Engineered *Thermoanaerobacterium aotearoense* with *nfnAB* knockout for improved hydrogen production from lignocellulose hydrolysates

Yang Li\(^1\), Jialei Hu\(^1\), Chunyun Qu\(^1\), Lili Chen\(^1\), Xiaolong Guo\(^1\), Hongxin Fu\(^1\) and Jufang Wang\(^1,2\)*

**Abstract**

**Background:** As a renewable and clean energy carrier, the production of biohydrogen from low-value feedstock such as lignocellulose has increasingly garnered interest. The NADH-dependent reduced ferredoxin:NADP\(^+\) oxidoreductase (NfnAB) complex catalyzes electron transfer between reduced ferredoxin and NAD(P)\(^+\), which is critical for production of NAD(P)H-dependent products such as hydrogen and ethanol. In this study, the effects on end-product formation of deletion of *nfnAB* from *Thermoanaerobacterium aotearoense* SCUT27 were investigated.

**Results:** Compared with the parental strain, the NADH/NAD\(^+\) ratio in the ∆nfnAB mutant was increased. The concentration of hydrogen and ethanol produced increased by (41.1 ± 2.37)% (*p* < 0.01) and (13.24 ± 1.12)% (*p* < 0.01), respectively, while the lactic acid concentration decreased by (11.88 ± 0.96)% (*p* < 0.01) when the ∆nfnAB mutant used glucose as sole carbon source. No obvious inhibition effect was observed for either SCUT27 or SCUT27/∆nfnAB when six types of lignocellulose hydrolysate pretreated with dilute acid were used for hydrogen production. Notably, the SCUT27/∆nfnAB mutant produced 190.63–209.31 mmol/L hydrogen, with a yield of 1.66–1.77 mol/mol and productivity of 12.71–13.95 mmol/L h from nonsterilized rice straw and corn cob hydrolysates pretreated with dilute acid.

**Conclusions:** The *T. aotearoense* SCUT27/∆nfnAB mutant showed higher hydrogen yield and productivity compared with those of the parental strain. Hence, we demonstrate that deletion of *nfnAB* from *T. aotearoense* SCUT27 is an effective approach to improve hydrogen production by redirecting the electron flux, and SCUT27/∆nfnAB is a promising candidate strain for efficient biohydrogen production from lignocellulosic hydrolysates.

**Keywords:** Metabolic engineering, *Thermoanaerobacterium aotearoense* SCUT27, NfnAB, Biohydrogen, Lignocellulose hydrolysate

**Background**

Fossil fuels such as natural gas, coal and petroleum have dominated the energy supply for long time. In 2017, renewable energy sources accounted for < 3% of the worldwide primary energy supply, while fossil fuels accounted for > 80% [1]. The use of carbon-based non-renewable fossil fuels causes serious environmental problems. To address these problems, many efforts have been made to explore and produce renewable and environmental-friendly energy [2, 3], especially biofuels (e.g., hydrogen, bioethanol and methane).

Biofuels can be produced from biomass via biological or thermochemical processes and they generally exist in liquid (biodiesel and bioethanol) or gaseous form (biohydrogen and biomethane) [4]. The production of biofuels using corn, wheat and sugarcane results in a substantial increase of food prices, and nonfood lignocellulosic resource could be an alternative to solve this problem [5, 6]. As clean energy carriers, hydrogen has
the potential to replace traditional fuels because of its high energy capacity and environmental friendliness [7, 8]. In 2018, the main sources for hydrogen were natural gas (approximate 48%), oil (30%) and coal (18%), while only 1.0% of hydrogen was derived from the conversion of biomass by microorganisms [9–11]. There are four hydrogen-producing methods using organisms, including the microbial electrolysis cell [12], biophotolysis [13], photofermentation [14], and dark fermentation [15–17]. Dark fermentation has been a research focus because of its wide substrate range, simple operation and easy industrialization, but it still suffers from major technical problems such as high substrate cost and low hydrogen yield [8, 18]. Theoretically, as shown in Eqs. 1 and 2, 4 and 2 mol H₂ could be produced from 1 mol glucose when the volatile fatty acids are acetic acid and butyric acid, respectively.

\[
C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)
\]

\[
C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (2)
\]

Glucose (usually produced from grains) is the most used feedstock for hydrogen production, which greatly decreases the economic viability of this process. Therefore, the employment of low-value feedstock is becoming a focus. Lignocellulose is the most abundant organic component in the biosphere, with an annual production of 1–5 \( \times 10^{13} \) kg [19]. However, the incineration of lignocellulose resources such as corn stalks and wheat straw is the main disposal approach, which causes serious environmental issues (global warming and air pollution) and wastes resources (10.75–11.25 billion tons per year) [20]. As cellulose and hemicellulose in lignocellulose can be hydrolyzed to glucose and xylose, lignocellulosic biomass (e.g., sugarcane bagasse [SCB], corn cob, corn stalk, and wheat straw) has the potential to be used for hydrogen production [15, 21, 22].

To improve the hydrogen yield from dark fermentation, many studies on process engineering and metabolic engineering of hydrogen-producing organisms have been carried out [16, 22–26]. Thermophilic hydrogen producers are considered ideal hydrogen-producing factories, as higher temperature is beneficial for hydrogen production [27, 28]; for example, thermophilic hydrogen production has higher hydrolysis and reaction rates, lower viscosity and energy consumption, and lower risk of contamination [29].

As shown in our previous studies, *Thermoanaerobacterium aotearoense* SCUT27 shows great potential for conversion of low-value feedstocks to biohydrogen [22]. The *ldh* deletion mutant of SCUT27 could produce 2.28 mol H₂ from 1 mol xylose in 125-mL serum bottles, and various sugars (mannose, cellobiose, glucose, trehalose, maltose, arabinose, galactose, lactose, fructose, and others.) could be used as carbon sources [22]. The hydrogen production reached 1.86 mol H₂/mol total sugar when SCB hydrolysate was used as the carbon source, and glucose and xylose in the hydrolysate could be used simultaneously without obvious carbon catabolic repression [6].

Electron carriers such as ferredoxin and NAD(P)H play an important role in biohydrogen production during dark fermentation. The NADH-dependent reduced ferredoxin:NADP⁺ oxidoreductase (NfnAB) complex containing two subunits was first discovered in *Clostridium kluiveri*, and transfers electrons from reduced ferredoxin and NADH to NADP⁺ (\( \text{Fd}^+ \text{red} + 2 \text{NADP}^+ + \text{NADH} + \text{H}^+ \rightarrow \text{Fd}^+ \text{ox} + 2 \text{NADPH} + \text{NAD}^+ \)) [30]. Hydrogen production in *Thermoanaerobacterium* is NADH-dependent, and blocking pathways competing for NADH or altering the intracellular NADH level could affect hydrogen production [17, 31, 32]. Deletion of *nfnAB* blocks direct NADPH production from NADH, which facilitates the accumulation of NADH-dependent products (i.e., hydrogen, lactic acid and ethanol) (Fig. 1).

Deletion of *nfnAB* had different effects on hydrogen and ethanol formation in different strains of *T. saccharolyticum*, which may be attributed to the cofactor (i.e., NADH or NADPH) specificity of alcohol dehydrogenase. For example, hydrogen production was increased by 46% and 900% after *nfnAB* was deleted from strains JW/SL-YS485 and M1442, respectively, while little effect was observed in strain M0353 [26]. To the best of our knowledge, the cofactor specificity of alcohol dehydrogenase and the role of *nfnAB* in end-product formation by *T. aotearoense* have never been reported so far.

In this work, the effect of *nfnAB* deletion on end-product formation in hydrogen-producing strain *T. aotearoense* SCUT27 was investigated. Then, six types of lignocellulose hydrolysate were selected and evaluated as low-value feedstocks for hydrogen production. Finally, the feasibility of hydrogen production from nonsterilized lignocellulose hydrolysates was investigated.

**Results and discussion**

**Hydrogen production using various substrates and intracellular NADH/NAD⁺ ratio of *ΔnfnAB* mutants**

To evaluate the effect of deletion of *nfnAB* on end-product formation, batch fermentation was first performed in serum bottles with glucose, xylose or glucose/xylose mixture as the substrate. In general, when SCUT27/ΔnfnAB and its parental strain SCUT27 were inoculated into fresh MTC medium (10 g/L total sugar), no obvious difference was observed in sugar use and acetic acid formation (fig. 2, Table 1. Additional
The deletion of nfnAB resulted in an increase of ethanol production from \((8.19 \pm 0.66) \%\) to \((14.28 \pm 1.25) \%\) \((p < 0.01)\) and a decrease of lactic acid production from \((9.63 \pm 0.74) \%\) to \((14.62 \pm 1.12) \%\) \((p < 0.05)\). Hydrogen production by SCUT27/ΔnfnAB using glucose, xylose and glucose/xylose mixture as substrates increased by \((41.1 \pm 2.37) \%\) \((p < 0.01)\), \((43.8 \pm 3.18) \%\) \((p < 0.01)\) and \((38.7 \pm 2.65) \%\) \((p < 0.01)\), respectively, compared with SCUT27. It should be noted that both SCUT27 and SCUT27/ΔnfnAB could use glucose and xylose simultaneously during sugar fermentation, although glucose was consumed at a faster rate. Figure 2 shows that the maximum glucose consumption rate of SCUT27 and SCUT27/ΔnfnAB were \((660.71 \pm 40.29) \%\) \((p < 0.01)\) and \((679.31 \pm 52.18) \%\) \((p < 0.01)\) higher than those of xylose, respectively. To evaluate the effect of pH on metabolite distribution, CaCO₃ was selected for pH control in serum bottles. As shown in Additional file 1: Table S3, CaCO₃ had little effect on the product distribution in SCUT27 and SCUT27/ΔnfnAB, but it increased sugar use because of a buffering effect.

In *T. aotearoense* SCUT27, NADH is consumed by the production of lactic acid, ethanol and hydrogen [22]. Deletion of nfnAB from SCUT27 led to a significant increase in hydrogen production. To explain this phenomenon, cells were collected at a 3-h interval from 6 to 18 h after inoculation, then the NADH/NAD⁺ ratio in strains SCUT27 and SCUT27/ΔnfnAB was determined. As shown in Fig. 3, there was a rapid decrease in the NADH/NAD⁺ ratio from 6 to 12 h, then the NADH/ NAD⁺ ratio remained stable beyond 12 h in both strains. However, SCUT27/ΔnfnAB displayed a much higher NADH/NAD⁺ ratio than the parental strain. For example, at 6 h after inoculation, the NADH/NAD⁺ ratio in SCUT27/ΔnfnAB was \((21.87 \pm 2.16) \%\) \((p < 0.01)\) higher than that in SCUT27 strain (Fig. 3). The elevated NADH/ NAD⁺ ratio disturbed the balance of reducing equivalents, and more NADH was used for hydrogen formation. This result was in accordance with a 46% increase of hydrogen production after nfnAB gene deletion in *T. saccharolyticum* strain JW/SL-YS485 [26]. Similarly, down-regulation of ferredoxin–NADP⁺ reductase in green alga *Chlamydomonas reinhardtii* increased the electron supply to the hydrogenases, resulting in a 2.5-fold higher hydrogen production rate [33]. In another study, when a fusion gene encoding ferredoxin–hydrogenase was expressed in *C. reinhardtii*, the hydrogen production rate of the mutant was 4.5-fold higher than that of the wild type [34]. Furthermore, our study demonstrated...
for the first time that *T. aotearoense* SCUT27 primarily uses NADH-dependent aldehyde dehydrogenase/alcohol dehydrogenase for ethanol production, as the decrease of NADPH caused by deletion of *nfnAB* did not significantly affect the formation of ethanol (Table 1). The carbon and electron recoveries of SCUT27 and SCUT27/Δ*nfnAB* using glucose, xylose and glucose/xylose mixture as carbon source were between (91.59±4.92)% and (98.21±6.75)%, consistent with the reports of Li and Zhou [22, 35].

**Hydrogen production from lignocellulose hydrolysates in serum bottles**
Thermochemical pretreatment of lignocellulose materials has been widely used since it is capable of achieving high solubilization and it is a low-cost process [36]. Both acidic and alkaline solutions are used for the pretreatment of lignocellulose materials. When alkaline-pretreated *Miscanthus* hydrolysate was used as the carbon source, the co-culture of *Caldicellulosiruptor saccharolyticus* and *Thermotoga neapolitana* produced 3.2–3.3 mol H₂/mol hexose [37]. Many kinds of agricultural residues have been pretreated using dilute acid for biohydrogen production, and dilute acid pretreatment is more successful than other pretreat methods and can improve the hydrolysis of hemicelluloses [38, 39]. Comparison of acid- and alkaline-pretreated grass for hydrogen production indicates that acid pretreatment was better than alkaline pretreatment for hydrogen production [40]. When thermal NH₃-pretreated rice straw and thermal dilute H₂SO₄-pretreated rice straw were used as substrates, the hydrogen production rates of *Thermotoga neapolitana* were 57.64±3.12 and 89.02±5.14 mL H₂/L/
Table 1 Comparison of sugar consumption and product formation in batch fermentation of strains SCUT27 and SCUT27/ΔnfnAB with different carbon sources

| Strain          | Carbon source | Sugar uptake (mmol/L) | Lactic acid concentration (mmol/L) | Acetic acid concentration (mmol/L) | Ethanol concentration (mmol/L) | Hydrogen concentration (mmol/L) | Hydrogen yield (mol/mol) | Biomass (mmol/L) | Carbon recovery (%) | Electron recovery (%) |
|-----------------|---------------|-----------------------|-----------------------------------|-----------------------------------|-------------------------------|---------------------------------|------------------------|-------------------|---------------------|----------------------|
| SCUT27          | Glucose       | 29.17 ± 2.06          | 25.44 ± 1.89                      | 15.17 ± 1.33                      | 28.48 ± 2.61                 | 34.92 ± 3.04                    | 1.20 ± 0.09             | 37.67 ± 2.79      | 91.59 ± 4.92        | 96.71 ± 6.98         |
|                 | Xylose        | 32.73 ± 2.2           | 20.78 ± 1.56                      | 13.67 ± 0.67                      | 26.52 ± 1.96                 | 31.14 ± 2.13                    | 0.95 ± 0.06             | 32.56 ± 2.33      | 94.67 ± 7.29        | 93.69 ± 7.16         |
|                 | GX = 2:1      | 30.77 ± 1.95          | 24.33 ± 1.33                      | 14.67 ± 0.83                      | 27.39 ± 1.52                 | 33.15 ± 1.96                    | 1.12 ± 0.07             | 33.95 ± 3.26      | 92.18 ± 6.81        | 93.59 ± 7.62         |
| SCUT27/ΔnfnAB   | Glucose       | 28.94 ± 1.78          | 22 ± 1.67**                       | 14.67 ± 1.01                      | 32.17 ± 2.39**               | 49.28 ± 2.58**                  | 1.70 ± 0.12**           | 36.74 ± 2.79      | 93.72 ± 5.49        | 98.21 ± 6.75         |
|                 | Xylose        | 32.53 ± 1.73          | 18.78 ± 1.44*                     | 13.5 ± 0.83                       | 28.7 ± 2.39*                 | 44.79 ± 3.78**                  | 1.37 ± 0.08**           | 32.09 ± 2.33      | 93.67 ± 6.19        | 92.38 ± 5.88         |
|                 | GX = 2:1      | 30.14 ± 2.36          | 20.78 ± 1.56**                    | 14.5 ± 0.67                       | 31.3 ± 1.96*                 | 45.97 ± 2.86**                  | 1.63 ± 0.13**           | 35.35 ± 2.79      | 94.75 ± 7.16        | 96.91 ± 6.25         |

Asterisks indicate significant differences between SCUT27 and SCUT27/ΔnfnAB (***p ≤ 0.01; *p ≤ 0.05; t test). The carbon in biomass was estimated using the general empirical formula for cell composition of CH_{2.5}N_{0.25}O_{0.5} [22]. mol/mol means mol H\textsubscript{2}/mol consumed sugar (glucose and/or xylose).
day [38]. Therefore, thermal acid pretreatment was used in this study.

Six types of lignocellulose hydrolysate were selected to evaluate their potential application for hydrogen production in serum bottles. The compositions of the hydrolysates after pretreatment are listed in Additional file 1: Tables S4, S5. As expected, compared to SCUT27, strain SCUT27/ΔnfnAB produced more hydrogen (34.8–43.8)% (p < 0.01) with higher productivity and yield (Table 2, Figs. 4 and 5 and Additional file 1: Tables S6–S17). Considering the various lignocellulose hydrolysates, the highest hydrogen yields, 1.21 ± 0.05 mol/mol and 1.65 ± 0.09 mol/mol for SCUT27 and SCUT27/ΔnfnAB, respectively, were obtained when dilute acid-pretreated corn cob hydrolysate was used as carbon source (Fig. 4c, d), consistent with the results obtained using glucose/xylose mixture (Table 1). In addition, when dilute acid-pretreated rice straw hydrolysate was used as carbon source, the hydrogen yields of SCUT27 and SCUT27/ΔnfnAB reached 1.16 ± 0.06 mol/mol and 1.48 ± 0.11 mol/mol, respectively (Fig. 4a, b). However, the hydrogen concentration, productivity and yields were much lower when using other hydrolysates (Figs. 4e, f, 5). When dilute acid-pretreated corn straw hydrolysate was used, the fermentation period extended to 15 h, while the fermentation finished in 9–12 h for other hydrolysates (Fig. 4e, f), indicating the relatively high toxicity of corn straw hydrolysate toward T. aotearoense SCUT27. As shown in Additional file 1: Table S5, the concentrations of inhibitors in corn straw hydrolysate were much higher than in other hydrolysates, resulting in the longer fermentation period and decreased hydrogen productivity (Table 2). It should be noted that the glucose/xylose ratio in the hydrolysates ranged from 1:1.5 to 1:6.04 (Additional file 1: Table S5). As higher xylose ratio in the hydrolysates may lead to lower hydrogen yield (Table 1), the relatively high hydrogen yield obtained in this study may result from other sugars in the hydrolysates (Additional file 1: Table S4).

Table 2 Comparison of sugar consumption and product formation by strains SCUT27 and SCUT27/ΔnfnAB using dilute acid pretreated substrate in serum bottles with 20 mM/L CaCO3

| Strain          | Dilute acid pretreated substrate | Sugar uptake (mmol/L) | Hydrogen concentration (mmol/L) | Hydrogen productivity (mmol/L) | Hydrogen yield (mol/mol) | Lactic acid production (mmol/L) | Acetic acid production (mmol/L) | Ethanol production (mmol/L) |
|-----------------|----------------------------------|-----------------------|---------------------------------|--------------------------------|--------------------------|-------------------------------|-------------------------------|-----------------------------|
| SCUT27          | Rice straw                       | 37.45 ± 1.82          | 43.38 ± 2.63                    | 3.61 ± 0.22                    | 1.16 ± 0.06               | 30.44 ± 1.56                  | 15.17 ± 1.17                  | 35.2 ± 2.39                  |
|                 | Corn cob                         | 41.29 ± 2.25          | 49.47 ± 2.57                    | 4.12 ± 0.21                    | 1.21 ± 0.05               | 31.89 ± 2.2                   | 20.33 ± 2.16                  | 38.48 ± 2.83                 |
|                 | Corn straw                       | 44.56 ± 2.11          | 39.79 ± 2.11                    | 2.65 ± 0.14                    | 0.91 ± 0.06               | 33.11 ± 1.78                  | 17.33 ± 1.83                  | 40.65 ± 2.39                 |
|                 | Wheat straw                      | 47.72 ± 2.56          | 38.35 ± 2.23                    | 3.20 ± 0.19                    | 0.81 ± 0.05               | 35.11 ± 2.2                   | 19.2 ± 2.17                   | 45.43 ± 2.39                 |
|                 | Soybean straw                    | 39.02 ± 1.95          | 33.69 ± 2.03                    | 2.81 ± 0.17                    | 0.84 ± 0.07               | 28 ± 1.33                     | 15 ± 1.67                     | 38.48 ± 2.61                 |
|                 | Sorghum straw                    | 40.82 ± 2.37          | 39.59 ± 1.87                    | 3.63 ± 0.16                    | 0.99 ± 0.04               | 31 ± 1.56                     | 14 ± 2.5                      | 38.91 ± 2.61                 |
| SCUT27/ΔnfnAB   | Rice straw                       | 41.51 ± 2.13*         | 62.39 ± 3.45**                  | 5.20 ± 0.29**                  | 1.48 ± 0.11**             | 28.67 ± 1.78**                | 15.33 ± 1.5                   | 43.26 ± 2.61**               |
|                 | Corn cob                         | 43.23 ± 2.59          | 69.76 ± 2.78**                  | 5.81 ± 0.23**                  | 1.65 ± 0.09**             | 27.67 ± 1.22**                | 20.33 ± 1.5                   | 44.78 ± 3.26**               |
|                 | Corn straw                       | 43.98 ± 1.84          | 54.33 ± 2.15**                  | 3.62 ± 0.14**                  | 1.25 ± 0.07**             | 29.44 ± 1.56**                | 17.83 ± 1.67                  | 47.39 ± 2.61**               |
|                 | Wheat straw                      | 48.35 ± 2.67          | 54.26 ± 2.83**                  | 4.52 ± 0.24**                  | 1.16 ± 0.05**             | 31 ± 1.67**                   | 18.67 ± 2.67                  | 51.96 ± 2.39**               |
|                 | Soybean straw                    | 42.38 ± 2.23*         | 46.35 ± 2.85**                  | 3.86 ± 0.24**                  | 1.09 ± 0.07**             | 26.11 ± 2.11**                | 14.83 ± 1.33                  | 41.52 ± 3.04*                |
|                 | Sorghum straw                    | 41.69 ± 2.51          | 53.37 ± 2.97**                  | 4.45 ± 0.25**                  | 1.28 ± 0.09**             | 26.89 ± 1.89**                | 13.83 ± 1.67                  | 45.65 ± 3.04**               |

Asterisks indicate significant differences between SCUT27 and SCUT27/ΔnfnAB (*p ≤ 0.01; **p ≤ 0.05; t test). Biomass was not measured due to the addition of CaCO3, mol/mol means mol H2/mol consumed sugar (mainly glucose and xylose)
Hydrogen production from nonsterilized lignocellulose hydrolysates in pH-controlled bioreactor

As thermophilic strains have the advantage of lower risk of contamination, hydrogen production by SCUT27/ΔnfnAB from sterilized (as the control) and nonsterilized hydrolysates of corn cob and rice straw was evaluated in a pH-controlled bioreactor. The fermentation using undiluted hydrolysates was performed at pH 6.0, because the pH value in serum bottles decreased from the initial 6.8 to ~5.0 and thus stopped the fermentation due to pH limitation. As shown in Fig. 6 and Additional file 1: Tables S18–S21, all the fermentation processes were finished within 15 h, and no obvious difference in cell density, sugar consumption, or product
Formation was observed between the fermentation with sterilized and nonsterilized hydrolysates. 16S rDNA sequence analysis showed that there was no bacterial contamination during the fermentation, indicating that it is feasible to produce hydrogen in a nonsterilized fermentation using strains SCUT27 and SCUT27/ΔnfnAB. In addition, glucose and xylose were totally consumed, which is necessary for efficient hydrogen production from lignocellulosic hydrolysates [41, 42]. For example, the concentration, yield and productivity of hydrogen produced by SCUT27/ΔnfnAB reached 209.31 mmol/L, 1.77 mol/mol consumed sugar and 13.95 mmol/L h from rice straw hydrolysate, and 195.71 mmol/L, 1.71 mol/mol consumed sugar and 13.04 mmol/L h from corn cob.
hydrolysate during the fermentation. These values were slightly higher than those using glucose, xylose or mixed sugar as substrate (from 1.37 ± 0.08 to 1.70 ± 0.12 mol/mol consumed sugar) (Table 1), probably due to the presence of other carbon sources other than glucose and xylose in the hydrolysates.

The hydrogen yield and productivity obtained in this study were comparable to or better than those in recent studies of thermophilic hydrogen production from low-value feedstock. For example, Lai et al., reported a hydrogen yield of 1.86 mol/mol consumed sugar using T. aotearoense SCUT27/Δldh from SCB hydrolysate, which was slightly higher than the values obtained in this study (1.68–1.79 mol/mol consumed sugar), but the hydrogen productivity (12.43 mmol/L h) of T. aotearoense SCUT27/Δldh was lower than that of SCUT27/ΔnfnAB [43]. In addition, the newly isolated strain T. thermostascharolyticum MJ1 showed a hydrogen yield of 2.52 mol/mol consumed sugar from SCB, but the relatively low hydrogen productivity of 6.55 mmol/L h restricted large-scale production of hydrogen by this strain [21]. The results obtained in the present study indicate that the engineered strain SCUT27/ΔnfnAB was able to directly use dilute acid-pretreated lignocellulose hydrolysates for hydrogen production with high productivity, yield and substrate use ratio, suggesting that it has great potential to produce hydrogen from nonsterilized lignocellulose hydrolysates.

**Conclusions**

The effect of nfnAB deletion from T. aotearoense SCUT27 on end-product formation was investigated. Deletion of nfnAB increased the NADH/NAD⁺ ratio and changed the product distribution, resulting in a significant increase (41.1 ± 2.37)% (p < 0.01) in hydrogen production when glucose was the sole carbon source. Our results demonstrate that strain SCUT27/ΔnfnAB was able to use both glucose and xylose in six types of dilute acid-pretreated biomass hydrolysate for hydrogen production. Furthermore, the possibility of nonsterilized
fermentation was verified using corn cob and rice straw hydrolysates. Taken together, these characteristics make *T. aotearoense SCUT27/ΔnfnAB* a promising target strain to produce hydrogen from lignocellulose hydrolysates.

**Methods**

**Strains and cultivation media**
The strictly anaerobic bacterium *T. aotearoense SCUT27* was isolated in Guangdong Province, China [22], and stored in anaerobic tubes at −80 °C with 25% glycerol as protective agent. Cells were grown in modified MTC medium [22]. The medium contained (per liter): 2.0 g citric acid tripotassium salt, 5.0 g urea, 1.0 g cysteine–HCl monohydrate, 1.25 g citric acid monohydrate, 1.5 g ammonium chloride, 0.1 g ferrous chloride tetrahydrate, 1.0 g sodium sulfate, 1.0 g yeast extract, 1.0 g magnesium chloride hexahydrate, 1.0 g potassium phosphate, 0.2 g calcium chloride, 2.5 g sodium bicarbonate, 0.02 g pyridoxamine dihydrochloride, 0.004 g p-aminobenzoic acid, 0.002 g d-biotin, 0.002 g cobalamin, and 0.004 g thiamine chloride. For hydrogen production in a 5-L bioreactor, the 1.0 g/L yeast extract in MTC medium was replaced by 5.0 g/L yeast extract and 2.0 g/L peptone. The effect of yeast extract concentration in the hydrolysate on hydrogen production was negligible (Additional file 1: Table S22). The carbon sources used were glucose, xylose, glucose/xylose mixture or lignocellulosic hydrolysate. Serum bottles were vacuumed and filled with nitrogen three times to guarantee anaerobic conditions, while the bioreactor was sparged with nitrogen for ~30 min to reach anaerobiosis. Serum bottle and the bioreactor (containing medium) were sterilized by autoclaving at 121 °C for 30 min. For mutant selection, transformants were grown in modified DSMZ 640 medium [22] with 2.0% agar, in which cellobiose was replaced by xylose. *Escherichia coli* DH5α was used for DNA manipulation and grown in Luria–Bertani medium with 100.0 µg/mL ampicillin.

**Gene cloning and vector construction**
The strains, vectors and primers used in this study are listed in Table 3. The genomic DNA of strain SCUT27 was extracted using TIANamp Bacteria DNA Kit (TIANGEN).

Bacterial plasmid DNA was purified using a TIANprep Mini Plasmid Kit (TIANGEN). The kanamycin resistance gene encoding 3′,5′-aminoglycoside phosphotransferase type III (*aph*, Genebank accession no. V01547) was amplified from plasmid puKAd stored in our laboratory using primers *aph*-F and *aph*-R, in which *Bam*HI and *Eco*RI sites were added at the 3′-end and the 5′-end, respectively. The purified PCR products were digested with *Bam*HI and *Eco*RI and then ligated into pBlue-script II SK (+) (Stratagene, CA, USA) digested with the same enzymes, yielding pBlue-aph. To disrupt the genes encoding NfnA (accession no. V518_0931) and NfnB.

### Table 3 Strains, plasmids and primers used in this study

| Description | Reference or source |
|-------------|---------------------|
| **Strains** |                     |
| *T. aotearoense SCUT27* | Wild-type          |
| *T. aotearoense SCUT27/ΔnfnAB* | *nfnAB* deletion mutant of SCUT27 |
| *E. coli DH5α* | Used for plasmid screening and propagation |
| **Plasmid** |                       |
| pBluescript II SK+ | Standard cloning vector |
| pPuKAd | As the template for kanamycin resistance gene amplification [44] |
| pBlumAB | Disrupts *nfnAB* with kanamycin resistance gene |
| **Primer** | **Sequence (5′–3′)** |
| *nfnA*-F | GGT ACC CCT TGC AGG CAT TTC TTC C |
| *nfnA*-R | GAA TTC GAA GGT TGC CCT GTT CAC G |
| *nfnB*-F | GGA TCC CCA ACT GTG ACT CTG CAT C |
| *nfnB*-R | GAG CTC AGA AGA AGC AAT TGA ATC C |
| *aph*-F | GGA TCC GAT AAA CCC AGC GAACC |
| *aph*-R | GAA TTC ATC GAT ACA AAT TCC TCG TAGG |
| M13-F | CGC CAG GGT TTT CCC AGT CAC GAC |
| M13-R | AGC GGA TAA CAA TTT CAC ACA GGA |
| probe-F | TTT GCT CGG AAG AGT ATG AA |
| probe-R | GCCACTTACTTTGCCATCT |
the Amplite™ SCUT27 and the mutant were detected using \( \Delta nfnAB \) USA). As the OD\(_{600} \) value of the sample would reflect the test sample. The following steps were performed in clear-bottomed 96-cell microplates. For NAD\(^+\) extraction assay, 25 μL of NAD\(^+\) extraction solution was added into the wells containing the test sample. After incubation at room temperature for 15 min, 25 μL of NADH neutralization solution were added to the well. For total NAD\(^+\) and NADH assay, NAD\(^+\)/NADH control solution was used instead of NAD\(^+\) extraction solution and NADH neutralization solution. After that, 75 μL of NADH reaction mixture were added into each well containing test sample to make the total volume 150 μL/well. The samples were incubated in the dark room at room temperature for 1 h, followed by measuring the absorbance at 460 nm. Phosphate-buffered saline was used as a blank control. Determinations were carried out in triplicate and all the data were the average of three independent experiments.

**Hydrogen production from different substrates in serum bottles**

In this study, dilute sulfuric acid was chosen for pretreatment of the six lignocellulosic biomasses. The acid concentration, temperature and duration of pretreatment were determined depending on our former experiments. The low-value lignocellulose materials (rice straw, corn cob, wheat straw, soybean straw, and sorghum straw) used in this study were purchased in Lianyungang City, Jiangsu Province, China. They were milled to 1–2 mm and dried at 65 °C for 24 h before tests. Acid hydrolysis of the feedstock was then performed. The samples were pretreated in dilute sulfuric acid (2.0%, w/v) at 121 °C for 60 min. The hydrolysis was performed in 1-L conical flasks with a solid to liquid ratio of 1:10 (g dry weight to mL). Then the solid residue was removed by centrifugation (10,000 \( \times g \), 15 min). The pH of the supernatant was adjusted to 7.0 with Ca(OH)\(_2\) and the precipitate formed was removed by centrifugation. Then, vacuum filtration was used to remove solid particles from the supernatant. Soluble sugars (glucose and xylose) and by-products (furfural, 5-methylfurfural, vanillin, etc.) were analyzed using high-performance liquid chromatography (HPLC).

Batch fermentation was performed in MTC medium with 10 g/L glucose, xylose, mixed sugar (glucose:xylose = 2:1), or lignocellulose hydrolysate as the carbon source. Since the optimum sugar concentration for hydrogen production by strain SCUT27 was 10 g/L in serum bottles [22], the hydrolysates were diluted accordingly before inoculation. Then, the fermentation performance of SCUT27 and SCUT27/\( \Delta nfnAB \) using these hydrolysates was evaluated. First, SCUT27 and SCUT27/\( \Delta nfnAB \) were transferred into 50-mL sterile medium and cultivated at 150 rpm and 55 °C for 12 h. Then, batch fermentation was carried out in serum bottles at 55 °C and 150 rpm, with 5% (v/v) inoculum. For hydrogen production from
lignocellulose hydrolysates, 20 mM CaCO₃ was also added to the serum bottle. Samples were collected at 3-h intervals until the end of the fermentation, and the hydrogen production, residual sugar and other metabolic products in the broth were determined. All treatments were carried out in 125-ml serum bottles with a nitrogen gas headspace. The determinations were carried out in triplicate and the data are the average of three independent experiments.

**Hydrogen production from sterilized and nonsterilized lignocellulose hydrolysates in 5-L bioreactor**

Hydrogen fermentation from sterilized and nonsterilized dilute acid-pretreated rice straw and corn cob hydrolysates by SCUT27/ΔnfnAB were carried out in a 5-L fermentor (BIOSTA A plus, Sartorius Stedim Biotech, Germany) with a working volume of 1 L. The bioreactor was equipped with a stirrer, heating element, and temperature and pH sensors. Seed culture (0.1 L) was transferred into 0.9 L hydrolysate containing the nutrients from modified MTC medium other than the carbon source. The fermentation was performed in anaerobic conditions at 150 rpm and 55 °C. The pH was kept at 6.0 by addition of 5 M of NaOH. The gas phase was collected in a 30-L aluminum foil gasbag (John Long biotech, Beijing, China). Every 3 h, the volume of gas in the gasbag was determined by water displacement. Culture (5 mL) was collected at 3-h intervals, and the residual sugar and products in the supernatant were analyzed using HPLC.

After fermentation, samples were taken from the bioreactor, cells were collected, and the genomic DNAs of the obtained microorganisms were extracted using the TIANamp Bacteria DNA Kit. Then the extracted genomic DNAs were used as template for 16S rDNA amplification. The fragments obtained were verified by DNA sequence analysis.

**Analytical methods**

Cell density was determined by measuring turbidity at 600 nm using a spectrophotometer (PERSEE T6, Beijing, China). When hydrolysates were used as carbon source for hydrogen production in serum bottles, the cell density was not monitored due to the interference from CaCO₃. Soluble sugars and metabolites in the medium were measured using a high-performance liquid chromatography (HPLC) (Waters 2695, Waters, US) equipped with an Amines HPX-87H column (Bio-Rad, Hercules, CA, USA). The column temperature was 60 °C, and 5.0 mM H₂SO₄ was used as mobile phase at a flow rate of 0.6 mL/min. The concentrations of by-products produced during the hydrolysis of lignocellulose were analyzed by HPLC equipped with an Agilent HC-C18 column (Agilent, USA). The column temperature was 25 °C, with 10% (v/v) acetonitrile as the mobile phase at a flow of 1.0 mL/min.

Hydrogen concentration was determined by measuring the hydrogen percentage in the headspace or gas stored in the aluminum foil gasbag by gas chromatography (GC; Fuli 9790, Fuli, China) equipped with a flame ionization detector, a thermal conductivity detector, and a TDX-01 column. The column temperature was 60 °C. Nitrogen was used as the carrier gas with a flow rate of 35 mL/min and 10–20 mL sample (including H₂, CO₂ and N₂) was injected into the GC, but only 1.0 mL sample was collected automatically for detection. The percentages of H₂ and CO₂ in the sample were calculated according to the corresponding peak areas and standard curves. The standard curves for H₂ and CO₂ were obtained as follows: H₂ or CO₂ was serially diluted to concentrations of 5%, 10%, 20%, 30%, 40% and 50% with N₂. The diluted gas samples were injected into the GC and the peak areas of each sample were detected. Then the percentage H₂, CO₂ and N₂ could be obtained from the following equations:

\[
W_{H_2} = -1.37 + A_{H_2} \times 6.53 \times 10^{-6},
\]

\[
W_{CO_2} = 7.02 + A_{CO_2} \times 9.71 \times 10^{-5},
\]

\[
W_{N_2} = 100 - W_{H_2} - W_{CO_2},
\]

where \(A\) and \(W\) are the peak area and the percentage of the corresponding gas.

The initial \(N_2\) quantity (\(n_{N_2}\)) in the serum bottle headspace gas was evaluated as 4.31 mmol (\(n_{N_2}=pv/RT, p=0.14\) Pa, \(v=75\) mL, \(T=298.15\) K, \(R=8.314\) m³ Pa/K mol).

H₂ quantity (\(n_{H_2}\), mmol) in the serum bottles was deduced as:

\[
n_{H_2} = n_{N_2} \times R_{H_2/N_2} = n_{N_2} \times W_{H_2}/W_{N_2},
\]

where \(R_{H_2/N_2}\) is the molar ratio of H₂ to N₂ in the detected sample.

H₂ quantity (\(n_{H_2}\), mmol) in the bioreactor was calculated as:

\[
n_{H_2} = pV_{H_2}/RT = pW_{H_2} \times V_g/RT,
\]

where \(V_g\) is the volume of the gas collected in the gasbag (\(p=0.1\) MPa, \(T=298.15\) K, \(R=8.314\) m³ Pa/K mol).

H₂ yield (\(Y_{H_2}\), mol H₂/mol sugar) was calculated as:

\[
Y_{H_2} = n_{H_2}/n_{sugar},
\]

where \(n_{sugar}\) is the amount of sugar consumed during the fermentation, in mmol.

For hydrogen production using hydrolysates or glucose/xylan mixture, the quantity of each consumed sugar was calculated separately and summed to give total consumed sugar.

Carbon recovery calculation was based on Li [22]:
\[ C_t = 0.4 \text{ sugar} + 0.4 \text{ lactate} + 0.6 \text{ acetate} + 0.78 \text{ ethanol} + 0.47 \text{ CDW}, \]

where \( C_t \) = total carbon; sugar = glucose, xylose or glucose/xylose mixture; CDW = cell dry weight. All units are g/L.

Electron recovery calculation was based on Zhou [35]. The general empirical formula for cell composition was \( \text{CH}_2\text{N}_2\text{O}_{3.5} \). Carbon contained in yeast extract was not included in the carbon and electron recovery calculations.

Statistical analysis
Statistical tests were performed using Statistical Package for the Social Sciences (SPSS vers. 19; IBM Corporation, USA). The data presented in the manuscript and Additional file are the means and standard deviations (**p \leq 0.01; *0.01 \leq p \leq 0.05; t test).
11. Sinha P, Pandey A. An evaluative report and challenges for fermentative biohydrogen production. Int J Hydrogen Energy. 2011;36(13):7460–78.
12. Khan M, Nizami A, Rehan M, Oudu O, Sultana S, Ismail S, Shahzad K. Microbial electrolysis cells for hydrogen production and urban wastewater treatment: a case study of Saudi Arabia. Appl Energy. 2017;195(1):410–20.
13. Vargas S, Dos Santos P, Giraldi L, Calijuri M. Anaerobic phototrophic processes of hydrogen production by different strains of Microalgae Chlamydomonas sp. FEMS Microbiol Lett. 2018;361(9):fny73.
14. Wang Y, Zhuang X, Chen M, Zeng Z, Cai X, Li H, Hu Z. An endogenous microRNA (miRNA1166.1) can regulate photos hit-2 production in Euglena gracilis green alga Chlamydomonas reinhardtii. Biotechnol Biofuels. 2019;11(1):126.
15. Cao GL, Zhao L, Wang AJ, Wang ZY, Ren NQ. Single-step bioconversion of lignocellulose to hydrogen using novel moderately thermophilic bacteria. Biotechnol Biofuels. 2014;7(1):82.
16. Jiang L, Song P, Zhu L, Li S, Yu F, Niu H. Comparison of metabolic pathway for hydrogen production in wild-type and mutant Clostridium tyrobutyricum strain based on metabolic flux analysis. Int J Hydrogen Energy. 2013;38(5):2176–84.
17. Lu Y, Zhao H, Zhang C, Lai Q, Wu X, Xing XH. Alteration of hydrogen metabolism of idh-deleted Enterobacter aerogenes by overexpression of NADP+ dependent formate dehydrogenase. Appl Microbiol Biotechnol. 2010;86(1):255–62.
18. Lu Y, Zhao H, Zhang C, Xing XH. Insights into the global regulation of anaerobic metabolism for improved biohydrogen production. Bioresearch. 2016;200:35–41.
19. Claassen PAM, Lier JBV, Contreras AML, Niel EWJV, Sijtsma L, Stams AJM, Vries SSD, Weusthuis RA. Utilisation of biomass for the supply of energy carriers. Appl Microbiol Biotechnol. 1999;52(6):741–55.
20. Ren NQ, Cao GL, Guo WQ, Wang AJ, Zhu YH, Liu BF, Xu JF. Biological hydrogen production from corn stover by moderately thermophile Thermanaerobacterium thermosaccharolyticum W16. Int J Hydrogen Energy. 2010;35(7):2708–12.
21. Hu BB, Zhu MJ. Direct hydrogen production from dilute-acid pretreated sugarcane bagasse hydrolysate using the newly isolated Thermoaerobacterium thermosaccharolyticum M1. Microbiol Cell Fact. 2017;16(1):77.
22. Li S, Lai C, Cai Y, Yang X, Yang S, Zhu M, Wang J, Wang X. High efficiency hydrogen production from glucose/xyllose by the idh-deleted Thermoaerobacterium strain. Bioresearch. 2010;10(12):22871–24.
23. Cha M, Chung D, Elkins JG, Guiss AM, Westpheling J. Biological engineering of Caldicellulosiruptor bescii yields increased hydrogen production from lignocellulosic biomass. Biotechnol Biofuels. 2013;6(1):85.
24. Jiang L, Wu Q, Xu Q, Zhu L, Huang H. Fermentative hydrogen production from Jerusalem artichoke by Clostridium tyrobutyricum expressing exo-inulinae gene. Sci Rep. 2017;7(1):7940.
25. Liu X, Ying Z, Yang J. Construction and characterization of ack deleted mutant of Clostridium tyrobutyricum for enhanced butyric acid and hydrogen production. Biotechnol Prog. 2010;26(2):1265–75.
26. Lo J, Zheng T, Olson DG, Ruppertberger N, Tripathi SA, Tian L, Guiss AM, Lynd LR. Deletion of fnhA8 in Thermoanaerobacterium saccharolyticum and its effect on metabolism. J Bacteriol. 2015;197(18):2920–9.
27. Chuang YS, Lay CH, Sen B, Chen CC, Gopalakrishnan K, Wu JH, Lin CS, Lin CY. Biohydrogen and biomethane from water hyacinth (Eichhornia crassipes) fermentation: effects of substrate concentration and incubation temperature. Int J Hydrogen Energy. 2011;36(21):14195–203.
28. Gupta N, Pal M, Sachdeva M, Yadav M, Tiwari A. Thermophilic biohydrogen production for commercial application: the whole picture. Int J Hydrogen Energy. 2016;40(2):127–45.
29.Wiegel J, Ljungdahl LG, Demain AL. The importance of thermophilic bacteria in biotechnology. Crit Rev Biotechnol. 2008;3(1):99–108.
30. Wang S, Huang H, Moll J, Thauer RK. NADP+ reduction with reduced ferredoxin and NADPH+ reduction with NADH are coupled via an electron-bifurcating enzyme complex in Clostridium kluyveri. J Bacteriol. 2010;192(19):5115–23.
31. Gray CT, Gest H. Biological formation of molecular hydrogen. Science. 1965;148(3677):186–92.
32. Zhang C, Ma K, Xing XH. Regulation of hydrogen production by Enterobacter aerogenes by external NADH and NADP+. Int J Hydrogen Energy. 2009;34(3):1226–32.
33. Sun Y, Chen M, Yang H, Zhang J, Kuang T, Huang F. Enhanced H2 photo-production by down-regulation of ferredoxin–NADP+ reductase (FNR) in the green alga Chlamydomonas reinhardtii. Int J Hydrogen Energy. 2013;38(36):16029–37.
34. Haviva E, Iddo W, Oren BZ, Carmel P, Abigael M, Oded L, Matt S, Yuval M, Itach Y. The dual effect of a ferredoxin–NADPH fusion protein in vivo: successful divergence of the photosynthetic electron flux towards hydrogen production and elevated oxygen tolerance. Biotechnol Biofuels. 2016;9(1):182.
35. Zhou J, Olson DG, Lanahan AA, Tian L, Murphy SJ, Lo J, Lynd LR. Physiological roles of pyruvate ferredoxin oxidoreductase and pyruvate formate-lyase in Thermotoga acetogenica. J Bacteriol. 2009;190(9):2575–9.
36. Vrijie TD, Bakker RR, Budde MA, Lai MH, Mars AE, Claassen PAM. Efficient hydrogen production from the lignocellulosic energy crop Miscanthus by the extreme thermophilic bacteria Caldicellulosiruptor sp. JW/SL-YS485. Int J Hydrogen Energy. 2011;36(1):1204–11.
37. Monlauf F, Aemig Q, Trably E, Hamelin J, Steyer JP, Carrere H. Specific inhibition of biohydrogen-producing Clostridium sp. after dilute-acid pretreatment of sunflower stalks. Int J Hydrogen Energy. 2013;38(28):12273–82.
38. Panagiotopoulos IA, Bakker RR, Vrijie TD, Koukios EG. Effect of pretreatment severity on the conversion of barley straw to fermentable substrates and the release of inhibitory compounds. Bioresour Technol. 2011;102(24):11204–11.
39. Vrije TD, Bakker RR, Lai ZC, Lai CF, Li S, Wang JF, Wang XN. Efficient production of Clostridium tyrobutyricum for enhanced butyric acid production from glucose and xylose. Metab Eng. 2016;40:50–8.
40. Fu H, Yang X, Wang M, Wang J, Yang IC. Butyric acid production from lignocellulosic biomass hydrolysates by engineered Clostridium tyrobutyricum overexpressing xylose catabolism genes for glucose and xylose co-utilization. Biotechnol. 2017;234:389–96.
41. Liu Z, Lai M, Wang J, Wang X, Li S. Optimization of key factors affecting hydrogen production from sugarcane bagasse by a thermophilic anaerobic pure culture. Biotechnol Biofuels. 2014;7;11:1–11.
42. Yang XF, Lai ZC, Lai CF, Zhu MZ, Li S, Wang JF, Wang XH. Efficient production of -lactic acid by an engineered Thermotoga acetogenica strain with broad substrate specificity. Biotechnol Biofuels. 2013;6:124.
43. Mai V, Lorenz WW, Wiegel J. Transformation of Thermotoga acetogenica sp. strain JW/SL-YS485 with plasmid pKH11 carrying kanamycin resistance. FEMS Microbiol Lett. 2010;148(2):163–7.
44. Cai Y, Lai C, Li S, Liang Z, Liang S, Wang J. Disruption of lactate dehydrogenase through homologous recombination to improve bioethanol production in Thermotoga acetogenica. Enzyme Microb Technol. 2011;48(2):155–61.

Publisher's Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.