Supplementation of green-synthesized nickel oxide nanoparticles enhances biohydrogen production of *Klebsilla* sp. using lignocellulosic hydrolysate and elucidation of the regulatory mechanism

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Abstract

Background: In recent years, adding nanoparticles to fermentative hydrogen production system has become an effective way to increase the biohydrogen yield, however, the application of green synthesized nanoparticles to hydrogen production system is rarely studied, even to the interpretation of the regulatory mechanism, there are few reports on the regulation of hydrogen production pathway and related gene expression by addition of nanoparticles. Thus, we herein reported the green synthesis of nickel oxide nanoparticles (NiO-NPs) from *Eichhornia crassipes* (Ec) extract for the first time, and evaluated the regulatory effect of these NPs on fermentative hydrogen production.

Results: Characterization of the Ec-NiO-NPs revealed their spherical shape, small diameter (9.1 ± 2.6 nm) and high purity. The maximum cumulative hydrogen production and hydrogen yield $Y(H_2/S)$ reached 4842.19 ± 23.43 mL/L and 101.45 ± 3.32 mL/g_{substrate}, respectively, in the presence of 20 mg/L Ec-NiO-NP, which were 47.29% and 37.78% higher than the control without NPs addition. Evaluation of glucose and xylose utilization efficiency as well as key node metabolites further established the potential of Ec-NiO-NP to improve the reducing sugar utilization and metabolic flux distribution in hydrogen synthesis pathway. Furthermore, addition of 20 mg/L Ec-NiO-NP resulted in enhanced hydrogenase activity with a maximum increase of 623% comparing to the control, and led to changes in the gene expression of both hydrogenase and formate-hydrogen lyase, which play important roles in promoting hydrogen production at different stages of fermentation.

Conclusions: The results prove that supplementation with green-synthesized Ec-NiO-NP effectively improves fermentative hydrogen production and regulates key node metabolites

alteration and functional gene expression. This study provides a cheap and eco-friendly method to enhance fermentative hydrogen production and new insights to reveal the regulatory mechanism underlying NP mediated increases in biohydrogen synthesis.

Key words: Green synthesis, Fermentative hydrogen production, *Klebsiella* sp., Nickel oxide nanoparticles, Lignocellulosic hydrolysate, Hydrogenase activity

Background

Energy shortage and environmental degradation have become major obstacles to economic development and social progress [1,2]. Hydrogen is a type of green energy known for its high energy storage capacity, clean and renewable production, and its utility as an alternative to traditional fossil fuels [3]. While there have been a number of approaches to its production, lignocellulosic hydrogen production from dark fermentation has risen to prominence because of its various advantages including the use of a wide range of raw materials, low cost, and simple operation [4,5]. The production of lignocellulosic hydrogen is often influenced by many factors, such as inoculum, temperature, pH value, and inoculation size [6,7]. In recent years, some researchers have discovered that supplementation with certain metal additives, especially metal nanoparticles, significantly improve hydrogen production [8-10]. Nickel is an important element that binds to the [Ni-Fe] hydrogenase active site, and has been recently used in the preparation of several different types of nanoparticles for use in dark fermentative hydrogen production systems [11-15]. In addition, these nickel-related nanoparticles also exhibit surface and quantum size effects which promote biohydrogen synthesis [16]. Therefore, supplementation of dark fermentation processes with these metal nanoparticles continues to be a focus area in developing

more efficient biological hydrogen production.

The beginning of this century has seen the wide application of nanomaterials in various fields, which has promoted a major revolution in global industry. To date, most metal nanoparticles have been synthesized using chemical and physical methods. However, there are many drawbacks to these methods, including the use of toxic, expensive chemicals, and flammable organic solvents, and the production of toxic by-products, making it difficult to meet the environmental requirements around improved environmental protection [17,18]. Therefore, recent developments have focused on the green synthesis of metal oxide nanoparticles in an effort to capitalize on their economic and environmental benefits [18]. Plant extracts play an important role in the green synthesis of metal nanoparticles, as they are rich in active compounds, such as phenols, organic acids, flavonoids, and alkaloids [19]. These active substances can act as stabilizers and promoters during their reactions with metal ions, effectively controlling the synthesis of metal nanoparticles ensuring greater consistency in terms of both shape and size [20,21].

Eichhornia crassipes is a widely distributed, invasive aquatic weed with strong adaptability, fast propagation, and wide invasion range [22,23]. At present, *E. crassipes* is seen as a major obstacle in environmental water treatment [24,25]. As biorefinery has become the most important industrial production method in this century, *E. crassipes* can be used as raw material for high-value product production, which not only increases our capacity to dispose of this waste product but can also convert it into high value-added products [25]. *E. crassipes* extracts contain phenols, sterols, flavonoids, terpenoids, anthraquinones, phenylene compounds, and other secondary metabolites, which act as stabilizers and promoters in the green synthesis of metal oxide nanoparticles [26-28]. At present, these extracts have been applied in the green synthesis of some metal nanoparticles.

For example, an *E. crassipes* extract was used as a reducing and stabilizing agent in the synthesis of spherical nano platinum [29]; the extract has also been used as a reducing and capping agent in the synthesis of spherical ZnO nanoparticles [28]. Besides, spherical copper oxide nanoparticles synthesized from *E. crassipes* extract showed good inhibitory effects against various fungal pathogens in plants [30]; and spherical silver nanoparticles synthesized from *E. crassipes* extract exhibited inhibitory effects in cancer cells [31]. To date, there have been no reports describing the green synthesis of NiO nanoparticles using *E. crassipes* extract.

Nickel oxide nanoparticles are rare p-type semiconductors built from transition metal oxides, which demonstrate high stability and a wide band gap [32]. NiO nanoparticles have attracted much attention and have been widely used in the fields of biology, catalysis, food, medicine, energy, artificial intelligence, and information [33]. There have been a limited number of studies evaluating the use of nickel oxide NPs to enhance biohydrogen production [12,33,34]. This includes the study from Mishra et al. (2018) who evaluated the impact of adding NiO and CoO NPs on hydrogen production during the anaerobic digestion of palm oil mill effluent. They found that the addition of these nanoparticles had a significant impact on the system increasing the hydrogen yield by 1.51 fold compared to the control [34]. Gadhe et al. (2015) evaluated the effects of hematite and nickel oxide nanoparticles on biohydrogen production using dairy and complex distillery wastewater, respectively, and discovered that both the NPs exerted some individual effect on the production of biohydrogen but that their combination induced the best hydrogen yield [13,14]. However, in all of these reports the NiO NPs were synthesized via traditional chemical methods and only evaluated hydrogen production from wastewater. To date, there have been no reports describing the use of green-synthesized NiO NPs for hydrogen production from

lignocellulosic hydrolysate.

In this study, we describe the novel production of nickel oxide (NiO) NPs using a green synthesis method. This method used *Eichhornia crassipes* extract as a raw material and the NiO NPs were then used to enhance biohydrogen production from lignocellulosic hydrolysate. To the best of our knowledge, this is the first study to explore and discuss the potential of adding green-synthesized NiO NPs to enhance lignocellulosic hydrogen production. Therefore this study serves to expand our current understanding of the use of green synthesized NiO NPs in lignocellulosic hydrogen production and enhances our understanding of the underlying process and the partner metabolic pathways needed for successful biohydrogen production.

Results

Characterization of the Ec-NiO-NP following green synthesis

The XRD pattern obtained for the synthesized Ec-NiO-NPs is shown in Fig. 1a, which displays strong well defined diffraction peaks, indicating the crystalline nature of the NPs. The sample was confirmed to produce diffraction peaks centered at 37.25°, 43.28°, 62.88°, 75.42°, and 79.41° corresponding to the 111, 200, 220, 311, and 222 crystalline structures, respectively, reflecting the face-centered cubic phase structure of the NiO phase with a lattice parameter of 4.17710 Å, which is in accordance with the standard card of JCPDS NO. 47-1049. No additional peaks were observed in the XRD patterns, indicating the purity of the sample. The crystalline structure of the Ec-NiO-NP was similar to that of the NiO nanorods synthesized using *hoenix dactylifera* (Dates) extract in a previous study [35]. Fig. 1b shows the FTIR spectrum for the green synthesized Ec-NiO-NPs, where the main bands were identified at 426 cm⁻¹ and 926 cm⁻¹ and ascribed to the stretching mode associated with Ni-O vibrations [36,37], confirming the crystalline nature of these

NiO NPs. The band at 3418 cm^{-1} corresponds to the vibration (stretching mode of H–O–H) of absorbed water molecules [37], while the band at 1625 cm^{-1} can be attributed to the bending vibration of water molecules, and the band at 1022 cm^{-1} was assigned to the C–O stretching vibration [38]. In general, the FTIR spectrum was in good agreement with the XRD data, which confirmed the fact that this synthesis produced high quality NiO NPs. The magnetization behavior of the synthesized Ec-NiO-NP was investigated using a sweeping external magnetic field from +30 to -30 kOe (Fig.1c). The Ec-NiO-NP exhibited slight curves in the low magnetic field and a linear portion at higher magnetic force. Saturation magnetization was not reached even at a maximum of 30 kOe, which may confirm the presence of superparamagnetism in these samples. The hysteresis curve from Ec-NiO-NP was similar to that of the NiO NPs synthesized using *Aegle marmelos* leaf extracts [39].

The SEM micrograph (Fig. 1d) shows that the Ec-NiO-NPs were uniformly distributed and adopted slightly agglomerated and spherical shapes. A typical TEM image is shown in Fig. 1e, where the Ec-NiO-NPs were shown to be both cubic and spherical in shape with slight agglomeration. This agglomeration is likely the result of the heat produced during the combustion process and subsequent gas evolution, low density, weak interparticle forces, and magnetic interactions between the particles [40]. Given this, we measured the particle sizes from the TEM image using ImageJ and calculated the distribution frequency for each. The particle size distribution diagram in Fig. 1f shows that the Ec-NiO-NPs presented with a small diameter of approximately $9.1 \pm 2.6\text{ nm}$. The synthesized Ec-NiO-NP possesses features such as small particle size, high dispersity, and large specific surface area suggesting that they may be a promising material for biological application.

Ec-NiO-NP supplementation enhanced the hydrogen production

To investigate the effect of green synthesized Ec-NiO-NP on hydrogen production, we detected and calculated the daily hydrogen and cumulative hydrogen production (Fig. 2). Fig. 2a illustrates that supplementation with Ec-NiO-NP at different concentrations exerted varying effects on daily hydrogen production. The peak values for daily hydrogen production were obtained at 24 h following supplementation with reasonably low concentrations (5, 10, and 20 mg/L) of Ec-NiO-NP. The peak value obtained at 20 mg/L was 18.7% higher than that of the control. Conversely, when the concentration reached 30 mg/L, the peak value was obtained at 48 h. Notably, every Ec-NiO-NP concentration enhanced the daily hydrogen production at 48 h with these improvements ranging from 173.8% to 302.6%. The daily hydrogen production showed a declining trend after 72 h of fermentation and ultimately produced hydrogen values that were close to or lower than the control despite changes in Ec-NiO-NP concentration. Only the 20 mg/L treatment retained its enhanced hydrogen production between 96 and 120 h when compared to the control. This suggests that only supplementation with 20 mg/L Ec-NiO-NP favors hydrogen production over the whole fermentation period. This was supported by the cumulative hydrogen production values that show the group treated with 20 mg/L Ec-NiO-NP produced 4842.19 ± 23.43 mL/L hydrogen over a 120 h period (Fig. 2b), reflecting a $47.29\% \pm 1.15\%$ improvement (R_{im}) (Table 1) when compared to the control. The other Ec-NiO-NP treatments also enhanced cumulative hydrogen production and resulted in above 10% improvement in their hydrogen yield (R_{im}) (Table 1) when compared to the control, confirming the significant positive effects of Ec-NiO-NP on hydrogen production, which further supports its beneficial biological effects in biohydrogen production. The cumulative hydrogen production described here was in good

agreement with its dynamically fitted results produced using a modified Gompertz model, which suggested that Ec-NiO-NP supplementation would increase hydrogen production potential (P) and hydrogen production rate (R_m) (Table 1). In addition, the optimal P and R_m values were obtained using 20 mg/L, further supporting our hypothesis that green-synthesized Ec-NiO-NP is a good accelerator for hydrogen production. Moreover, lag phase times (λ) in all Ec-NiO-NP treated samples increased compared to the control treatment, likely due to the disturbance effects of these additives on microbial metabolism, as explained by Engliman et al. (2017) [41].

Ec-NiO-NP supplementation improved reducing sugar utilization

Klebsiella sp. WL1316 has been reported to be an efficient hydrogen-producing bacterium with the ability to produce hydrogen gas via the fermentation of lignocellulosic hydrolysate [6], which intrinsically uses both glucose and xylose as substrates. Fig.3a shows that the addition of Ec-NiO-NP improved both glucose and xylose utilization efficiencies with those improvements increasing use by at least 4% or 6%, respectively. Notably, an optimum glucose utilization efficiency of 94.7% and xylose utilization efficiency of 96.9% were obtained when the system was supplemented with 20 mg/L Ec-NiO-NP. The hydrogen promoting effect of the Ec-NiO-NPs was similar to that described for ferrihydrite, where the addition of these ferrous NPs increased glucose conversion in hydrogen producing systems [42]. Our Ec-NiO-NPs were unique however in their ability to increase the use of both glucose and xylose, which is of vital importance for lignocellulosic hydrogen production.

On this basis, we calculated the hydrogen yield based on the reducing sugar consumption ($Y(H_2/S)$) (Fig. 3b), and showed that the highest $Y(H_2/S)$ yield, 101.45 ± 3.32 mL/g_{substrate}, was also obtained at 20 mg/L and increased by 37.78% compared to the control. This suggests that

more reducing sugars were converted to H₂ gas at this concentration, adding more support to our hypothesis that 20 mg/L is the optimal concentration for Ec-NiO-NP supplementation allowing maximal hydrogen production and substrate conversion. On the other hand, the values for Y(Δ OD/S), was higher in all of the supplemented treatments compared to the control, indicating that the addition of Ec-NiO-NP effectively promotes bacterial growth. In addition, the maximum Y(Δ OD/S) was obtained at 30 mg/L, which illustrated that a relatively high concentration of Ec-NiO-NP might facilitate a higher reducing sugar conversion rate encouraging even more bacterial growth. Taking into account the high hydrogen yield, 20 mg/L was determined to be the optimum concentration of Ec-NiO-NP for this application.

Ec-NiO-NP supplementation caused alteration of metabolites profile

As a facultative anaerobic bacterium, *Klebsiella* sp. typically generates H₂ gas via a mixed acid fermentation pathway, metabolites such as succinic, lactic, acetic and formic acids, and ethanol, are regarded as the key node metabolites and indirect measures of hydrogen synthesis [43-45]. Some studies have reported that the addition of nanoparticles can affect metabolic pathways and cause changes in metabolite profiles [8,41,42]. Fig. 4 shows a time course evaluation of the key node metabolite profiles in the presence of 20 mg/L Ec-NiO-NP. Pyruvic acid is an important intermediate in the initiation of the mixed acid fermentation pathway and is a basic requirement of H₂ gas generation. The dynamic concentrations of pyruvic acid during the fermentation stage are illustrated in Fig. 4a; the addition of Ec-NiO-NP increased the pyruvic acid concentration by 24% at 24 h compared to the control, which might benefit the metabolic flux in pyruvate-initiated metabolic pathways. The addition of Ec-NiO-NP caused a slight decrease in pyruvate concentration compared to the control after 48 h, indicating that Ec-NiO-NP accelerated the

conversion of pyruvic acid rather than changing the pathway initiated by its presence. Formic acid is split into H_2 and CO_2 and is critical to hydrogen production in facultative anaerobes [46,47]. Fig. 4b shows that the addition of Ec-NiO-NP causes a decrease in formate concentration between 24 and 48 h, and then an increase in this substrate between 72 and 96 h, and finally a decrease at 120 h when compared to the control. This decrease in formic acid concentration might lead to the generation of H_2 gas. In addition, these changes in formic acid concentration coincided with the daily hydrogen production trend (Fig. 2a).

From the perspective of the biohydrogen synthesis pathway, succinic, lactic and acetic acid and ethanol are the key node metabolites for competitive pathways, so understanding the dynamic concentrations of these metabolites is helpful in effectively regulating the biohydrogen synthesis process. Fig. 4c shows that the addition of Ec-NiO-NP led to an increase in succinic acid at 24 h, and then a sharp decrease after 48 h reaching a stable concentration with minimal variation over the remaining time frame when compared to the control. This indicates that the addition of Ec-NiO-NP causes the succinic acid production branch to compete for the metabolic flux distribution of biohydrogen synthesis during the early stages of fermentation, while this disturbance is reduced during the mid-to-late stages of this process. In addition, when we compared the accumulation of lactic and acetic acids in each group we noted only a minor change in these metabolites following the addition of Ec-NiO-NP (Fig. 4d and e), while there was a distinct change in ethanol, where ethanol concentrations were decreased following the addition of Ec-NiO-NP. This decrease was most significant at 120 h (Fig. 4f), suggesting that the addition of Ec-NiO-NP decreases ethanol production increasing the metabolic flux in the biohydrogen synthesis pathway.

Evaluation of hydrogenase activity and functional gene expression following the addition of Ec-NiO-NP

Hydrogenase plays an important catalytic role in regulating fermentative hydrogen production by hydrogen-producing bacteria [48,49]. In this study, we evaluated the hydrogenase activity of the batch fermentation over a 120 h period in the presence of 20 mg/L Ec-NiO-NP (Fig. 5). The hydrogenase activity at 20 mg/L Ec-NiO-NP was similar to that of the control, with a peak value at 24 h. The peak hydrogenase activity obtained at 24 h was in good agreement with the daily hydrogen production values for each treatment group (Fig. 2a). Moreover, the hydrogenase activity was enhanced following the addition of Ec-NiO-NP with increased activity between 11% and 623%. The maximum enhancement was observed at 48 h, and the increase in activity remained above 150% even between 96 and 120 h, which was consistent with the enhanced daily hydrogen production exhibited by these treatment groups (Fig. 2a). In summary, hydrogenase activity should be considered as a promising accelerator in biohydrogen production and evaluated as a key driver in increasing hydrogen yields.

Hydrogen-producing bacteria produce biohydrogen via several mechanisms including, but not limited to hydrogenase catalysis. These pathways include the formate-hydrogen splitting pathway, where catalysis by formate-hydrogen lyase is particularly important. To understand the regulatory effect of Ec-NiO-NP on fermentative hydrogen production, the relative expression of four hydrogenase and three formate-hydrogen lyase genes during the fermentation process were evaluated by RT-qPCR. Fig. 6 shows that the addition of Ec-NiO-NP enhanced the expression levels of most of the functional genes with $2^{-\Delta\Delta C_T} > 1$ at 24 h, when compared to the control. This was most obvious in hydrogenase maturation factor *hypD*, which demonstrated a 21.79 ± 0.23 fold

change at 24 h. The hydrogenase maturation factors have been reported to be responsible for the synthesis of hydrogenase precursors and facilitate nickel insertion into hydrogenase, which is helpful for hydrogenase gene expression [50]. In this study, the increased expression of the *hypD* gene might promote other hydrogenase related gene expression, and it should also be noted that the hydrogenase small subunit and hydrogenase expression *hypA* genes, also underwent a significant increase, more than 3 fold, in response to this supplementation. Increased expression of these hydrogenase genes may result in increased hydrogenase activity and daily hydrogen production, with all three peaking at 24 h. We also noted increases in the expression of the formate-hydrogen lyase genes in response to Ec-NiO-NP at 24 h, especially the formate-hydrogen lyase transcriptional activator gene which demonstrated a 7.79 ± 1.51 fold change, suggesting that this enzyme is also linked to the increased hydrogen production described in this study. Some studies have reported that formate-hydrogen lyase plays an important role in biohydrogen production [47], and overexpression of the formate-hydrogen lyase transcriptional activator gene could improve hydrogen production during fermentation [51,52]. Obviously, the expression of formate-hydrogen lyase-related genes is crucial for hydrogen production. Our findings suggest that the expression of the formate-hydrogen lyase transcriptional activator gene was maintained at a high level over the entire course of fermentation, reaching the highest fold change, 13.98 ± 2.22 , at 96 h, suggesting that these changes may contribute to the enhanced hydrogen production observed in the late fermentation stages of these experiments. In addition, formate-hydrogen lyase subunit 6 also showed an increase in expression levels at 48h and 96 h, which may contribute to the increase in hydrogen yield at the same time points. By comparison, the hydrogenase-related genes presented with a changing expression profile at different fermentation times, which might

be related to the regulation of hydrogenase maturation factor. In general, the expression of all these functional genes plays an important role in promoting hydrogen production at different fermentation stages.

Discussion

In the present study, we successfully synthesized NiO-NP using *E. crassipes* extract, which was supplemented into the fermentative hydrogen production system of *Klebsiella* sp. WL1316 and distinctly enhanced the fermentative hydrogen production. Table S1 summarizes the green-synthesized nanoparticles using *E. crassipes* extract in recent years, it shows that the iron related NPs were the most synthesized nano materials, and applied in anti-microorganism and pollutant removal fields [27, 54-57]; while some NPs were synthesized without specific application [28,29,53]; up to now, the synthesis of Ec-NiO-NP in the present study was the first report of the NiO-NP synthesized using *E. crassipes* extract, which was also the first attempt to apply the *E. crassipes* -based NPs into fermentative hydrogen production system.

The remarkable effects of the green-synthesized Ec-NiO-NP on fermentative hydrogen production included the enhanced daily and cumulative hydrogen production, and increased hydrogen production potential (P) and hydrogen production rate (R_m). The existing literature suggests that nickel and its oxide nanoparticles have been used to enhance hydrogen production in both pure hydrogen-producing microbial cultures and anaerobic mixed consortia systems (Table 2). However, the Ni-related NPs applied in these studies were synthesized via chemical methods, and no study has described the application of green-synthesized Ni-related NPs in fermentative hydrogen production. Several studies have used green-synthesized Fe-related NPs in hydrogen production systems and shown that these NPs exerted positive effects on hydrogen production

[59-61]. A similar effect was also recorded for the green-synthesized Ec-NiO-NPs in this study. Thus, this study is the first attempt to use green-synthesized Ni-related NPs for fermentative hydrogen production. Moreover, when we compared these technologies (Table 2), the application of Ec-NiO-NP produced higher hydrogen yield with lower dose and smaller particle size. Given this data, it may be possible that the unique effect experienced in this system was a result of the smaller particles. Nevertheless, it is difficult to evaluate these effects as there are also significant differences in the inocula and cultivation conditions used in these studies. We did however obtain a higher optimum hydrogen yield in this study when compared to our previous study [8] using chemically synthesized Ni⁰ NPs.

Furthermore, our findings suggest that the addition of Ec-NiO-NP not only promoted the expression of hydrogenase-related genes, but also promoted the expression of formate-hydrogen lyase-related genes, all of which play a positive role in promoting the synthesis of biohydrogen. In recent years, several groups have studied the expression of hydrogenase genes following the addition of nanoparticles in an effort to understand the underlying mechanism facilitating NP mediated increases in hydrogen production [62,63]. However, these studies only focused on the analysis of single hydrogenase gene. In fact, hydrogen-producing bacteria possess diverse hydrogenase-related genes; therefore, the analysis of a single gene will also be incapable of explaining the full regulatory mechanism. For example, *Klebsiella* sp.WL1316 processes more than ten hydrogenase-related genes within its genome each likely to perform some specialized task [64]. Here we used this genome information to develop a multigene RT-qPCR assay and found that the addition of Ec-NiO-NP exerted a range of regulatory effects on the expression of different hydrogenase-and formate-hydrogen lyase-related genes. This data serves to broaden the range of

genes evaluated in hydrogen production systems and suggests that both hydrogenase and formate-hydrogen lyase-related gene expression is important to improve overall hydrogen production. However, there is no literature describing the effects of NPs on hydrogen production via its regulation of the formate-hydrogen lyase genes, although there are some studies describing the effect of increased hydrogenase gene expression on the expression of formate-hydrogen lyase gene [65]. There is still some work to be done to identify and describe the nuances of this mechanism. In this study, we were able to identify some of the regulatory effects of Ec-NiO-NP supplementation on both hydrogenase-and formate-hydrogen lyase-related gene expression as well as its effects on hydrogen production. These observations may be beneficial to future investigations designed to reveal the regulatory mechanism underlying NP mediated increases in biohydrogen synthesis.

Conclusions

This study broadens our knowledge of green synthesized Ec-NiO-NP as H₂-generation-accelerant and provides a cheap and eco-friendly method to enhance fermentative hydrogen production. The maximum cumulative hydrogen production and hydrogen yield Y(H₂/S) increased by 47.29% and 37.78%, respectively, in the presence of 20 mg/L Ec-NiO-NP, when compared to the control. Meanwhile, supplementation with Ec-NiO-NP also led to enhanced glucose and xylose utilization and altered the metabolite profile of these reactions to be more conducive to increased metabolic flux in the biohydrogen synthesis pathway. In addition, the enhanced hydrogenase activity and improved hydrogenase and formate-hydrogen lyase-related gene expression resulting from Ec-NiO-NP supplementation are likely to be at least partially responsible for the increased hydrogen production described in these systems. Thus, this study provides new insights to reveal

the regulatory mechanism underlying NP mediated increases in biohydrogen synthesis.

Methods

Inoculum and Medium

Klebsiella sp. WL1316 was used as the inoculum, and was previously reported to possess efficient hydrogen production from lignocellulosic hydrolysates [6]. These cells were cultured in the following medium: 10 g/L xylose, 10 g/L glucose, 5 g/L beef exact, 10 g/L peptone, 5 g/L NaCl, 0.5 g/L KH_2PO_4 and 1 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ [6].

Green synthesis and characterization of NiO NPs

About 5 g of fresh *E. crassipes* stems and leaves were collected, rinsed completely with distilled water, sliced into pieces, ground into paste, added to 100 mL of distilled water, boiled for 20 min, and filtered to obtain the pure *E. crassipes* extract used in the next steps. About 50 mL of 0.1 mol/L NiCl_2 was added to 50 mL of *E. crassipes* extract under constant and continuous stirring over a 30 min period. The resulting precipitate was collected and centrifuged at 4000 rpm, and then washed with distilled water and ethanol three times to remove any remaining residue. This product was then annealed at 400 °C for 6 h in a muffle furnace, producing a black powder and naming Ec-NiO-NP.

The crystal structure of green-synthesized Ec-NiO-NP was examined on a Bruker AXS D8 diffractometer at $2\theta = 10^\circ - 80^\circ$ using Cu $K\alpha$ radiation at $\lambda = 0.154$ nm. The functional groups were identified using a Fourier infrared spectrometer (Nicolet IS10, USA) in the range of 4000–400 cm^{-1} . The surface morphology was determined using a Hitachi SU8020 microscope and transmission electron microscopy (TEM) performed on a Tecnai G2 F30 S-TWIN electron microscope with an accelerating voltage of 200 kV. The size distribution of the Ec-NiO-NPs was

determined using the TEM micrographs and analyzed using image processing software ImageJ 1.52, while the particle size was calculated using Origin 8.0. The room-temperature magnetization of Ec-NiO-NP was detected using a SQUID-VSM, MPMS-3 vibrating sample magnetometer.

Preparation of rice straw hydrolysate

Dried rice straw was cut short, crushed with a pulverizer, and then sifted using a 20-mesh sieve to obtain the straw powder, which was mixed with 4% (v/v) sulfuric acid in a solid-liquid ratio of 1:5, and hydrolyzed at 121°C for 30 min. After hydrolysis, the mixture was filtered and centrifuged at 4,000 rpm, the supernatant was collected, detoxified and decolorized using a calcium hydroxide solution and macroporous resin, as reported in our previous study [66,67], resulting in a product with main reducing sugar components were glucose and xylose, while other components (eg. arabinose, galactose) were barely detected. Finally, the concentration of the reducing sugars in the lignocellulosic hydrolysate was adjusted to 50 g/L before use in the fermentative hydrogen production experiment.

Fermentative hydrogen production procedure

The batch fermentations used to produce hydrogen were carried out in 500 mL self-designed fermenters containing 350 mL of medium with the following composition: 1 L rice straw hydrolysate (reducing sugar concentration was 50 g/L), 5 g/L beef extract, 10 g/L peptone, 5 g/L NaCl, 0.5 g/L KH_2PO_4 , and 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Fermentations were then supplemented with 0, 5, 10, 20, or 30 mg/L Ec-NiO-NP and the effects of these NiO NPs on the hydrogen production process were monitored over the full 120 h fermentation process. All experiments were conducted in triplicate. The volume of fermentative hydrogen was measured three times every 24 h, which were added together and defined as daily hydrogen production. The hydrogen production volume

measured at each fermentation time point was accumulated and converted into the corresponding cumulative hydrogen production. In addition, the optical density at 600 nm (OD_{600}) of the fermentation broth was also examined every 24 h on a 7230G UV–visible spectrophotometer to reveal bacterial growth. The concentrations of glucose and xylose were also monitored during this period to facilitate the evaluation of reducing sugar utilization during fermentation. The glucose concentration was measured using a glucose detection kit (Comin, China) which relied on glucose oxidation and colorimetry to produce a readout on the 7230G UV–visible spectrophotometer. Xylose concentration was evaluated using a xylose detection kit (ZZStandard, China) via the dehydrogenation of xylose and measurement of NADH content on a Multiskan FC automatic microplate reader.

Hydrogenase activity assay

Hydrogenase activity of the purified protein samples from *Klebsiella* sp. WL1316 was determined using the production of H_2 via its reaction with methyl viologen (MV) and sodium dithionite ($Na_2S_2O_4$) (NaDT) where reduced MV is able to transfer electrons onto metal-containing proteins [68]. The assay procedure was as follows: the purified protein was added to a mixture of buffer A1 (1 mL, 20 mM Tris-HCl, pH 8, 20 mM NaCl, 5% glycerol, 1 mM DTT, 1 mM EDTA) containing 5 mM MV and 50 mM $Na_2S_2O_4$, and incubated for 15 min at room temperature [69]. The concentration of H_2 was measured with a handled hydrogen detector (KP810H20, China) and the protein of *Klebsiella* sp. WL1316 was extracted and purified using a Bacterial Protein Extraction kit (Sangon Biotech, China), as per the manufacturer's instructions.

Metabolites monitoring and analysis

The fermentation broth was sampled every 24 h and centrifuged at 8000 rpm for 10 min to obtain

the supernatant, which was then filtered through 0.22 μm membranes and used to test for changes in the concentrations of node metabolites present in the metabolic network and related to hydrogen gas generation. Succinic, pyruvic, lactic, acetic, formic acids, and ethanol, which are typically present in mixed acid fermentation pathway, were all measured using their relevant detection kits (ZZStandard, China) based on the reaction of dehydrogenation of succinic, pyruvic, lactic, acetic, formic acids, and ethanol, and the amount of NADH produced was then measured using a Multiskan FC automatic microplate reader according to the manufacturer's instructions.

Real-Time Quantitative PCR (RT-qPCR) analysis

The relative expression of four hydrogenase genes and three formate-hydrogen lyase genes associated with hydrogen synthesis were assayed by RT-qPCR. The primers for these genes were designed using Beacon Designer software version 7.0, according to the genomic information for *Klebsiella* sp. WL1316 [64], and the sequencing data had been deposited into the NCBI database (BioProject accession number PRJNA611005). The hydrogenase- and formate-hydrogen lyase-related genes in the genome of *Klebsiella* sp. WL1316 are illustrated in Fig. S1, and the designed primers are listed in Table S2.

Total RNA from *Klebsiella* sp. WL1316 was extracted and purified using a Bacterial total RNA rapid extraction kit (Sangon). Single-stranded cDNA was reverse-transcribed using PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Japan). TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) reagent (TaKaRa, Japan) was used for the RT-qPCR analysis using the Bio-Rad CFX Connect Real-Time PCR Detection System. Each reaction was comprised of 2X TB Green Premix Ex Taq II (10 μL), single-stranded cDNA (2 μL), and 0.4 μM of forward and reverse primers in a final volume of 20 μL , following an initial denaturation at 95°C for 30 s,

samples were subjected to 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 30 s, and extension at 72°C for 10 s. RT-qPCR assays were performed in three replicates. Relative gene expression was measured using the $2^{-\Delta\Delta C_T}$ method [70], and the fold change in the target gene was normalized against the 16S rDNA gene expression in the control [71]. Where $\Delta\Delta C_T = (C_{T, \text{Target}} - C_{T, 16S \text{ rDNA}})_{\text{Treatment}} - (C_{T, \text{Target}} - C_{T, 16S \text{ rDNA}})_{\text{Control}}$, 16S rDNA was used as the internal control gene. Treatment was used to describe samples treated with 20 mg/L NiO NPs, and Control was used to describe samples who received no NPs (0 mg/L).

Calculation and kinetic analysis

The modified Gompertz mode was used to fit the cumulative hydrogen production for each batch using equation (1) [72]:

$$H = P \times \exp \left\{ -\exp \left[\frac{R_m \times e}{P} (\lambda - t) + 1 \right] \right\} \quad (1)$$

where H is the cumulative hydrogen production (mL), P is the hydrogen production potential (mL), R_m is the maximum hydrogen production rate (mL/(L·h)), $e = 2.71828$, λ is the lag phase time (h), and t is the culture time (h). The kinetic parameters (P , R_m , and λ) were estimated using Sigmaplot software 12.0, and the correlation coefficient R^2 for each treatment was also calculated.

The glucose utilization efficiency (R_{glucose}), xylose utilization efficiency (R_{xylose}), hydrogen yield based on reducing sugar consumption ($Y(H_2/S)$), the increment in OD₆₀₀ (ΔOD) based on the reducing sugar consumption ($Y(\Delta OD/S)$), and the improvement in hydrogen production with each NP supplementation (R_{im}) were calculated and analyzed using equations (2) – (6):

$$R_{\text{glucose}} = \frac{(C_{\text{glucose},i} - C_{\text{glucose},f})}{C_{\text{glucose},i}} \times 100\% \quad (2)$$

$$R_{\text{xylose}} = \frac{(C_{\text{xylose},i} - C_{\text{xylose},f})}{C_{\text{xylose},i}} \times 100\% \quad (3)$$

$$Y(H_2 / S) = \frac{V_{H_2, cumulative, 120h}}{(C_{glucose, i} + C_{xylose, i} - C_{glucose, f} - C_{xylose, f})} \quad (4)$$

$$Y(\Delta OD / S) = \frac{(OD_{600, f} - OD_{600, i})}{(C_{glucose, i} + C_{xylose, i} - C_{glucose, f} - C_{xylose, f})} \quad (5)$$

$$R_{im} = \frac{(P_{cut} - P_{cuc})}{P_{cuc}} \times 100\% \quad (6)$$

where $C_{glucose, i}$ is the initial concentration of glucose, $C_{glucose, f}$ is the final concentration of glucose, $C_{xylose, i}$ is the initial concentration of xylose, $C_{xylose, f}$ is the final concentration of glucose, $V_{H_2, cumulative, 120h}$ represents the cumulative hydrogen production at 120 h, $OD_{600, i}$ is the initial optical density at 600 nm of each experiment and $OD_{600, f}$ is the final optical density at 600 nm. P_{cut} represents the final cumulative hydrogen production following NP supplementation and P_{cuc} is the final cumulative hydrogen production following control treatment (0 mg/L). The equation (6) was also described by Li et al. (2020) [8].

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Authors' contributions

QZ proposed, designed, and supervised the study and revised the manuscript. SX designed and performed most of the experiments, and drafted the manuscript. YL co-supervised the study and revised the manuscript. PD carried out the green synthesis and characterization experiments and edited manuscript. YZ did the hydrogen fermentation experiments and carried out the RT-qPCR assays. PZ examined the hydrogen volume, hydrogenase activity and concentrations of the key node metabolites. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Additional file

Additional file 1: **Table S1.** Green synthesis of nanoparticles using aqueous extracts of *Eichhornia crassipes*. **Table S2.** Details of the primers used in the RT-qPCR assays. **Figure S1.** Hydrogenase- and formate-hydrogen lyase-related genes in the genome of *Klebsiella* sp. WL1316. **a** The direction and relative position of hydrogenase-related gene cluster; **b** The direction and relative position of formate-hydrogen lyase-related gene cluster; **c** The distributions of gene clusters of hydrogenase and formate-hydrogen lyase in the genome.

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Tables

Table 1 Kinetic parameters describing the cumulative hydrogen production and improved hydrogen yield following supplementation with Ec-NiO-NP at various concentrations.

Conc. of nanoparticles (mg/L)	Dynamically fitted parameters					Improvement of hydrogen yield (R_{im} , %)
	P (mL/L)	R_m (mL/(L·h))	λ (h)	R^2		
CK	0	3290.81	52.73	2.14	0.9645	—
NiO NPs	5	3682.73	75.50	2.36	0.9932	13.78 ± 1.23
	10	3812.10	77.87	4.18	0.9961	17.93 ± 1.71
	20	4623.38	97.84	3.99	0.9958	47.29 ± 1.15
	30	3545.42	89.88	8.23	0.9982	10.03 ± 1.15

Table 2 Application of Nickel and its oxide nanoparticles in different fermentative hydrogen production systems.

Inoculum	Substrate	Nanoparticles			Optimum hydrogen yield	References
		Type	Concentration	Size		
Anaerobic sludge	Glucose	Ni ⁰ NPs	2.5 mg / L	—	270mL/g glucose	[58]
<i>Klebsiella</i> sp. WL1316	Cotton stalk hydrolysate	Ni ⁰ NPs	30 mg/L	50 nm	92.82± 0.25mL/g reducing sugar	[8]
Mixed culture bacteria	Industrial wastewater	NiONPs	20 mg / L	—	130±6.0 mL / L	[12]
Anaerobic sludge	Dairy wastewater	NiONPs	10 mg/L	—	15.7 mmol /gCOD	[13]
Anaerobic sludge	Distillery wastewater	NiONPs	5 mg/L	23 nm	8.83 mmol /gCOD	[14]
<i>Bacillus anthracis</i> PUNAJAN 1	Palm oil mill effluent	NiONPs	1.5 mg/L	14 nm	0.56 LH ₂ /gCOD	[34]
Anaerobic sludge	Glucose	NiNPs	5.67mg/L	13.64 nm	4400mL	[15]
Anaerobic sludge	Industrial wastewater	NiNPs	60 mg/L	60nm	24.73±1.12 mL/g	[11]
<i>Klebsiella</i> sp. WL1316	Rice straw hydrolysate	NiO NPs	20 mg/L	9.1±2.6 nm	101.45 ± 3.32 mL/g _{substrate}	This study

Figures

Fig. 1 Characterization of the green-synthesized NiO NPs. **a**: XRD pattern; **b**: FTIR analysis; **c**: Magnetization curve; **d**: SEM micrograph; **e**: TEM micrograph; **f**: Particle size distribution.

Fig. 2 The effect of green-synthesized Ec-NiO-NP on daily hydrogen production (**a**) and cumulative hydrogen production (**b**).

Fig. 3 The effect of Ec-NiO-NP on reducing sugar utilization (**a**) and the hydrogen yield and increment of OD₆₀₀ based on the reducing sugar consumption (**b**).

Fig. 4 Time course of key node metabolites profile in the presence of 20 mg/L Ec-NiO-NP. **a**: Pyruvic acid; **b**: Formic acid; **c**: Succinic acid; **d**: Lactic acid; **e**: Acetic acid; **f**: Ethanol.

Fig. 5 Time-dependent hydrogenase activity and the influence of 20 mg/L Ec-NiO-NP.

Fig. 6 Time-dependent changes in the relative expression of hydrogenase and formate-hydrogen lyase-related genes following supplementation with 20 mg/L Ec-NiO-NP. All experiments were performed in triplicate. The y-axis represents the functional genes while the x-axis represents the relative expression level from the RT-qPCR analysis.