

Phytosynthesized iron nanoparticles: effects on fermentative hydrogen production by *Enterobacter cloacae* DH-89

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Abstract. In recent years the application of metal nanoparticles is gaining attention in various fields. The present study focuses on the additive effect of 'green' synthesized iron nanoparticles (FeNPs) on dark fermentative hydrogen (H₂) production by a mesophilic soil bacterium *Enterobacter cloacae*. The FeNPs were synthesized by a rapid green method from FeSO₄ using aqueous leaf extract of *Syzygium cumini*. The synthesized FeNPs showed a characteristic surface plasmon resonance peak at 267 nm. The transmission electron microscopy images confirm that the formation of FeNPs was mainly porous and irregular in shape, with an average particle size of 20–25 nm. The presence of iron (Fe) in the synthesized FeNPs was confirmed by energy-dispersive X-ray spectroscopy. The comparative effect of FeSO₄ and FeNPs on batch fermentative H₂ production from glucose was investigated. The fermentation experiments reveal that the percentage and yield of H₂ in FeNPs supplementation were increased significantly than the control (no supplementation) and FeSO₄ containing media. The maximum H₂ yield of 1.9 mol mol⁻¹ glucose utilized was observed in 100 mg l⁻¹ FeNPs supplementation, with two-fold increase in glucose conversion efficiency. Thus, the result suggests that FeNPs supplementation in place of FeSO₄ could improve the bioactivity of H₂ producing microbes for enhanced H₂ yield and glucose consumption.

Keywords. Iron nanoparticles; green synthesis; *Syzygium cumini*; dark fermentation; biohydrogen production; *Enterobacter cloacae*.

1. Introduction

The green method of nanoparticle synthesis has received immense attention, and is a burning area of research since the last decade. Owing to their unique size-dependent physicochemical properties, metal and metal oxide nanoparticles get varied range of applications in biomedical, optics, mechanics and energy science.^{1–3} A variety of physical and chemical methods have been reported for the synthesis of metal nanoparticles.^{4–7} The necessity of expensive equipment or high energy in physical methods and use of toxic chemicals as well as hazardous by-products formation during chemical synthesis, upsurge the curiosity to find an alternate and safe approach. The green synthesis of nanoparticles using biological materials is considered as a cost-effective, non-toxic and eco-friendly approach.⁸ Biological resources like microorganisms and plant extracts can be used as reducing agents.^{9–11} The plant extract-mediated synthesis of nanoparticles is potentially advantageous over microorganisms because of simple, cost-effective, readily available and relatively reproducible.^{12,13} Plant extracts generally reduce the metal ions in a shorter period of time than microbes. Depending upon the plant type and phytoconstituents concentration, nanoparticles are synthesized within a few

minutes to hours.¹⁴ In recent times, green synthesis of iron nanoparticles (FeNPs) of various shapes and sizes have been reported using green tea,¹⁵ *Murraya koenigii* leaf extract,¹⁶ soya bean sprouts and sorghum bran extract.¹⁷ The above-mentioned nanoparticles were successfully applied in various fields like dye degradation, biohydrogen production and waste water treatment. In recent times, the synthesis of silver nanoparticles using leaf and bark extract of *Syzygium cumini* as reducing agent has been reported.^{18,19} However, there are very few reports on the synthesis of FeNPs using aqueous leaf extract of *S. cumini*.

Hydrogen (H₂) a sustainable energy source, considered as a promising substitute of fossil fuel. It is a clean fuel, as it produces only water instead of green house gases on combustion.²⁰ Among various methods of H₂ production, dark fermentative process has been recognized as the most suitable because of its ecofriendly and energy efficient status.²¹ However, the major challenge in this method is the low yield and slow production rate. Therefore, the search for a novel approach is required to accelerate the H₂ production rate and enhance the yield. Micronutrients like iron and magnesium are well known for their involvement in the activation or function of many enzymes and co-enzymes, and are also vital for the growth of most microorganisms.²² Hydrogenases, a group of metalloenzyme catalyses the H₂ formation in a variety of microorganisms. They have very unique

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active sites and are classified based upon the active site metal composition as: [Fe], [Fe–Fe], and [Ni–Fe] hydrogenases.²³ Thus, the supplementation of suitable micronutrients is likely to enhance the activity of these enzymes for high H₂ yield. Iron is generally used as a media component in the fermentation medium for H₂ production. Mainly, iron is a component of ferredoxin which acts as an electron carrier in hydrogenases.¹⁷ A few studies have reported on improved H₂ yield and more substrate utilization by supplementing production media with metal nanoparticles.^{2,3,24,25} In the present study a clean method is applied for the synthesis of FeNPs using water extract of the *S. cumini* leaf. The synthesized FeNPs is used as media supplement to enhance the dark fermentative H₂ production by the isolated strain DH-89 in glucose-based batch fermentation.

2. Materials and methods

2.1 H₂ producing bacterial strain

The H₂ producing bacterial strain was isolated from soil sample of paddy field from North-East India. For this, 1 g of the soil sample was serially diluted with 0.85% NaCl (w/v) and then plated on nutrient agar plates, incubated at 37°C for 24 h. Different bacterial colonies based on their colony morphology were obtained and subsequently maintained as pure cultures for screening of potent H₂ producers.

2.2 Preparation of leaf extract

Freshly collected *S. cumini* leaves were washed three times with water and air dried. The dried leaves were then grinded into fine powder. The extract was prepared by mixing 50 g of finely powdered leaf with 1 litre distilled water, followed by boiling for 10 min in boiling water bath. The extract was then filtered through Whatman No. 1 filter paper and the filtrate was used as reducing agent for nanoparticle synthesis.

2.3 Synthesis of FeNPs using leaf extract

Different concentrations of leaf extracts and FeSO₄ solution were reacted to obtain the optimum concentration for synthesis of FeNPs. In a typical synthesis, 25 ml of plant extract was slowly added to 475 ml of 1 mM FeSO₄ solution with constant stirring for 10 min at room temperature. A visible colour change of FeSO₄ from light yellow to dark black indicates the formation of FeNPs. The colloidal mixture was then centrifuged (12,000 rpm, 20°C) for 10 min. The pellet was washed with distilled water and freeze dried for further use.

2.4 Characterization of the synthesized FeNPs

UV–visible spectrophotometer (Thermo Fischer Scientific, Evolution 201) was used to perform optical measurements. The UV–vis spectrum of FeNPs was taken in quartz cuvettes, using distilled water as a reference solvent. UV–visible

analysis was carried out by continuous scanning from 200 to 600 nm. X-ray diffraction (XRD) patterns of the FeNPs were obtained by X-ray diffractometer (Miniflex, Rigaku Corporation Japan). The scanning was performed from 10° to 70° 2θ with 2 min⁻¹ of scanning rate. For surface morphology study, scanning electron microscopy (SEM–JEOL JSM-6390 LV, Japan) and transmission electron microscopy (TEM–TECNAI G 20 S-TWIN, 200 kV) analysis were done. The elemental composition of the synthesized FeNPs was determined using energy-dispersive X-ray (EDX) spectrophotometer (JEOL-JSM 6390, Japan). Fourier transform infrared (FTIR) spectra of the aqueous leaf extract and FeNPs were analysed by FTIR spectrophotometer (Perkin Elmer 1000 FT-IR spectrometer).

2.5 Experimental procedure for dark fermentative H₂ production

Batch experiments were conducted for dark fermentative H₂ production in 125 ml BOD bottle with a working volume of 100 ml under anaerobic condition at 37°C. The fermentation medium was mineral salt medium (MSM), consisted of macronutrients (g l⁻¹): (NH₄)₂SO₄, 2; Na₂HPO₄, 2.61; KH₂PO₄, 4.75; MgSO₄, 0.8; glucose, 10; and micronutrients (μg l⁻¹): CaCl₂, 500; MnSO₄, 100; H₃BO₃, 10; ZnSO₄, 70; and MoO₃, 50. The pH of the medium was adjusted to 7.0 before autoclaving. The supplementation of FeSO₄ and FeNPs in MSM was done in the range from 0 to 200 mg l⁻¹ to evaluate and compare the effect on H₂ production. After bacterial inoculation, the bottles were made air tight using glass stoppers and initial anaerobic condition was established by flushing nitrogen gas. The evolved gases from fermenters were collected by the water displacement method under acidic water. The gas collection and analysis were carried out till the gas production was ceased.²⁶

2.6 Analytical methods

The evolved biogas during fermentation was measured through the water displacement method and analysed by gas chromatograph (Nucon GC5765) equipped with Porapak-Q and molecular sieve columns using thermal conductivity detector.²⁷ The operating temperatures of the oven, injector and the detector were set at 60, 80 and 110°C, respectively. Argon served as carrier gas at a flow rate of 20 ml min⁻¹. The concentration of glucose was estimated by the DNS method.²⁸ Cell growth was measured indirectly by measuring the protein content of whole-cell extract at the end of fermentation.²⁹

3. Results and discussion

3.1 Screening and identification of H₂ producing bacterial strain

Purified bacterial isolates were screened for H₂ production by the method described earlier for fermentation. In this

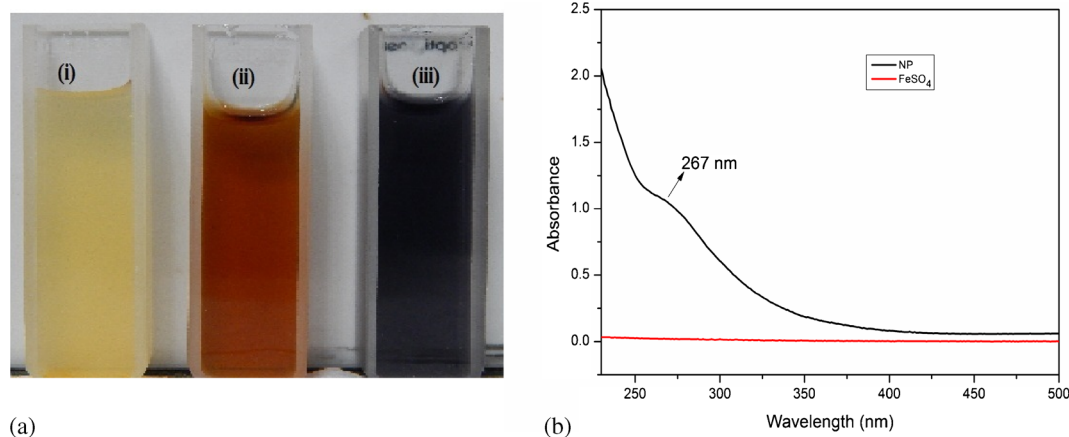


Figure 1. (a) Visible colour change during the synthesis of FeNPs: (i) cuvette containing FeSO_4 solution, (ii) leaf extract and (iii) synthesized colloidal nanomaterial, and (b) UV-visible absorption spectra of synthesized FeNPs and FeSO_4 .

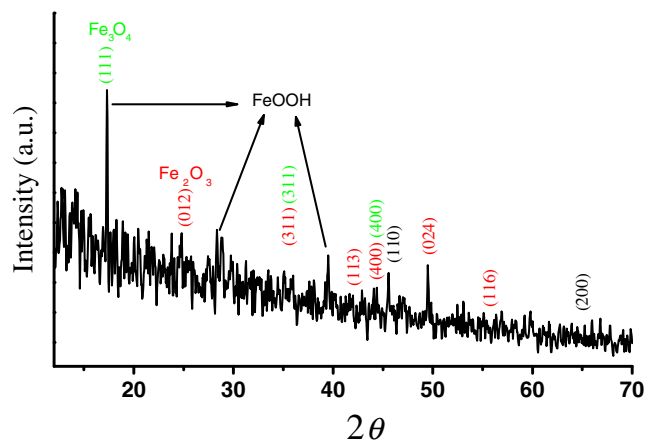


Figure 2. XRD patterns of synthesized FeNPs.

study, strain DH-89 was selected on the basis of maximum biogas production during screening. Identification of the selected strain DH-89 was carried out by 16S rDNA gene sequencing analysis. Finally the sequence was submitted to the NCBI genebank and the strain was named as *Enterobacter cloacae* DH-89 strain (NCBI Accession no. KP723362).

3.2 Characterization of FeNPs

3.2a UV-vis spectroscopy and XRD study: The formation of FeNPs was visually observed by change in colour of the parent precursor and reducing agent mixture (figure 1a) during the synthesis. Figure 1b shows the UV-vis spectra of the aqueous solution of FeSO_4 and the synthesized colloidal material. A prominent difference can be observed in the spectra. The synthesized FeNPs showed absorption from 350 nm with a peak at 267 nm, which is typical for iron nanomaterials. The XRD pattern of the FeNPs (figure 2) is complex and shows several small intensity

diffraction peaks. The prominent diffraction peaks can be indexed to α - FeOOH (JCPDS no. 29-0713), Fe_2O_3 (JCPDS no. 33-0664) and Fe_3O_4 (JCPDS no. 19-0629). Most of the peaks from the phases overlap each other. Since the peaks are not intense, it is difficult to determine the crystallite size using the Scherrer formula.

3.2b SEM, TEM, EDX and FTIR analysis: The morphology of the FeNPs was investigated by electron microscopy. Figure 3a shows the SEM image. It indicated the formation of random shaped aggregates of micron-sized particles. It can be attributed to uncontrolled nanoparticle agglomeration during the synthesis. This fact is clearly evident from the TEM images (figure 3b). It showed agglomerated clusters of nanoparticles of primary sizes in the range of 20–25 nm. Careful observation of the TEM images indicated that the material is porous. The EDX spectrum (figure 4) shows the elemental profile of FeNPs, primarily composed of C, O, S and Fe. The C and O are mainly from the compounds present in plant extracts, while Fe and S from the FeSO_4 precursor. The elemental percentage was observed as 40.54% C, 50.82% O, 2.45% S and 6.19% Fe. The FTIR spectra of the plant extract and FeNPs are shown in figure 5. After comparing the spectra, it was observed that some peaks were shifted and missed. However, few additional peaks were also emerged in case of synthesized FeNPs. The peak positioned at 3428 cm^{-1} in the plant extract was shifted to 3421 cm^{-1} in case of FeNPs, similarly peaks at 1739, 1623, 1392 and 1052 cm^{-1} were shifted to 1732, 1630, 1334 and 1020 cm^{-1} , respectively. The peak at 1456 cm^{-1} in plant extract was found missing in the synthesized FeNPs and emergence of new peaks were observed at 618, 520 and 468 cm^{-1} . The absorption peaks observed in the range of 1800 – 1500 cm^{-1} can be assigned as peak due to double bond stretching between C=O and C=C. The peaks observed in the range of 1400 – 1000 cm^{-1} can be assigned due to stretching vibration of single bond between C–O and N–H. These stretching's arises from the organic moieties of the plant extract present

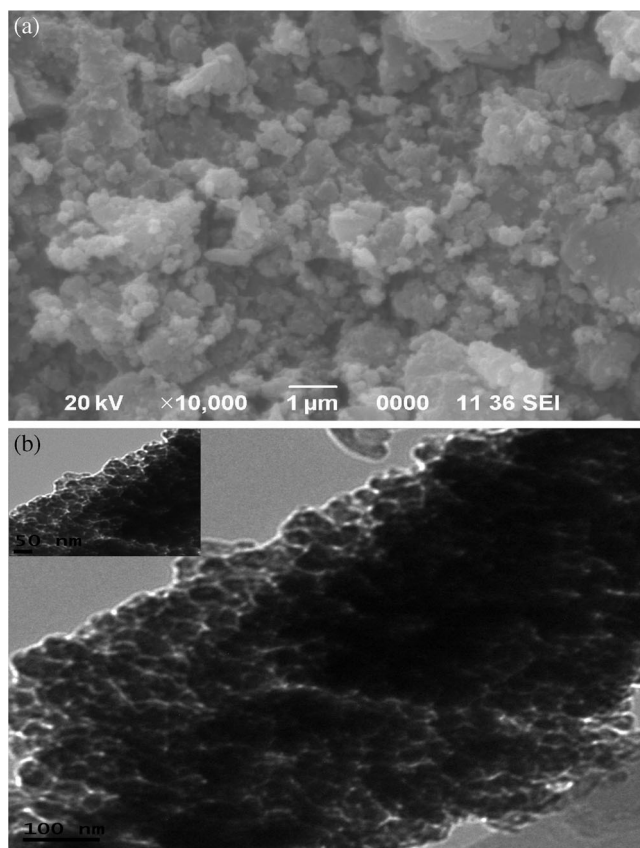


Figure 3. (a) SEM image of the synthesized FeNPs and (b) TEM images of the synthesized FeNPs at bar scale of 50 and 100 nm.

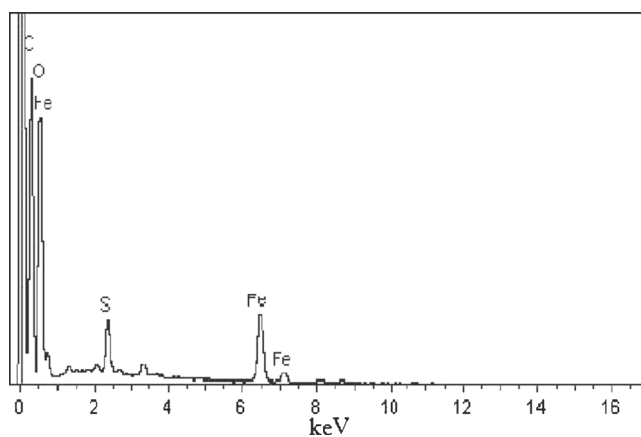


Figure 4. EDX spectrum of the synthesized FeNPs.

in the synthesized FeNPs. The absorption band at 468 and 520 cm^{-1} , in case of FeNPs correspond to the Fe–O stretches of Fe_2O_3 and Fe_3O_4 .^{30,31}

3.3 Effects of FeSO_4 and FeNPs on batch fermentative H_2 production

The effects of FeSO_4 and FeNPs on fermentative H_2 production using *E. cloacae* DH-89 are shown in figure 6. It

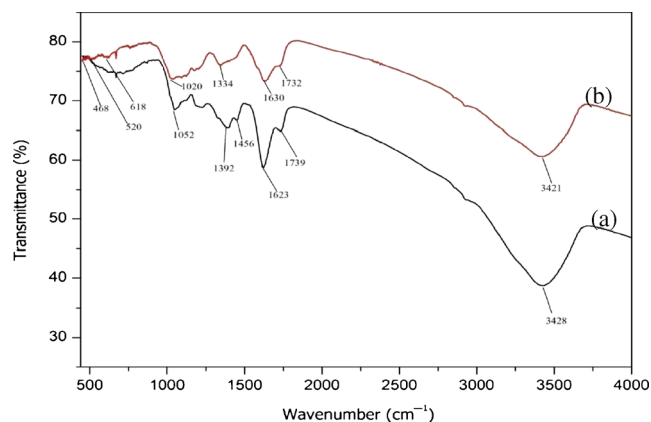


Figure 5. FTIR spectra of (a) *S. cumini* leaf extract and (b) synthesized FeNPs.

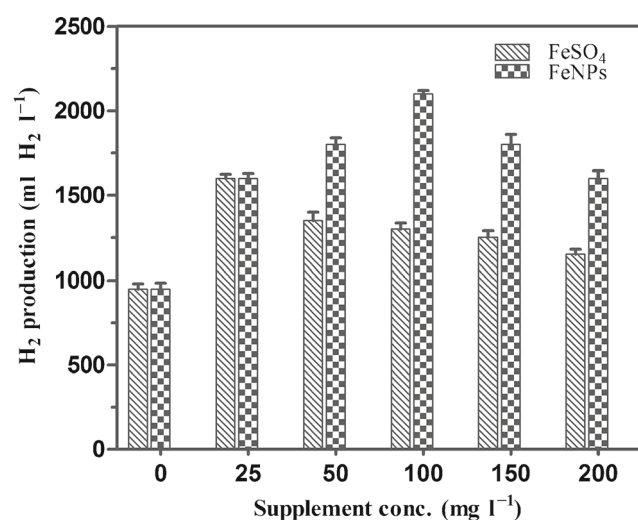


Figure 6. H_2 production at different concentrations of FeSO_4 and FeNPs supplementation.

was found that the H_2 production increases on the addition of both the form of iron. The H_2 production in the control (no supplementation) was 950 ml l^{-1} of fermentation media, which was noticeably increased by supplementation of FeSO_4 at a concentration of 25 mg l^{-1} and then decreased with further increase in the concentration from 50 to 200 mg l^{-1} . With the optimum concentration of 25 mg l^{-1} FeSO_4 supplementation the H_2 production was increased to 1600 ml l^{-1} . The addition of high concentration of FeSO_4 (above 25 mg l^{-1}) suppressed the activity of bacterium, consequently a low H_2 production. A similar kind of result, i.e., the higher concentration of FeSO_4 addition decreases the H_2 production has been reported.¹⁶ However, supplementation of green synthesized FeNPs in place of FeSO_4 was found to further increase the H_2 production. The maximum H_2 production of 2100 ml was achieved at 100 mg l^{-1} FeNPs supplementation and it was decreased by increasing the concentration above 100 mg l^{-1} . The present finding suggests that the supplementation of iron can be replaced by iron-based nanoparticles for better H_2 production. The fact is that,

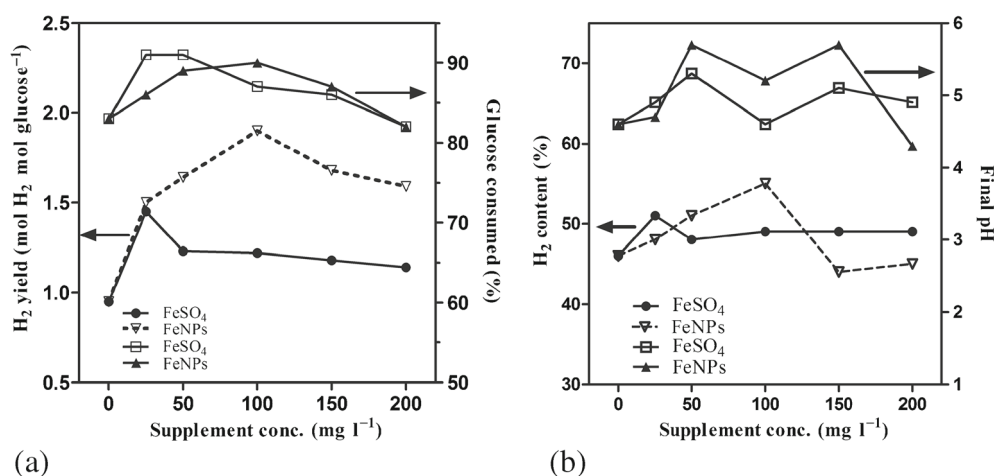


Figure 7. Effects of FeSO₄ and FeNPs supplementation on (a) H₂ yield and glucose consumption and (b) H₂ content and final pH.

Table 1. Effects of FeSO₄ and FeNPs supplementation on cell growth and glucose conversion efficiency.

Supplement type	Control	FeSO ₄					FeNPs				
Conc. (mg l ⁻¹)	0	25	50	100	150	200	25	50	100	150	200
Conversion efficiency ^a (%)	23.8	36.3	30.8	30.5	29.5	28.5	37.5	41	47.5	42	39.8
Cell growth ^b	52.7	55.45	55.45	54.95	54.7	52	53.7	59.45	61.7	56.95	54.7

^aYield of 4 mol H₂ mol glucose⁻¹ is equal to 100%.

^bProtein concentration mg dl⁻¹ of whole-cell extract.

iron is a fundamental component of ferredoxin, which acts as an electron carrier in the hydrogenases, responsible for H₂ production. Hence, at a certain concentration of iron or FeNPs supplementation increases the ferredoxin activity during the fermentation process, which leads to enhanced H₂ production.^{32,33}

3.4 Effects of FeSO₄ and FeNPs on H₂ yield, glucose conversion efficiency and cell growth

The experimental data for the effects of FeSO₄ and FeNPs on H₂ yield and glucose consumption efficiency are shown in figure 7a. Without any iron supplementation, the H₂ yield was 0.95 mol mol⁻¹ glucose utilized, which was increased to 1.45 mol mol⁻¹ glucose utilized with supplementation of 25 mg l⁻¹ FeSO₄. Further it was decreased with increasing the FeSO₄ concentration above 25 mg l⁻¹. However, supplementation of FeNPs results into two-fold increase in the H₂ yield as compared to control. The yield was observed 1.9 mol mol⁻¹ glucose utilized with 100 mg l⁻¹ FeNPs. The percentage of H₂ in the evolved biogas was found maximum (55%) with optimum concentration of FeNPs supplementation (figure 7b). Table 1 shows the experimental data for cell growth and glucose conversion efficiency. The cell growth was observed maximum in case of FeNPs supplementation as compared to control as well as FeSO₄ supplementation. The conversion efficiency (%) is a measure of practical

conversion of glucose to H₂ during fermentation. Theoretically, 1 mol of glucose produce 4 mol of H₂, considered as conversion efficiency value of 100%.³⁴ A two-fold increases in conversion efficiency was observed in FeNPs supplementation as compared to the control. The maximum conversion efficiency of 47.5% was observed at 100 mg l⁻¹ FeNPs supplementation. The results suggest that the FeNPs supplementation also improves the metabolic process of glucose by the bacterial strain resulted into higher carbon flux for H₂ production.

4. Conclusions

In this present study, isolation and identification of soil bacterium *E. cloacae* DH-89 was done for fermentative H₂ production. The strain can produce H₂ by batch fermentation with a yield of 0.95 mol mol⁻¹ glucose consumed. The efficiency of the strain for H₂ production was increased by iron supplementation in the fermentation media. The iron supplementation was done with FeSO₄ and FeNPs. The successfully synthesized FeNPs using aqueous leaf extract of *S. cumini* was characterized and this method can be used for rapid green synthesis of FeNPs. The synthesized FeNPs supplementation with a concentration of 100 mg l⁻¹ was observed to enhance the H₂ production potential of the bacterium by two-fold with increasing glucose consumption rate and cell biomass yield.

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