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ORIGINAL PAPER

Influence of different factors on the nitrogenase activity of the engineered *Escherichia coli* 78-7

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Abstract The engineered *Escherichia coli* 78-7 is a derivative of *E. coli* JM109 carrying a nitrogen fixation (*nif*) gene cluster composed of nine genes (*nifB, nifH, nifD, nifK, nifE, nifN, nifX, hesA and nifV*) and its own σ^{70}-dependent *nif* promoter from a gram-positive bacterium *Paenibacillus* sp. WLY78. The physiological and biochemical characteristics of the engineered *E. coli* 78-7 were analyzed by using Biolog GEN III MicroPlate, with *E. coli* JM109 and JM109/pHY300PLK (*E. coli* JM109 carrying empty vector) as controls. Analysis of 94 phenotypic tests: 71 carbon source utilization assays and 23 chemical sensitivity tests showed that the engineered *E. coli* 78-7, *E. coli* JM109 and JM109/pHY300PLK gave similar patterns of utilization of various substrates as carbon and energy sources. Furthermore, the effect of carbon source, nitrogen source, culture temperature on the nitrogenase activity of the engineered *E. coli* 78-7 was investigated. Our study demonstrates that the *nif* capacity of *E. coli* 78-7 was affected significantly by the different culture condition. The significant nitrogenase activity of *E. coli* 78-7 were obtained when cells were cultivated in the medium containing 4 g/l glucose (carbon source) and 2 mM glutamate (nitrogen source) and at 30 °C.

Keywords Engineered *Escherichia coli* · Nitrogenase activity · Nitrogen fixation (*nif*) gene cluster · Physiological and biochemical characteristics

Introduction

Biological nitrogen fixation, the conversion of atmospheric nitrogen into ammonia by nitrogen-fixation organism, is an energy-intensive process. The energy requirement for nitrogenase synthesis could be met by an exogenously supplied carbon source. The utilization of various carbon sources as energy sources for N_2 fixation has been studied in different nitrogen-fixing organisms, and the energy source availability significantly differences (Bhargava et al.2013; Haahtela et al.1983; Privalle and Burris 1984; Zuberer and Silver 1978; Selao et al. 2011).

Lots of studies have shown that ammonia inhibits synthesis and activity of nitrogenase in almost all of nitrogen-fixing organisms (Dixon and Kahn 2004; Huergo et al. 2012). In addition, some studies suggested that a variety of inorganic and organic nitrogen compounds such as amino acids and amino acid analogs had different influence on nitrogenase synthesis and activity in several nitrogen-fixing bacteria and cyanobacteria (Rawson 1985; Thomas et al. 1982; Thiel and Leone 1986; Helber et al. 1988; Nygren et al. 2000; Khumanthem et al. 2007).

Temperature sensitivity of nitrogen fixation had been reported (Brooks et al. 1984; Pankhurst and Craig. 1978; Zhu and Brill 1981). The high temperature repressed the transcription of nitrogenase. However, low temperature

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influenced the organism growth and nitrogenase synthesis.

Previous studies have shown that the carbon sources and nitrogen sources are very important to the nitrogenase activity for diazotroph. A comprehensive view of nitrogen-fixing bacterium requires that attention be paid to the carbon source, nitrogen source requirements and temperature (Eichner et al. 2014; Sulieman et al. 2014).

Paenibacillus sp. WLY78 is a nitrogen-fixing bacterium isolated from the bamboo rhizosphere by our laboratory. Our previous studies revealed that nitrogenase activity of this bacterium is inhibited by ammonium and oxygen. It did not exhibit nitrogenase activity at O_2 concentrations above 5 % or at NH_4^+ concentrations above 1 mM (Wang et al. 2013). The engineered *E. coli* strain 78-7 was a derivative of *E. coli* JM109 carrying a *nif* operon containing nine genes (*nifBHDKENXhesAnifV*) from *Paenibacillus* sp. WLY78 (Wang et al. 2013). The engineered *E. coli* strain 78-7 exhibited nitrogenase activity even in the presence of 200 mM NH_4Cl. However, the nitrogenase activity of *E. coli* 78-7 was much lower than that of the original *Paenibacillus* sp. WLY78. Similarly, the nitrogenase activity of the engineered *E. coli* carrying *Klebsiella oxytoca nif* genes is lower than that of the original *K. oxytoca* (Temme et al. 2012). In order to improve the nitrogenase activity of *E. coli* 78-7, physiological and biochemical characteristics of the engineered *E. coli* 78-7 are here analyzed by using Biolog GEN III MicroPlate. Also, the effects of carbon sources, amino acids and temperature on the nitrogenase activity of the engineered *E.coli* 78-7 are studied. Our results will be very useful to improve nitrogenase activity of the engineered *E. coli* carrying foreign *nif* genes.

Materials and methods

Strains and mediums

E. coli strains were grown in Luria broth (LB) medium or nitrogen-deficient medium, with shaking at 180 rpm 30 °C. Nitrogen-deficient medium contained (per liter) 10.4 g Na_2HPO_4, 3.4 g KH_2PO_4, 26 mg $CaCl_2{\cdot}2H_2O$, 30 mg $MgSO_4$, 0.3 mg $MnSO_4$, 36 mg Ferric citrate, 7.6 mg $Na_2MoO_4{\cdot}2H_2O$, 10 mg p-aminobenzoic acid, 5 mg biotin, 4 g glucose as carbon source and 2 mM glutamate as nitrogen source (Wang et al. 2013).

The engineered *E. coli* strain 78-7 used here was generated by cloning the 11-kb DNA fragment containing *nif* (σ^{70}-dependent) promoter and the contiguous nine genes (*nifBHDKENXhesAnifV*) from *Paenibacillus* sp. WlY78 into the multicopy plasmid pHY300PLK and then transformed this into *E. coli* JM109 (Wang et al. 2013). All strains and plasmids used in the experiments are shown in supplementary material Table 1.

BIOLOG assay

We used BIOLOG GEN III plates (Biolog, Hayward, CA, USA) to test the ability to utilize different carbon sources of *E. coli* 78-7 in triplicate experiments. The test was performed according to the manufacturer's instructions. The system indicated which isolates could not be identified after 20 h and required further incubation. Such isolates were re-incubated and re-read between 3 and 6 h later. Those which remained unidentified after 26 h were recorded as having no identification (Wragg et al. 2014).

Determination of nitrogenase activity

The nitrogenase activity was estimated using the acetylene reduction assay (ARA) as described by (Wang et al. 2013). The strains were cultured in 5 ml of LB media (with 20 μg/ml tetracycline) in 50 ml flasks shaken at 250 rpm for 16 h at 30 °C. The cultures were washed three times with sterilized water after centrifugation, and resuspended in nitrogen-deficient medium to a final OD_{600} of 0.3. Then, 1 ml of culture was injected to a 25-ml anaerobic tube and the anaerobic tube was sealed with robber stopper. The headspace in the tube was then exchanged with gas of agron. After incubating the cultures for 6–8 h at 30 °C with shaking at 250 rpm, C_2H_2 (10 % of the headspace volume) was injected into the test tube. After incubating the cultures for a further 3 h, 100 μl of culture headspace was withdrawn through the rubber stopper with a gas tight syringe and manually injected into HP6890 gas chromatograph to quantify ethylene production. All treatments were in three replicates and all the experiments were repeated three or more times.

For measuring the effect of carbon source on nitrogenase activity, 4 g glucose (carbon source) in nitrogen-deficient medium was replaced mannose, or fructose, sucrose, lactose, maltose, sodium citrate, arabinose, sodium lactate and sodium pyruvate. Four different temperatures 25, 30, 33 and 37 °C were used to measure the effect of culture temperature on nitrogenase activity. For measuring the effect of amino acid on the nitrogenase activity, 2 mM glutamate (nitrogen source) in nitrogen-deficient medium was replaced with individual amino acid. For measuring the effect of ammonium on the nitrogenase activity, 2 mM glutamate (nitrogen source) in nitrogen-deficient medium was replaced with NH_4Cl.

Statistical analysis

Software SPSS 15.0 (SPSS. Inc., USA) was for statistical analysis. When the analysis of variance showed significant

Table 1 Cultural characteristics of the engineered *E. coli* 78-7

Test	1	2	3	Test	1	2	3
Dextrin	w	w	w	Glycyl-L-Proline	+	+	+
D-Trehalose	+	+	+	L-Arginine	–	–	–
D-Cellobiose	–	–	–	L-Aspartic acid	+	+	+
Sucrose	+	+	+	L-Glutamic acid	w	w	w
D-Turanose	w	w	w	L-Histidine	–	–	–
Stachyose	–	–	–	L-Pyroglutamic acid	–	–	–
Vancomycin	+	+	+	L-Serine	+	+	+
Tetrazolium blue	+	+	+	Guanidine HCl	w	w	w
α-D-Lactose	+	+	+	Niaproof 4	+	+	+
N-Acetyl-D-Glucosamine	+	+	+	Pectin	–	–	–
N-Acetyl-β-D-Mannosamine	+	+	+	D-Galacturonic acid	+	+	+
N-Acetyl neuraminic acid	+	+	+	L-Galactonic acid lactone	+	+	+
D-Mannose	+	+	+	D-Gluconic acid	+	+	+
D-Fructose	+	+	+	D-Glucuronic acid	+	+	+
3-Methyl Glucose	w	w	w	Glucuronamide	+	+	+
L-Fucose	+	+	+	Mucic acid	+	+	+
L-Rhamnose	+	+	+	Quinic acid	–	–	–
Inosine	+	+	+	D-Saccharic acid	+	+	+
Fusidic acid	+	+	+	p-Hydroxy-Phenylacetic acid	–	–	–
D-Serine	w	w	w	L-Lactic acid	+	+	+
D-Sorbitol	–	–	–	α-Keto-glutaric acid	+	+	+
D-Mannitol	+	+	+	D-Malic acid	+	+	+
D-Arabitol	+	+	+	L-Malic acid	+	+	+
Glycerol	+	+	+	Nalidixic acid	+	+	+
D-Glucose-6-PO4	+	+	+	Lithium chloride	w	w	w
D-Fructose-6-PO4	+	+	+	Potassium tellurite	+	+	+
D-Aspartic acid	–	–	–	Tween 40	w	w	w
D-Serine	+	+	+	α-Hydroxy-Butyric acid	+	+	+
Troleandomycin	+	+	+	β-Hydroxy-D, L-butyric acid	w	w	w
Rifamycin SV	+	+	+	Acetoacetic acid	W	w	w
Minocycline	+	+	+	Acetic acid	+	+	+
Sodium butyrate	+	+	+	Formic acid	+	+	+
N-Acetyl-D-Galactosamine	+	w	w	Sodium bromate	+	w	w
D-Galactose	+	w	+	L-Alanine	w	+	+
D-Fucose	w	+	w	Lincomycin	w	+	+
1 % Sodium lactate	w	+	+	Methyl pyruvate	w	+	+
Myo-Inositol	w	+	w	D-Lactic acid methyl ester	–	w	w
D-Maltose	+	w	+	Citric acid	–	–	w
Gentiobiose	w	–	–	Bromo-succinic acid	w	w	+
Tetrazolium violet	+	w	+	γ-Amino-Butryric acid	–	–	w
D-Melibiose	+	+	w	α-Keto-Butyric acid	w	+	w
β-Methyl-D-Glucoside	w	+	+	Propionic acid	w	+	w
D-Salicin	+	+	w	Aztreonam	w	+	+
D-Raffinose	w	–	–	α-D-Glucose	+	w	+

+, positive; −, negative; w, weakly positive

1. *E.coli* JM109; 2. *E. coli* JM109/vector; 3. *E.coli* 78-7

treatment effects, Duncan test ($P < 0.05$) was applied to make comparisons between treatments.

Results

Physiological and biochemical characteristics of the engineered *E. coli* 78-7

Physiological and biochemical characteristics of the engineered *E. coli* 78-7 were analyzed by using Biolog GEN III MicroPlate, with *E. coli* JM109 and JM109/pHY300PLK (*E. coli*JM109 carrying empty vector) as controls. Analysis of 94 phenotypic tests: 71 carbon source utilization assays and 23 chemical sensitivity tests showed that the engineered *E. coli* 78-7, *E. coli* JM109 and JM109/pHY300PLK gave similar patterns of utilization of various substrates as carbon and energy sources (Table 1).

Effect of different carbon sources on nitrogenase activity of the engineered *E. coli* 78-7

The effects of different carbon sources on the nitrogenase activity of the engineered *E. coli* were investigated. Nitrogenase activity of *E. coli* 78-7 was measured in different carbon sources. As shown in Fig. 1, the highest nitrogenase activity was observed when glucose was used as carbon source. A significant nitrogenase activity was obtained when mannose, fructose and maltose were used as carbon source. However, no nitrogenase activity was observed when sodium citrate and sodium lactate were used as carbon source.

Effect of amino acids on the nitrogenase activity of the engineered *E. coli* 78-7

The effect of different amino acid on nitrogenase activity is here determined. As shown in Fig. 2, the highest nitrogenase activity was obtained in medium containing glutamate (Glu), and the significant activities were also obtained when with proline (Pro), or aspartate (Asp) or threonine (Thr). However, the amino acids tryptophan (Try), arginine (Arg), histidine (His), isoleucine (Iso) and lysine (Lys) repress nitrogenase activity.

Effect of culture temperature on the nitrogenase activity of the engineered *E. coli* 78-7

The nitrogenase activity of the engineered *E. coli* 78-7 was determined when it was cultivated in different temperatures 25, 30, 33 and 37 °C. As shown in Fig. 3, the optimal culture temperature is 30 °C for the highest nitrogenase activity. The activity at 25 °C is lower than at 30 °C, but higher than at both 33 and 37°C. The activity was lowest when the engineered *E. coli* 78-7 was grown at 37 °C. The data suggest that nitrogenase is sensitive to high temperature.

Effect of NH_4^+ concentration on the nitrogenase activity of the engineered *E. coli* 78-7

In most of diazotrophs, *nif* genes possess a σ^{54}-dependent promoter, whose expression is activated by NifA activator which is repressed by high concentration of ammonium, and thus nitrogenase activity was also repressed by

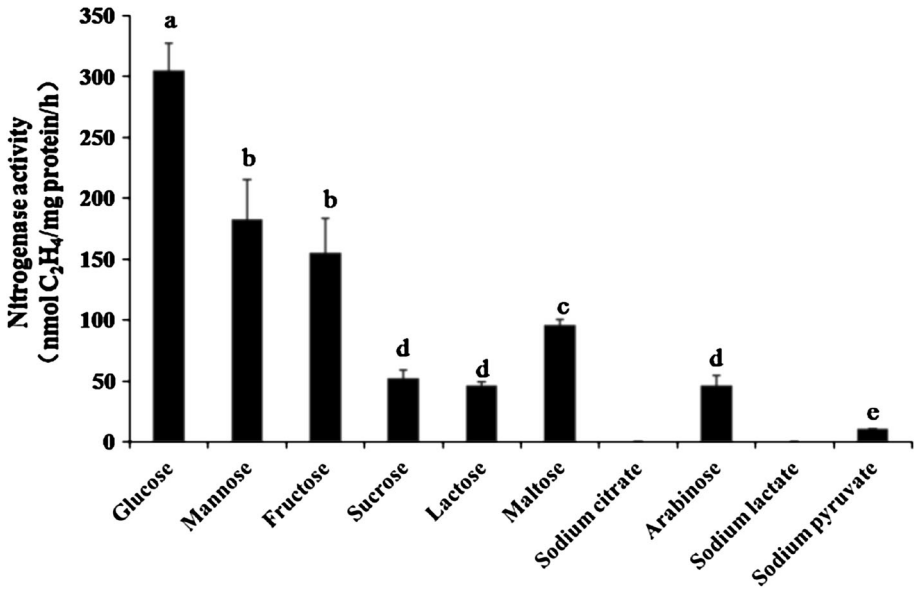

Fig. 1 Nitrogenase activity of the engineered *E. coli* 78-7 strain grown anaerobically in nitrogen-deficient conditions, supplemented with single carbon source and 2 mM glutamate as N source. *Error bars* indicate the standard deviation observed from at least two independent experiments. The same *lowercase letters* or part of the same means that the difference was not significant, otherwise significant. Significant when compared with control at $P < 0.05$ by ANOVA followed by Duncan test; n = three experiments per condition. Data are mean ± SEM

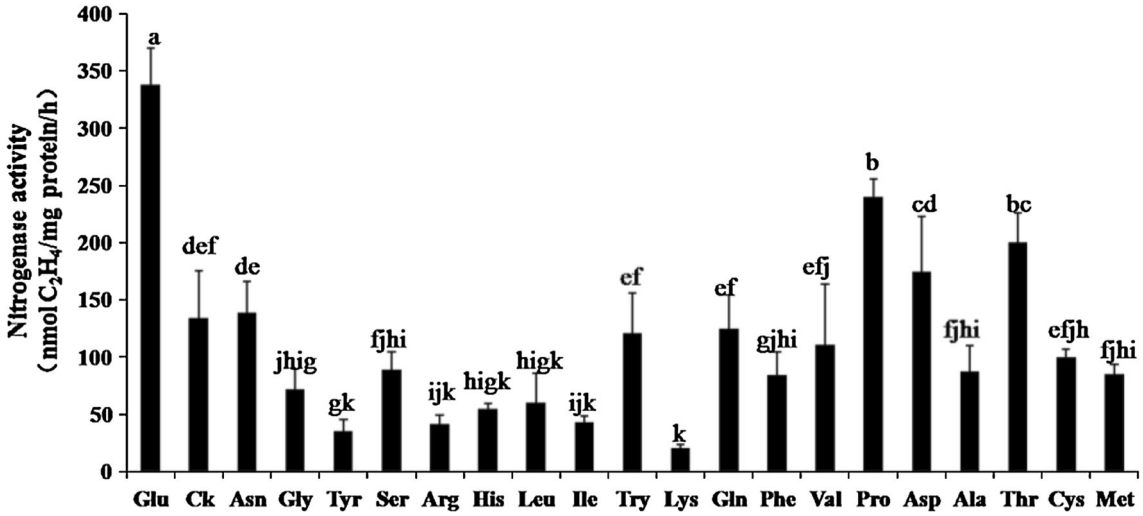

Fig. 2 The effects of amino acid (2 mM) on the nitrogenase activity of the engineered *E. coli* 78-7 strain grown under anaerobic conditions with supplement of 12 mM glucose. There is no nitrogen source in the control. *Error bars* indicate the standard deviation observed from at least two independent experiments. The same *lowercase letters* or part of the same means that the difference was not significant, otherwise significant. Significant when compared with control at $P < 0.05$ by ANOVA followed by Duncan test; n = three experiments per condition. Data are mean ± SEM

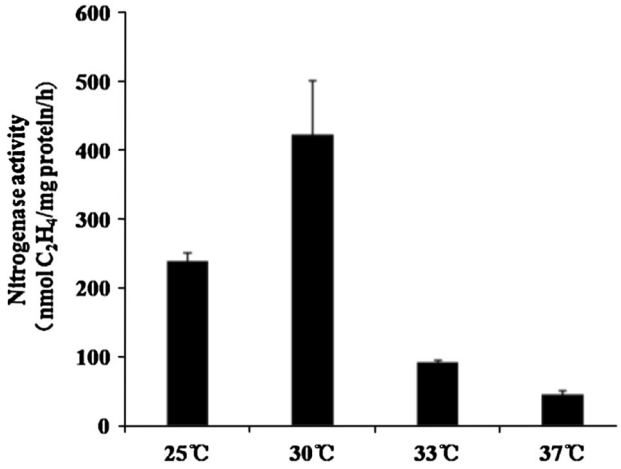

Fig. 3 Nitrogenase activity of the engineered *E. coli* 78-7 strain grown anaerobically in nitrogen-deficient conditions at different temperature. *Error bars* indicate the standard deviation observed from at least two independent experiments

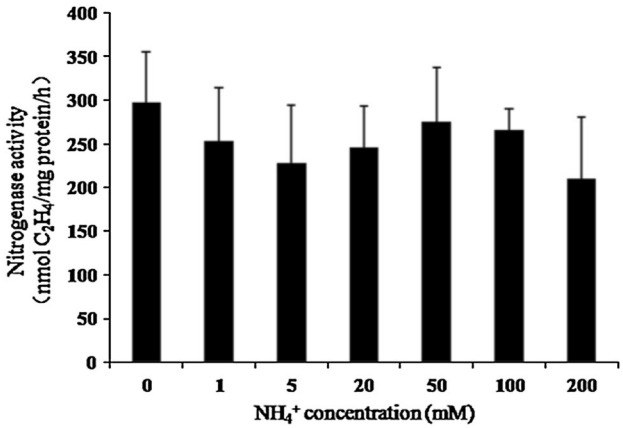

Fig. 4 Influence of NH_4^+ on nitrogenase activity of the recombinant strain 78-7. When cultures are grown in the presence of ammonium (at the initial concentrations shown on the x axis). *Error bars* indicate the standard deviation observed from at least two independent experiments

ammonium. Our previous studies revealed that the nitrogenase activity of *Paenibacillus* sp. WLY78 was inhibited by ammonium, which may be due to the transcription repression mediated by GlnR which is a global nitrogen regulator in Gram-positive bacteria (Xie et al. 2014). Since there is no GlnR in Gram-negative *E. coli*, the engineered *E. coli* 78-7 carrying the *nif* gene cluster under the control of its σ^{70}-dependent promoter of *Paenibacillus* sp. WLY78 exhibited high nitrogenase activity even in the presence of high concentration (200 mM) of ammonium (Fig. 4). The data suggest that transcription of *nif* genes is constitutive in *E. coli*, indicating that negative regulation of *nif* transcription is bypassed in the heterologous host.

Discussion

In this work, we demonstrate that the engineered *E. coli* 78-7, *E. coli* JM109 and JM109/pHY300PLK gave similar patterns of utilization of various substrates as carbon and energy sources analyzed by using Biolog GEN III MicroPlate. The data suggest that existence of the foreign *nif*

gene cluster does not produce much effect on the carbon and nitrogen utilization of the host *E. coli*.

Nitrogen fixation is an energy-expensive process and consequently it is tightly regulated at a variety of levels (Berthold et al. 2009; Fu and Burris 1989; Huergo et al. 2012; Zhang et al. 1997). Although the environmental factors affecting nitrogenase activity has been investigated in some native diazotrophs, their effects on the engineered *E. coli* have not been well studied. It was reported that nitrogenase activity was supported by proline in bacteroid from drought-stressed nodules (Pedersen et al. 1996). In the Cyanobacterium *anabaena cylindrical* PCC7122,alanine, arginine, asparagine, aspartate, glutamate, glutamine and serine caused reductions in nitrogenase activity, and cysteine and histidine were toxic at low concentration (Rawson 1985). Klassen et al (1997) indicated that all amino compounds inactivated nitrogenase activity except for L-glutamate and L-glutamine in *Herbaspirillum seropedicae* SMRl (Klassen et al. 1997). However, our results showed that glutamine acid and proline can improve the nitrogenase activity, but tryptophan, arginine, histidine, isoleucine and lysine repress nitrogenase activity (Fig. 2).

Regulation mechanism of nitrogen fixation has been extensively studied in Gram-negative diazotrophs such as *K. oxytoca* and *Azotobacter vinelandii.* The *nif* genes from these bacteria are generally transcribed from σ^{54}-dependent promoters that are subject to transcriptional activation by the enhancer binding protein NifA and are regulated in response to fixed nitrogen (Dixon and Kahn 2004). However, regulation mechanism of nitrogen fixation has not been well studied in the Gram-positive *Paenibacillus*. Our previous studies revealed that the nitrogenase activity of *Paenibacillus* sp. WLY78 was inhibited by ammonium, which may be due to the transcription repression mediated by GlnR which is a global nitrogen regulator in Gram-positive bacteria (Xie et al. 2014). The genome of *Paenibacillus* sp. WLY78 contains a *glnR* gene and there is a GlnR-binding site in *nif* promoter of this bacterium. Also, we demonstrates the binding of GlnR to the *nif* promoter of *Paenibacillus* sp. WLY78 by using electrophoretic mobility shift assays (EMSA) (results not published). Since there is no GlnR in Gram-negative *E. coli*, the engineered *E. coli* 78-7 carrying the *nif* gene cluster under the control of its σ^{70}-dependent promoter of *Paenibacillus* sp. WLY78 exhibited high nitrogenase activity even in the presence of high concentration (200 mM) of ammonium. The data suggest that transcription of *nif* genes is constitutive in *E. coli*, indicating that negative regulation of *nif* transcription is bypassed in the heterologous host. Our results will be very useful to improve nitrogenase activity of the engineered *E. coli* carrying foreign *nif* genes.

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