B). Morphology observation of Nanoparticles show that the Fe^{3+} nanoparticles (Figure 2A) had a spherical morphology with uniform distribution and the average size of 7-8 nm, also in TEM image (Figure 2B) Fe^0 nanoparticles had spherical morphology with uniform distribution and the size ranges from 5-35 nm (Image magnification is 350 nm).

3.4 | Biosurfactant production and surface tension reduction of selected strain 61 with and without Fe^{3+} nanoparticles in bioreactor

The amount of surfactin production and surface tension reduction of each bioreactor are presented in Tables 5 and 6. The results of culture in the 5 L bioreactor showed that the culture medium containing Fe^{3+} nanoparticles had higher biomass and surfactin production and lower surface tension compared to the culture medium without Fe^{3+}nanoparticles. The highest amount of biomass produced in the Fe^{3+} nanoparticle containing medium was 6.02 g/L with the lowest surface tension (10.30 mN/m) after

TABLE 4 Results of the average of the three replicates of hemolysis, biomass, biosurfactant (surfactin), emulsification rate, and surface tension reduction of samples containing medium without iron nanoparticles and containing iron nanoparticles for selected strains of 61 and 63 at 48, 72, 96 h postgrowth

No.	Sample	Biomass (mg%)	Biosurfactant (mg%)	Surface tension reduction (mN/m)	Emulsification	Hemolysis
1	61/48	235	0.132	36	83	+
2	61/72	446	0.436	32	84.5	++
3	61/96	470	0.023	36.2	84.5	+
4	63/48	153	0.05	37.5	84	++
5	63/72	135	0.12	34	84.4	++
6	63/96	150	0.064	35.2	83.4	+
7	61/0/48	420	0.444	32.25	84	++
8	61/0/72	481	0.47	30.1	88.2	+++
9	61/0/96	478	0.475	30.2	86	+
10	63/0/48	194	0.075	34	84.5	++
11	63/0/72	165	0.077	32	83.3	+++
12	63/0/96	189	0.099	33.5	83.5	+
13	61/F/48	429	0.475	25	86.5	+
14	61/F/72	497	0.463	23.95	88.9	++
15	61/ F/96	481	0.461	25.3	88.3	+++
16	63/ F/48	200	0.104	31.25	84.4	+
17	63/ F/72	168	0.1	30	84.5	++
18	63/ F/96	182	0.05	30.2	84.2	+++

0 corresponds to Fe^0 and Fe corresponds to Fe^{3+} (with starch coating).

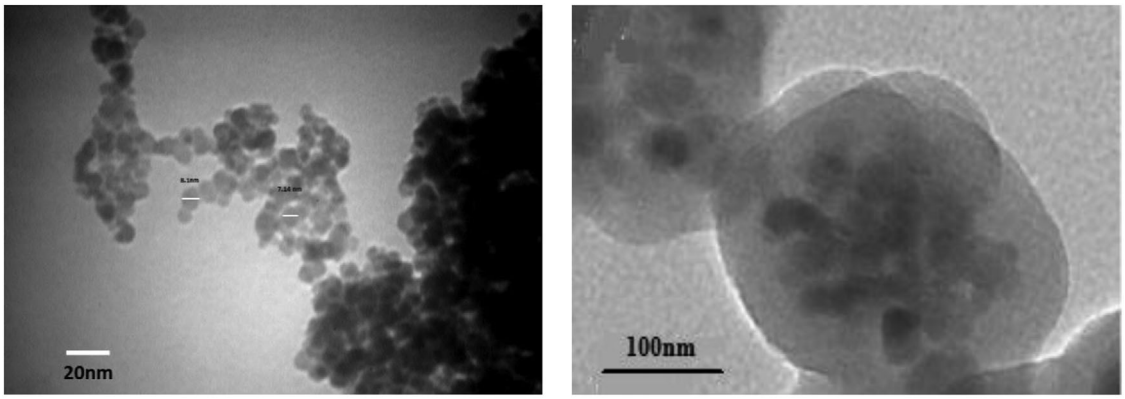

FIGURE 2 (A) TEM micrograph of magnetic nanoparticles Fe^{3+}, (B) TEM micrograph of magnetic nanoparticles Fe^0

TABLE 5 Quantities of biomass, surfactin, and surface tension reduction in the bioreactor without Fe^{3+} nanoparticles

Reduction of surface tension (mN/m)	Amount of biomass (g/L)	Amount of surfactin (g/L)	Time in hours (h)
34.5	5.65	2.01	48
34.21	6.32	2.7	72
34.35	5.7	2.7	98

TABLE 6 Quantities of biomass, surfactin, and reduction of surface tension in the bioreactor containing Fe^{3+} nanoparticles

Reduction of surface tension (mN/m)	Amount of biomass (g/L)	Amount of surfactin (g/L)	Time in hours (h)
15.41	7.56	4.76	48
10.3	8.66	6.02	72
10.32	7.8	6	98

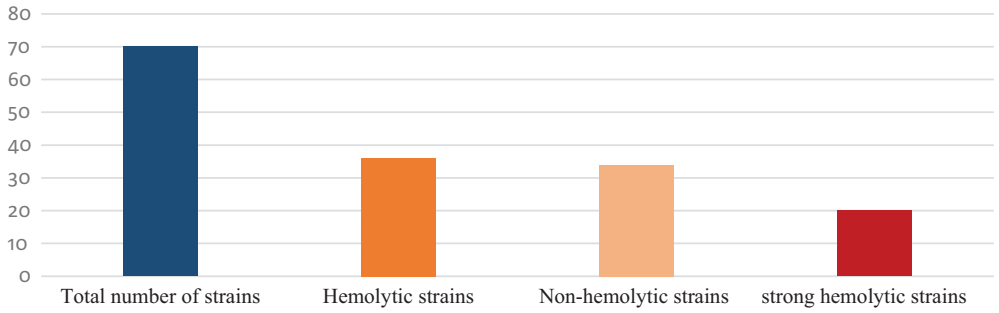

FIGURE 3 Comparison of soil isolated samples (horizontal surface of bacterial groups and vertical level of abundance)

72 h. The results show that the amount of surfactin produced by strain 61 cultured in Fe^{3+} nanoparticle containing bioreactor after 72 h of growth was higher than that of produced by of strain 61 cultured without Fe^{3+} nanoparticle after 72 h. In other words, the surface tension decreased significantly in environments containing Fe^{3+} nanoparticles at 48, 72, and 98 h. Also, in other researchers' studies, the levels of surface tension, pH, and cell mass volume were measured during testing. The results showed that with increasing cell mass, the surface tension decreased due to increased surfactin production. In this study, the best obtained operating condition for biosurfactant production in two bioreactors was at 37°C with a stirring speed of 150 rpm with an oxygenation rate of 0.75 v/v, which is consistent with results presented by other researchers such as Lee and Cooper [56, 57].

4 | DISCUSSION AND CONCLUSION

Due to the growing applications of biosurfactants in agriculture industry in recent years, the development of these biological products has attracted many researchers and they have used a variety methods to improve biosurfactant production. Bacteria, yeasts, and fungi are the main sources of biosurfactant production. The use of native bacteria can lead to a significant increase in biosurfactant production [58]. Several studies investigated the effect of different nanoparticles on the rate of biosurfactant production by different bacteria [59, 60]. The research of Alamdar et al., showed that, the effect of nanoparticles on the bacterial growth and biosurfactant production varied corresponding to the type and concentration of nanoparticles [50]. In this study, effect of starch-coated Fe^0 and Fe^{3+}nanoparticles on the biomass and biosurfactant production of *B. subtilis* was investigated. For screening the best biosurfactant-producing strains, hemolysis test, drop collapses test, emulsification, and surface tension reduction were used. Hemolytic activity of biosurfactants was first reported by Bernheimer and Avigad for biosurfactants produced by *B. subtilis* [61]. Two Bacillus strains [60, 62] selected by Kishore Das et al. method [62], were able to reduce the surface tension to less than 40 (32.2 mN/m). Mostafapour and Ahmady reduced the surface tension of the environment to less than 40 using a species of *B. subtilis* with hemolytic activity [63]. Also, in a study by Beata Koim–Puchowska et al., 45 hemolysis inducing *B. subtilis* strains were selected. A sum of 19 of them reduced surface tension to 40 mN/m; in six cases, the reduction was as much as 50%, which was consistent with the results of our research [64].

In the present study, three mediums (medium containing starch-coated Fe^0 nanoparticles, starch-coated Fe^{3+}nanoparticles, and culture medium without nanoparticles) were used for the culture of two selected strains. Optimal concentration 1 mg/L of starch-coated Fe^{3+} nanoparticles had positive effects on bacterial biosurfactant production. After 24, 48, and 72 h of addition of nanoparticles to shaking medium, the results were evaluated, strain 61 containing Fe^{3+} nanoparticles had higher levels of hemolysis, biomass, surfactin production, emulsification rate, and lower surface tension after 72 h of growth compared to nanoparticles-free medium, which seems with continuing the research, to have a good commercialization potential in the future.

In the similar study, the effect of Fe/SDS and Au nanoparticles on growth rate and biosurfactant production for application in oil industry was investigated. The results showed that low concentrations of nanoparticles Fe/SDS and Au have positive effects on bacterial biosurfactant production and suggested that they could serve as a good alternative to chemical surfactants for petroleum industry [50].

CONFLICT OF INTEREST

The autors have declared no conflict of interest.

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