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Abstract



Biohydrogen production under hyper salinity stress by an anaerobic sequencing batch reactor with mixed culture

Ensiyeh Taheri^{1,2,3} · Mohammad Mehdi Amin^{1,3} · Ali Fatehizadeh^{1,3} · Hamidreza Pourzamani^{1,3} · Bijan Bina^{1,3} · Henri Spanjers⁴

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Abstract

Background This study investigated the effect of organic loading rate (OLR) and NaCl concentration on biohydrogen production by preheated anaerobic sludge in a lab scale anaerobic sequencing batch reactor (ASBR) fed with glucose during long time operation.

Methods During ASBR operation, the OLR was increased in steps from 0.5 to 5 g glucose/L.d and NaCl addition started at an OLR of 5 g glucose/L.d, to obtain NaCl concentrations in the reactor in the range of 0.5–30 g/L.

Results With an increasing OLR from 0.5 to 5 g glucose/L.d, the biohydrogen yield increased and reached 0.8 ± 0.4 mol H₂/mol glucose at an OLR of 5 g glucose/L.d. A NaCl concentration of 0.5 g/L resulted in a higher yield of biohydrogen (1.1 ± 0.2 mol H₂/mol glucose). Concentrations above 0.5 g/L NaCl led to decreasing biohydrogen yield and the lowest yield (0.3 ± 0.1 mol H₂/mol glucose) was obtained at 30 g/L of NaCl. The mass balance errors for C, H, and O in all constructed stoichiometric reactions were below 5%.

Conclusions The modified Monod model indicated that $r(H_2)_{max}$ and C_{crit} values were 23.3 mL H₂/g VSS/h and 119.9 g/L, respectively. Additionally, ASBR operation at high concentrations of NaCl shifted the metabolic pathway from acidogenic toward solventogenic.

Keywords Biohydrogen · Electron equivalent balance · Monod model · Probit analysis · Stoichiometric reaction

Background

In the field of energy production from organic wastes by biological processes, the biohydrogen production is an attractive option because of its benefits. These include, high energy yield and combustion without any harmful byproducts.

Biohydrogen can be produced by photosynthetic processes, photo fermentation, dark fermentation, and microbial electrochemical cells. Anaerobic fermentative hydrogen production (dark fermentation) is a more favorable option because of easy operation and its suitability for the demands of a sustainable development strategy. The dark fermentation process with anaerobic acidogenic culture is an effective way of harvesting H₂ from organic wastes [1–3].

The major limitation of dark fermentation is the production of a high acid concentration when the cultures are fed with a high concentration of sugar (high OLR). This shifts the biohydrogen production pathway to solvent production and leads to a decreasing yield of biohydrogen [4]. On the other hand, high concentrations of metals, including magnesium, sodium, zinc and iron can limit the cell mass growth and change the biohydrogen production pathway [4].

The other factor that can significantly affect biohydrogen production is salinity. This is present in many industrial wastewaters, such as effluents from the production of organic peroxides, pharmaceutical production, tanneries, seafood

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processing, petroleum refineries, and textile industries [5]. A low concentration of Na^+ is essential, for bacterial cell growth, adjusting cellular buffers, electrolytes and energy production. Therefore, a properly adjusted concentration of NaCl maximizes cell activity and biohydrogen production [6, 7]. However, when the NaCl concentration of the environment is above 10.16 g/L *Clostridium Butyricum*, a biohydrogen-producing species, consumes more energy for maintenance than for cell generation. Under these conditions, the biohydrogen production pathway changes from butyric to acetic synthesis and biohydrogen production yield decreases [7]. Xia et al., (2015) reported that a high salt concentration (11.7 g NaCl/L) leads to high ethanol production and inhibits the substrate utilization and biohydrogen production [8]. The adverse effect of salinity on biohydrogen production and substrate utilization has been reported by researchers, but there is no general agreement on inhibition concentrations. Different NaCl concentrations of 2.5, 10, 35.1, and 81.34 g/L were reported as inhibitive in different studies [7–10].

For the prediction of the correlation between different variables, including influent carbohydrate concentration, F/M ratio, gaseous and soluble products and biohydrogen production, a mathematical model can be used. In anaerobic processes, mathematical models are used to describe the relationships between the design data and experimental results [11, 12]. Kinetic models can adequately describe the relationship between the different state variables and be used for analysis, design, and operation of any fermentation process [13, 14]. Bacterial growth kinetics are based on two fundamental relationships: growth rate and substrate utilization rate [15].

In general, kinetic models are classified as either structured or unstructured models. Structured models take metabolic pathways into consideration and are generally complicated. In these models, the biochemical and physiological aspects of growth and metabolite synthesis are considered simultaneously, and the key fermentation rates are expressed and evaluated with respect to substrate consumption and end-product inhibitory effects. The unstructured kinetic models usually consider microorganisms as a component or reactant, and they are much simpler than the structured ones [13, 16]. The unstructured kinetic models are frequently employed for modeling microbial systems because they are simple, yet can provide useful information about the process [14]. Using unstructured kinetic models, such as Michaelis–Menten and the Logistic model, Mu et al., studied the kinetics of the hydrogen production process by mixed anaerobic cultures in short batch experiments and reported that the Logistic model, Michaelis–Menten model, and modified Gompertz model can describe the kinetics of biomass growth, substrate utilization, and product formation very well [13]. Yuan et al., showed in short batch experiments that the products' formation kinetics can describe the formation of the main products of anaerobic

biohydrogen production (e.g. hydrogen, acetic and butyric acid) very well [16].

However, information about the effect of NaCl addition on biohydrogen production in an ASBR by pretreated mixed culture is still limited and a comprehensive kinetic and stoichiometric model might become a useful tool in assisting the identification of rate-determining factors. So, this study aimed:

- To evaluate different salt concentrations effects on biohydrogen production by mixed culture during long-term operation.
- To model inhibition of biohydrogen production by NaCl in an ASBR fed with glucose.
- To investigate the electron equivalence (e^- eq) balance and stoichiometric reaction of biohydrogen production from glucose.
- To evaluate the substrate utilization and microbial growth kinetics by kinetic models.

Materials and methods

ASBR start up and operation

A plexiglas column (internal diameter 18.8 cm, height 38 cm) with a total volume of 10 L (working volume 9 L and 1 L headspace) was used as an ASBR in this study. The ASBR was operated at a hydraulic retention time of 4.5 d and a 24 h cycle time (30 min feeding, 22 h reaction, 1 h sedimentation and 30 min decantation). During the reaction phase, the ASBR content was well mixed (150 s mixing at 80 rpm and 750 s idle) with an electrical mixer. The ASBR was enclosed within a hot water bath to maintain a temperature of 37 °C.

The anaerobic inoculum was harvested from a full scale anaerobic digester (South wastewater treatment plant, Tehran, Iran). Before inoculation, the anaerobic sludge was sieved through standard mesh No. 16 and then heated for 30 min at 100 °C to enrich biohydrogen producing bacteria, in accordance with findings from our previous study [17].

Synthetic wastewater was used that contained all the macro and micro elements needed for bacterial growth and activation, as found in our previous study [18]. Glucose was used as the sole carbon source and its concentration was varied to achieve OLRs of 0.5, 1, 2, 3, and 5 g glucose/L.d. When the OLR reached 5 g glucose/L.d, the NaCl addition was started to achieve reactor concentrations from 0.5–30 g/L. Each stage of ASBR operation (OLR and NaCl addition) was continued until steady state conditions, as judged by gas production and carbohydrate conversion.

Analysis

During operation, influent and effluent chemical oxygen demand (COD), pH, alkalinity, and carbohydrate concentration were routinely measured by closed reflux colorimetry, pre-calibrated glass body pH probe (CG 824 SCHOTT), titration, and phenol-sulfuric acid methods, respectively. In addition, total suspended solids (TSS), volatile suspended solids (VSS), and mixed liquid suspended solids (MLSS) were determined by weighing the glass fiber filter after being dried at 105 °C and burned at 550 °C [19]. In the headspace of the ASBR, the H₂ percentage of produced biogas was determined by a hydrogen analyzer (COSMOS-XP-3140 model, Japan).

The extracellular polymeric substances (EPS) were extracted following the heating method of Cosenza et al., [20]. The sample, which was taken from the ASBR during the reaction phase, was centrifuged at 5000 rpm for 5 min. To rinse the sludge pellet, it was resuspended in pure water to its original volume in a centrifugal tube and then centrifuged at 5000 rpm for 3 min after which the supernatant was discarded. After that, the sludge pellet was re-suspended in pure water to its original volume for the second time. The sludge suspension was heated at 80 °C in a water bath for 10 min, and then centrifuged at 5000 rpm for 5 min. After filtration through a 0.45 μm filter the supernatant was subjected to carbohydrate [21] and protein [22] analysis.

After filtration through filter paper (Whatman No. 42), the ASBR effluent was analyzed for soluble end products (SEPs); such as volatile fatty acids (VFAs), including acetic, propionic, butyric and valeric acid; and for solvents, including methanol, ethanol and acetone. The VFAs were extracted via liquid-liquid extraction by diethyl ether and analyzed with a gas chromatograph equipped with a flame ionization detector (GC-FID, Agilent 7890A GC with Varian CP- Sil5cb column) following the method of Manni et al. [23].

The extraction and quantification of solvents was done by first pouring a 2 mL sample into a standard vial (10 mL) that contained 1 g of NaCl, 70 μL of 1 g/L isobutanol solution and 200 μL of 2 M H₂SO₄ solution. The vial was then analyzed by a GC-FID equipped, be Combi-Pal auto sampler using 10 mL headspace vials and a 2.5 mL HD type Hamilton gas-tight syringe following the method of Adorno et al. [24].

Mathematic calculation

Inhibition model

To evaluate the non-competitive inhibitory effect of NaCl on biohydrogen production, the Modified Monod model was used (Eq. (1)).

$$r(H_2) = r(H_2)_{\max} \left(1 - \frac{C}{C_{crit}}\right)^n \left(\frac{S}{K_s + S}\right) \quad (1)$$

At a non-limiting glucose concentration, that is: $S > K_s$, Eq. (1) can be simplified to Eq. (2) [25, 26]:

$$r(H_2) = r(H_2)_{\max} \left(1 - \frac{C}{C_{crit}}\right)^n \quad (2)$$

where $r(H_2)$ and $r(H_2)_{\max}$ are biohydrogen production and maximum biohydrogen production and referring to divided amount of biohydrogen per each gram of VSS and cycle time (mL H₂/g VSS/h). Nonlinear least squares regression analysis was used to estimate the model parameters (n , $r(H_2)_{\max}$ and C_{crit}) by using the “Solver” function in Microsoft Excel 2013.

A previous study stated that the extent of inhibition is usually demonstrated by the relative activity and was the obtained biohydrogen production under various NaCl in comparison of control [26]. In this study, the hydrogen production activity of ASBR during operation (without and with NaCl) as a function of $r(H_2)$ was monitored. The obtained data was subjected to Probit analysis by using IBM SPSS statistics 20 to the assess $C_{I,50}$ concentration.

Substrate utilization kinetics

The substrate degradation was assessed by using the Monod model [13] according to Eq. (3).

$$v = \frac{v_m S}{K_s + S} \quad (3)$$

From Eq. (3), the v_m and K_s were calculated by a nonlinear method using the “Solver” function in Microsoft Excel 2013.

Microbial growth kinetics

The microbial growth during biohydrogen production can be expressed by Eq. (4) as the Logistic model [13].

$$\frac{dX}{dT} = k_c X \left(1 - \frac{X}{X_{\max}}\right) \quad (4)$$

Equation (5) is the integrated form of Eq. (4) and was used for prediction of microbial cell concentration.

$$X = \frac{X_0 \exp(k_c t)}{1 - (X_0/X_{\max})(1 - \exp(k_c t))} \quad (5)$$

Data availability The data will not be shared with a reason, in this section.

Results and discussion

Biohydrogen production

Figure 1 depicts the obtained data on biohydrogen production during ASBR operation as a function of the applied OLR and NaCl concentration in the reactor.

As shown in Fig. 1, the biohydrogen production increased as applied OLR rose. When the applied OLR increased from 0.5 to 5 g glucose/L.d, the biohydrogen production increased from 0.2 ± 0.1 to 6.99 ± 0.9 L/d. Shida et al., operated two anaerobic fluidized bed reactors with and without pH buffer and reported that the biohydrogen production increased from 10 to 95 and from 12 to 76 L/d, respectively, when OLR increased from 19 to 140.6 g glucose/L.d [27].

As shown in Fig. 1, the biohydrogen yield increased as a function of the applied OLR and the highest value (0.8 ± 0.4 mol H₂/mol glucose) achieved, occurred at the highest studied OLR (5 g glucose/L.d). This is 2.5 times more than its value at an OLR of 0.5 g glucose/L.d (0.32 ± 0.1 mol H₂/mol glucose). Lee and Rittman reported that, based on stoichiometric reactions, the biohydrogen yield increased when initial glucose increased from 0.8 to 6.0 g/L [28].

The variation in biohydrogen production yield as OLR increases does not have a same trend in different studies and the reason for this is not clear. The important parameters used to predict biohydrogen yield variation are type and concentration of VFAs and carbon conversion. High accumulation of VFAs in the solution led to inhibition of biohydrogen producing bacteria activity. Also, the highest biohydrogen yield is related to the highest substrate conversion [27, 29].

The reduction of biohydrogen production at high concentrations of glucose could be attributed to substrate inhibition and or product inhibition or could be due to solution pH reduction [30].

In this study, the highest biohydrogen yield (1.1 ± 0.2 mol H₂/mol glucose) was achieved at 0.5 g/L of NaCl. A higher yield at 0.5 g/L compared to 0 g/L may be related to the higher Na⁺ concentration outside the cell, which created the proper unbalance Na⁺ gradient and results in more substrate being actively transport into the cell [7]. Lee et al., (2012) reported that the low concentrations of Na⁺ induced microbial growth and substrate consumption enhancement and depicts less than 1.67 g/L of Na⁺ for acid pretreated anaerobic sludge [7].

An increase in the NaCl concentration from 0.5 g/L to 20 g/L led to decreases in the biohydrogen production from 6.5 ± 0.9 to 5.4 ± 0.1 L/d. At a NaCl concentration of 30 g/L, the biohydrogen production was only 2.1 ± 0.1 L/d and the corresponding biohydrogen yield was 0.3 ± 0.1 mol H₂/mol glucose.

The promotion of biohydrogen production at specific concentrations of metal ions was reported in a previous study. In that study, a significant reduction in biohydrogen yield occurred at NaCl concentrations of 35.1, 2.5, 10 and 81.3 g NaCl/L [7, 8, 10, 24]. When the NaCl concentration in the reactor is high, the bacteria cell pumps Na⁺ across the cell membrane to adjust the Na⁺ gradient and is an energy consuming process [7]. Therefore, the low biohydrogen production at high concentrations of NaCl may be attributed to the inhibitive effect of the salt, low substrate conversion, high energy consumption, and cell lysis [7, 8].

Fig. 1 Biohydrogen production (as biohydrogen production and average hydrogen yield) as a function of OLR and NaCl concentration in the reactor

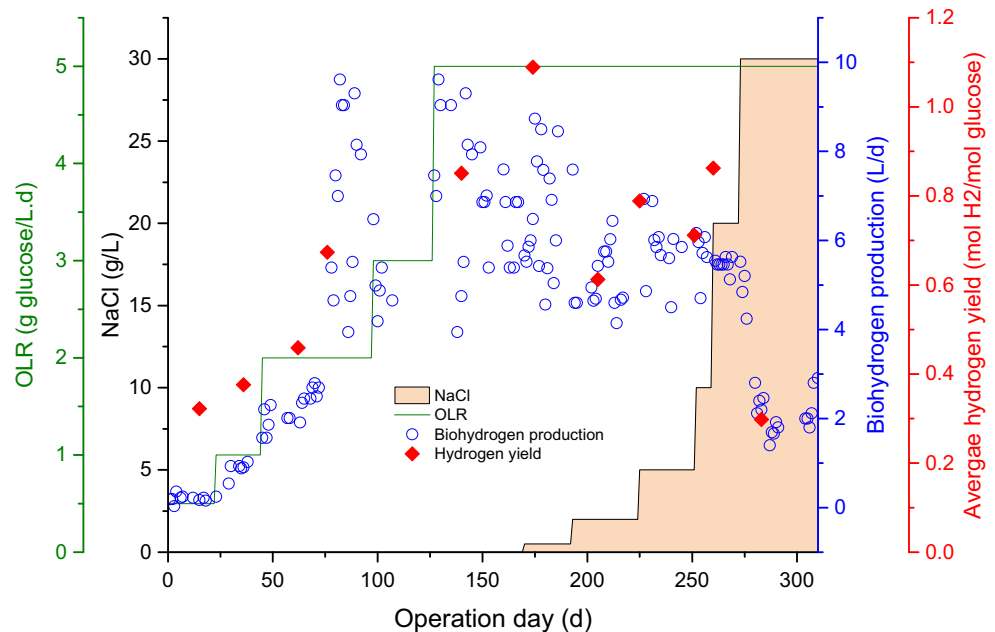
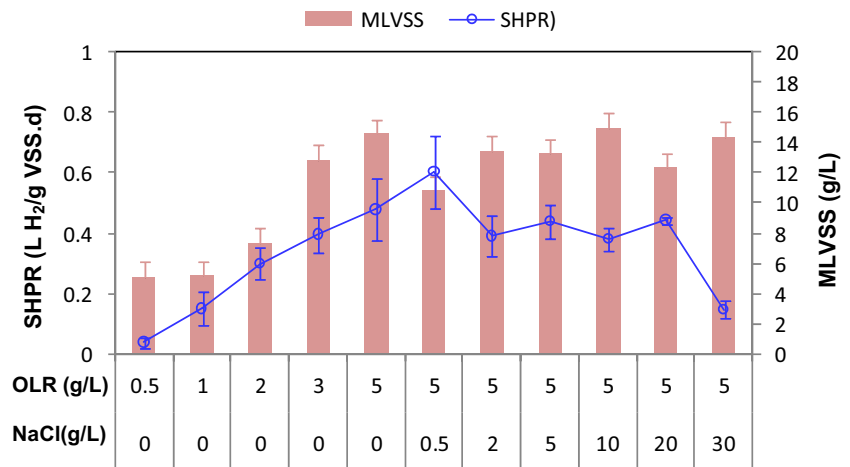


Fig. 2 SHPR and MLVSS as a function of OLR and NaCl concentration



The average of specific biohydrogen production rate (SHPR) and MLVSS during ASBR operation is shown in Fig. 2.

As shown in Fig. 2, when the applied OLR increased from 0.5 to 5 g glucose/L.d, the average SHPR increased from 0.04 ± 0.01 to 0.48 ± 0.1 L/g VSS.d. The highest SHPR (0.6 ± 0.1 L/g VSS.d) was obtained at 0.5 g/L of NaCl and the SHPR decreased to 0.15 ± 0.03 L/g VSS.d at a NaCl concentration of 30 g/L. The obtained SHPRs in this study are significantly lower than reported by Zheng et al., who reported from batch experiments that when the NaCl concentration increased from 0 to 29.22 g/L of NaCl the SHPR decreased from 3.382 to 0.502 L/g VSS.d [31]. These differences are presumably related to different reactor type, operating conditions, and the sole carbon source concentration.

Inhibition model

The $r(H_2)$ was evaluated as a function of NaCl concentration to find out the effect of NaCl on biohydrogen production (Fig. 3).

As seen in Fig. 3, the $r(H_2)$ during ASBR operation (OLR: 5 g glucose/L.d) with NaCl addition initially increased and

then decreased at higher NaCl concentrations. The obtained results show that with increasing NaCl concentrations from 0 to 0.5 g/L, $r(H_2)$ increased from 20.8 mL H₂/g VSS/L to 25.2 mL H₂/g VSS/L. However, when the NaCl concentrations were higher than 0.5 g/L, the biohydrogen production decreased. In addition, from the non-linear regression analysis (Fig. 3), the inhibition model parameters were calculated as $r(H_2)_{max}$ of 23 mL H₂/g VSS/L, C_{crit} of 120 g/L and n of 3.82. The relatively high determination coefficient (r^2 : 0.91) demonstrated that the inhibition model was able to well describe the trend of $r(H_2)$ with the inhibitory effect of NaCl.

As previously mentioned, in order to evaluate the inhibition caused by an inhibitor, the relative activity is used [31]. Figure 4 shows the relative activity of $r(H_2)$ as a function of NaCl concentration and also an estimation of the NaCl concentration at which the activity of biohydrogen production was reduced by 50% ($C_{I,50}$).

The Probit analysis estimated that at 60 g/L of NaCl, the relative activity of the hydrogen producing bacteria was only 2% of the activity without the presence of NaCl. From Fig. 4, the $C_{I,50}$ value of NaCl was estimated at 25 g/L, which was higher than that reported by Lee et al., (18.5 g/L of NaCl) [7] and lower than that reported by Zheng et al., (26.5 g/L of

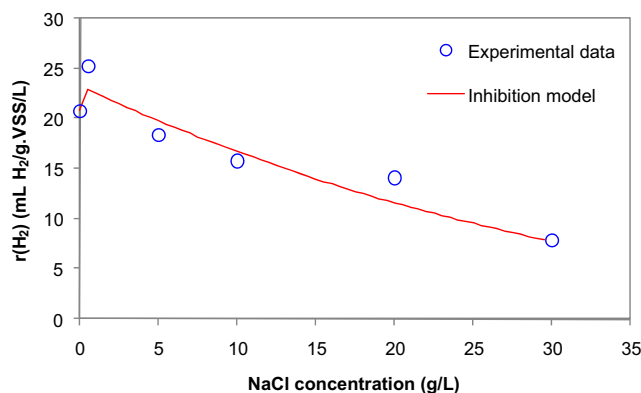


Fig. 3 Biohydrogen production rate as a function of NaCl concentration: experimental data and model (Eq. 2) fit

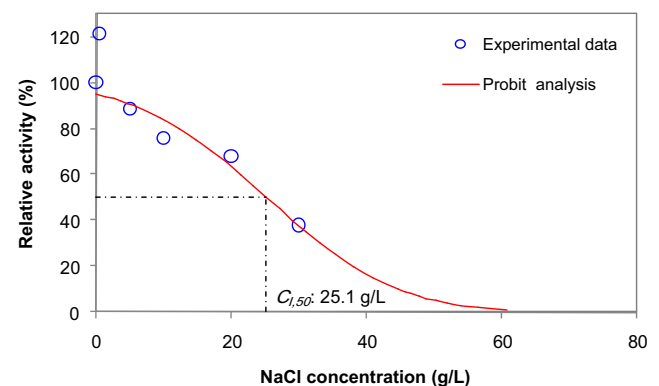


Fig. 4 Effect of NaCl addition on relative activity of SHPR

NaCl) [31]. These differences are presumably due to substrate type, reactor type and operation and adaptation period [7, 31].

COD removal and glucose conversion

The COD removal efficiency and glucose conversion efficiency during ASBR operation are depicted in Fig. 5.

As shown in Fig. 5, with increasing OLR from 0.5 to 5 g glucose/L.d, the average COD removal decreased from $30 \pm 8\%$ to $14 \pm 4\%$. The steepness in the trend of COD removal against OLR was in line with a previous study: Intanoo et al. (2012) demonstrated that the highest achieved COD removal (32%) was obtained at a COD loading rate equal to 68 g/L.d and the COD removal decreased as the COD loading rate increased to 79 g/L.d. This related to the toxic effect of VFA accumulation [32]. Overall, during ASBR operation, the COD removal was lower than 40%. Fermentation is an anaerobic redox process in which organic material is the electron donor and internal cell products are the electron acceptors [33]. If both the electron donor and acceptor are organic material, then only one organic compound is converted to another organic compound, hence the COD removal is not significant. Studies, such as by Ren et al. [34] and Mohan et al. [35] reported that in biohydrogen production processes, the COD removal was lower than 50%.

At constant OLR, equal to 5 g glucose/L.d, the NaCl concentration was increased stepwise from 0.5 to 2, 5, 10, 20 and 30 g/L. At this time, the obtained COD removal was 10.2 ± 0.9 , 8.1 ± 3 , 10.2 ± 4 , 24.8 ± 6.5 , 15.8 ± 3.8 and $13 \pm 5\%$, respectively. During the NaCl addition period, when the NaCl concentration was 10 and 20 g/L, the highest COD removal was obtained. These results are in line with Guo et al. [36]. The COD removal increase was presumably related to a higher energy requirement for adaptation to the saline environment [36, 37].

A higher NaCl concentration (30 g/L) resulted in a slight decrease of the COD removal efficiency ($13 \pm 5\%$) and was probably related to an inhibitory effect of the saline environment on bacterial activity.

In this study, glucose was used as the sole carbon source and glucose conversion was more than 80% during operation, except when NaCl concentration was 30 g/L. As shown in Fig. 5, when the applied OLR was 0.5 to 1, 2, 3, and 5 g COD/L.d, the average glucose conversion efficiency was 82.1 ± 0.9 , 90.6 ± 0.4 , 93.9 ± 0.7 , 87.7 ± 0.6 , and 92.7 ± 0.5 , respectively. This demonstrated that the increase in OLR does not have an adverse effect on the glucose conversion by biohydrogen producing bacteria.

At a constant applied OLR of 5 g glucose/L.d and NaCl concentrations of 0.5, 2, 5, 10 and 20 g/L, the averages of glucose conversion were 95.2 ± 0.6 , 94.2 ± 2.7 , 95.5 ± 0.3 , 94.9 ± 1.9 and 94.9 ± 0.5 percentage, respectively. However, when the NaCl was supplied at a concentration was increased to 30 g/L, the glucose conversion promptly dropped to $39.8 \pm 5.6\%$. This may be due to the inhibitory effect of high salinity on the hydrogen producing bacteria [36].

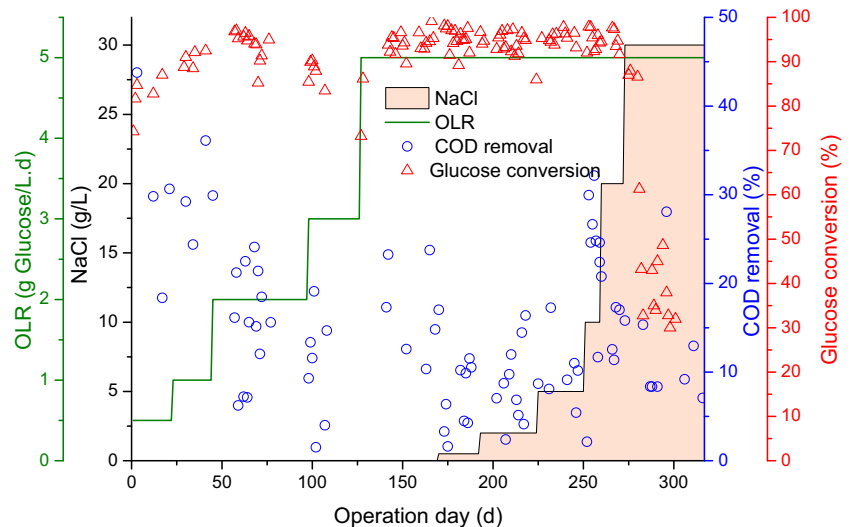
Anaerobic kinetic models

To assess the Monod kinetic parameters ν_m and K_s , the degradation of glucose under high NaCl concentrations in the ASBR was monitored (Fig. 6).

The parameters of the Monod model were fitted with Eq. (3) and are summarized in Table 1.

As seen in Table 1, the values of ν_m and K_s obtained in this study are relatively higher than those found in other studies. A higher K_s value indicates that the bacteria have a lower affinity to the substrate. As for the biohydrogen process, this showed that glucose was not as readily biodegradable compared to sucrose. The difference might be

Fig. 5 COD removal and glucose conversion efficiencies at different OLR and NaCl concentrations



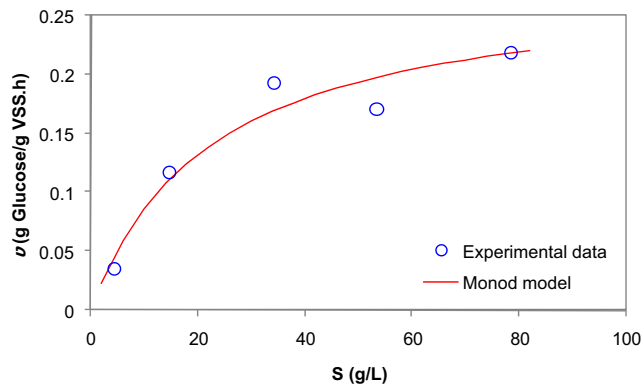


Fig. 6 Glucose degradation rate as a function of the glucose concentration: experimental data and model (Eq. 3) fit

attributed to type of process, operational conditions, substrate type and concentration [13, 39, 40].

In addition, the Logistic model (Eq. 5) was applied for the mixed culture growth during ASBR operation (Fig. 7).

The r^2 value for the Logistic model fit was >0.94 indicating satisfactory agreement of the Logistic model to experimental data from the ASBR. The estimated values of k_c and X_{max} for mixed culture growth were 0.01 1/h and 38.12 g VSS/L, respectively. Mu et al., reported k_c and X_{max} values of 0.07 1/h and 9.46 g VSS/L, respectively, in a batch experiment. The X_{max} value are much lower than those found in this study and relate to the fact that the Logistic model does not involve a substrate term [13].

Soluble end products (SEPs)

The quantity and type of SEPs formed by the fermentation process is highly related to the organisms involved and metabolic pathways [33]. In this study, the ASBR effluent was analyzed for VFAs and solvents as major liquid fermentation products. Figure 8 shows the VFA and solvent concentrations during ASBR operation.

It is observed from Fig. 8 that except for operation at OLR of 0.5 g glucose/L.d, ethanol was detected in the effluent. The average of SEPs produced at OLRs of 0.5, 1, 2, 3, and 5 g glucose/L.d, were 1529 ± 52 , 2050 ± 40 , 3274 ± 810 , 3540 ± 301 , and 6358 ± 720 g/L, respectively. When the OLR increased, the SEPs production increased, which is in line with Zhang et al., who studied biohydrogen production from saline wastewater by halophilic bacteria [29].

Table 1 Monod parameters present study compared with previous studies

Process type	ν_m (g/g VSS.h)	K_s (g/L)	Substrate	Reference
Aerobic	0.11	0.1–0.6	Domestic wastewater	[38]
Biohydrogen	0.28	13.5	Sucrose	[5]
Methanogenic	0.03	2.1	Glucose	[39]
Biohydrogen	0.28	22.5	Glucose	Present study

With increasing OLR from 0.5 to 5 g glucose/L.d, an increase in the concentration of acetic and butyric acid was observed. In the case of ethanol, for OLR increasing from 0.5 to 3 g glucose/L.d an increasing trend in concentration was observed. However, this did not hold for other OLR values. The increasing acetic and butyric acid and also ethanol concentration with OLR showed that biohydrogen production was especially related to the bacterial group but also to the metabolic pathway. The biohydrogen process followed by the acetate/butyrate pathway has acetic and butyric acid as dominant SEPs [28] and in this study, the biohydrogen production was coincided with acetate/butyrate pathway.

With NaCl addition (0.5 g/L), the SPES continued increasing and reached 9301 ± 91 mg/L. With continued addition of NaCl from 0.5 to 2, 5, 10, 20, and 30 g/L, the SEPs concentration decreased and reached the lowest concentration (2751 ± 550 mg/L as acetic acid) at 30 g/L NaCl addition.

Despite increasing SEPs production as a function of OLR during ASBR operation, the NaCl addition led to a varying SEPs production. This is presumably due to the increase of bacteria activity with increasing OLR and changing in the dominant bacteria species involved in biohydrogen production.

During ASBR operation, ethanol production started at an OLR of 1 g glucose/L.d and the lowest and highest concentrations of produced ethanol were 140 ± 38 and 870 ± 290 mg/L and these were obtained at an OLR of 1 g glucose/L.d without NaCl addition and an OLR of 5 g glucose/L.d with 30 g/L of NaCl, respectively. The production of other solvents (methanol and acetone) was monitored but not detected. This was likely due to the elimination of methanobacteria during anaerobic sludge heat treatment. The ethanol production by halo tolerant bacteria during biohydrogen fermentation is due to their enzymes' resistance to high salinity and their ability to utilize organic matter in a saline environment [41–44]. Biohydrogen production from food waste by *Enterobacter sp. T4384*, a NaCl tolerant bacterial strain, resulted in 947 mL H₂/L of reactor and in addition, 3.2 g/L, and 0.2 g/L, of ethanol and acetic acid, respectively [10].

Electron equivalence balance and stoichiometric reaction

The electron equivalence (e^- eq) balance during ASBR operation was evaluated following the procedure described in a

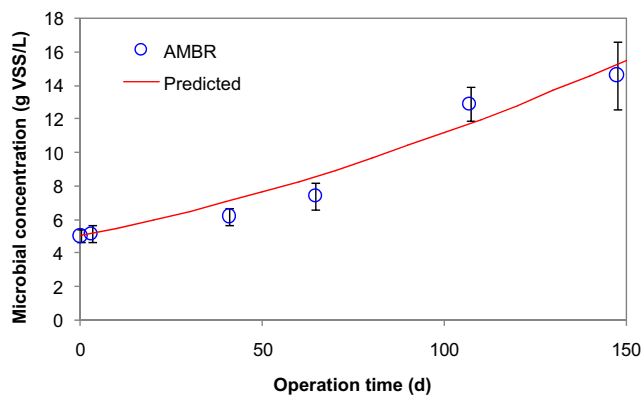


Fig. 7 Microbial concentration as a function of time: experimental data and fit of Logistic model for biohydrogen producing mixed culture growth

previous study [28]. All experimentally measured end products including, soluble and gaseous products, biomass and residual glucose were converted to e^- eq. The e^- eq and calculated fractions of end product e^- eq distributions (in percentage) are summarized in Table 2. Overall, during ASBR operation, the e^- eq balances closed within 4.6 to 24.2% which is relatively good [18]. During NaCl addition, the worst e^- eq balance closure was obtained and likely relates to not measured SEPs. The highest portion of electrons resulting in H_2 (13.8%) was obtained at 0.5 g/L of NaCl, which supports the promoting effect of NaCl on H_2 production. During ASBR operation, except at 30 g/L of NaCl, residual glucose e^- eq ranged between 1.5 and 15% and demonstrates higher glucose conversion. The relatively constant concentration of acetate during ASBR operation demonstrates that acetate production is universal for the conversion of ATP in biohydrogen fermentation processes [18].

During biohydrogen production, after accurate quantifying of end products, the stoichiometry of substrate conversion to products can be analyzed [18]. We used a procedure, proposed in previous research, for constructing the stoichiometric

reaction during ASBR operation [18, 28]. The overall stoichiometric reaction without biomass synthesis was constructed and is summarized in Table 3.

The mass balance errors for C, H, and O in all constructed stoichiometric reactions were below 5%. Moreover, the biohydrogen yields were slightly higher than the experimentally observed biohydrogen yield (Fig. 1), because the e^- eq of biomass was not considered in the stoichiometry due to the energetic concept of reaction construction.

Protein and carbohydrate as dominant portion of EPS

The measured associated protein and carbohydrate in EPS at various NaCl concentrations is depicted in Fig. 9.

The EPS is comprised of carbohydrates and proteins as dominant components that are released by microorganisms. As seen in Fig. 9, at an OLR of 5 g glucose/L.d, with an increasing NaCl concentration from 0 to 10 g/L, the amount of protein and carbohydrate increased, probably due to the ionic strength that is a function of salinity [45]. Vyrides and Stuckey (2009) reported that EPS production is a natural response to osmotic stress [38]. When the NaCl was increased from 10 to 30 g/L, the concentration of EPS decreased, which may have been due to biodegradation of EPS by the cell, EPS release to the medium [38], or deactivation of bacteria because of salt's inhibitory effect.

Variation of effluent TSS and VSS during ASBR operation

Figure 10 depicts TSS and VSS in ASBR effluent as function of OLR and NaCl addition. As shown in Fig. 10, with an increasing OLR from 0.5 to 5 g glucose/L.d, the TSS and VSS concentration in the ASBR effluent increased. As previously mentioned greater biohydrogen production was observed as the OLR increased (Fig. 1) and this led to more sludge resuspension, which appeared in the effluent. As the

Fig. 8 Concentration of SEPs during ASBR operation as function of OLR and NaCl addition

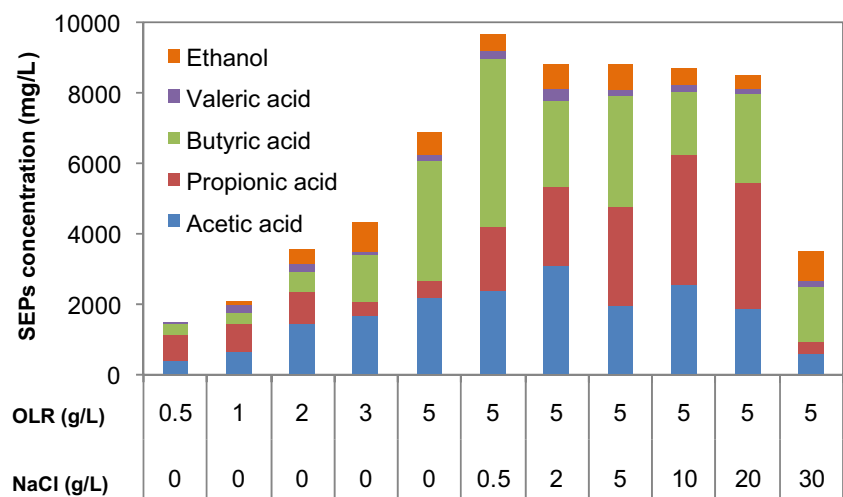


Table 2 Average of end product e^- eq distribution fractions during ASBR operation function of applied OLR and NaCl addition

Compounds	ASBR operation stage										
	0.5 g/L.d	1 g/L.d	2 g/L.d	3 g/L.d	5 g/L.d	5 g/L.d, 0.5 g NaCl/L	5 g/L.d, 2 g NaCl/L	5 g/L.d, 5 g NaCl/L	5 g/L.d, 10 g NaCl/L	5 g/L.d, 20 g NaCl/L	5 g/L.d, 30 g NaCl/L
Influent glucose	587 (100%)	1302.6 (100%)	3041.6 (100%)	3858.1 (100%)	5251.1 (100%)	5251.4 (100%)	5251.4 (100%)	5251.4 (100%)	5251.4 (100%)	5251.4 (100%)	5251.4 (100%)
Acetate	104.6 (18.7%)	439.1 (38.9%)	657.9 (27.2%)	718.3 (21.1%)	722.6 (17.3%)	643.1 (15.9%)	834.5 (22.5%)	526.5 (13.1%)	690.5 (18.5%)	770.2 (19.9%)	698.2 (17.9%)
Propionate	277.5 (49.6%)	312.9 (27.7%)	347.3 (14.4%)	154.6 (4.5%)	262.2 (6.3%)	685.5 (16.9%)	869.3 (23.4%)	1082.5 (27.1%)	1399.8 (37.6%)	1378.8 (35.6%)	117.7 (3.1%)
Butyrate	137.1 (24.5%)	149.6 (13.3%)	916.6 (37.9%)	1484.2 (43.5%)	1791.2 (42.9%)	1837.3 (45.5%)	1096.4 (29.6%)	1453.5 (36.3%)	843.8 (22.7%)	1148.1 (29.6%)	731.9 (18.7%)
Ethanol	0 (0%)	73.1 (6.5%)	215.2 (8.9%)	430.9 (12.6%)	315.2 (7.6%)	254.8 (6.3%)	358.1 (9.7%)	394.4 (9.8%)	264.2 (7.1%)	194.4 (5.1%)	453.7 (11.7%)
Biomass	0.351 (0.06%)	33.9 (3.1%)	51.9 (2.1%)	23.2 (0.7%)	12.9 (0.3%)	33.7 (0.8%)	9.8 (0.2%)	32.2 (0.8%)	0.3 (0.01%)	0.3 (0.01%)	46.8 (1.2%)
Residual glucose	21.58 (3.9%)	44.4 (3.9%)	46.1 (1.9%)	212.9 (6.2%)	599.3 (14.4%)	29.9 (0.8%)	113.7 (3.1%)	59.2 (1.5%)	80.6 (2.2%)	58.3 (1.5%)	1678.2 (42.9%)
Hydrogen	18.99 (3.4%)	75.2 (6.7%)	181.8 (7.5%)	385.9 (11.3%)	468.8 (11.2%)	557.4 (13.8%)	427.4 (11.5%)	460.2 (11.5%)	444.6 (11.9%)	327.4 (8.4%)	178.2 (4.7%)
Total	560.1	1128.2	2416.8	3410.3	4172.1	4041.7	3709.1	4008.4	3723.7	3877.5	3904.8
Δe^- meq	4.6%	13.4%	20.5%	11.6%	20.5%	17.4%	24.2%	18.1%	23.9%	20.8%	20.2%

Units are in e^- meq and in parenthesis as %

$$i(\%) = \left(\frac{e_{eq,i}^-}{e_t^-} \right) \times 100$$

$\Delta e^- meq(\%) = \left(\frac{e_{glu,i}^- - e_t^-}{e_{glu,i}^-} \right) \times 100$, $e_{glu,i}^-$ is e^- meq of initial glucose, e_t^- is the sum of e^- meq of SEP, biomass, bioH₂ and residual glucose. 1 mol glucose: 24 e^- eq, 1 mol acetate: 8 e^- eq, 1 mol propionate: 14 e^- eq, 1 mol butyrate: 20 e^- eq, 1 mol ethanol: 12 e^- eq, 1 mol biomass: 20 e^- eq, 1 mol hydrogen: 2 e^- eq

Table 3 Overall stoichiometric reactions of biohydrogen production by ASBR

OLR (g glucose/L.d)	Influent NaCl (g/L)	Balanced reaction
0.5	0	$C_6H_{12}O_6 + 0.14 H_2O = 0.58 C_2H_3O_2^- + 0.88 C_3H_5O_2^- + 0.31 C_4H_7O_2^- + \mathbf{0.42 H_2} + 0.96 CO_2 + 2.66 H^+$
1	0	$C_6H_{12}O_6 + 0.90 H_2O = 1.25 C_2H_3O_2^- + 0.51 C_3H_5O_2^- + 0.17 C_4H_7O_2^- + 0.14 C_2H_5OH + \mathbf{0.86 H_2} + 1.0 CO_2 + 2.96 H^+$
2	0	$C_6H_{12}O_6 + 0.53 H_2O = 0.85 C_2H_3O_2^- + 0.26 C_3H_5O_2^- + 0.47 C_4H_7O_2^- + 0.18 C_2H_5OH + \mathbf{0.94 H_2} + 1.26 CO_2 + 2.31 H^+$
3	0	$C_6H_{12}O_6 + 0.74 H_2O = 0.68 C_2H_3O_2^- + 0.08 C_3H_5O_2^- + 0.56 C_4H_7O_2^- + 0.27 C_2H_5OH + \mathbf{1.46 H_2} + 1.61 CO_2 + 1.96 H^+$
5	0	$C_6H_{12}O_6 + 0.69 H_2O = 0.61 C_2H_3O_2^- + 0.13 C_3H_5O_2^- + 0.60 C_4H_7O_2^- + 0.18 C_2H_5OH + \mathbf{1.58 H_2} + 1.64 CO_2 + 1.93 H^+$
5	0.5	$C_6H_{12}O_6 + 0.71 H_2O = 0.48 C_2H_3O_2^- + 0.30 C_3H_5O_2^- + 0.55 C_4H_7O_2^- + 0.13 C_2H_5OH + \mathbf{1.68 H_2} + 1.63 CO_2 + 1.98 H^+$
5	2	$C_6H_{12}O_6 + 0.87 H_2O = 0.70 C_2H_3O_2^- + 0.42 C_3H_5O_2^- + 0.37 C_4H_7O_2^- + 0.20 C_2H_5OH + \mathbf{1.43 H_2} + 1.50 CO_2 + 2.31 H^+$
5	5	$C_6H_{12}O_6 + 0.66 H_2O = 0.40 C_2H_3O_2^- + 0.47 C_3H_5O_2^- + 0.44 C_4H_7O_2^- + 0.20 C_2H_5OH + \mathbf{1.41 H_2} + 1.59 CO_2 + 2.08 H^+$
5	10	$C_6H_{12}O_6 + 0.88 H_2O = 0.57 C_2H_3O_2^- + 0.66 C_3H_5O_2^- + 0.28 C_4H_7O_2^- + 0.14 C_2H_5OH + \mathbf{1.46 H_2} + 1.49 CO_2 + 2.43 H^+$
5	20	$C_6H_{12}O_6 + 0.53 H_2O = 0.60 C_2H_3O_2^- + 0.62 C_3H_5O_2^- + 0.36 C_4H_7O_2^- + 0.10 C_2H_5OH + \mathbf{1.03 H_2} + 1.29 CO_2 + 2.42 H^+$
5	30	$C_6H_{12}O_6 + 0.81 H_2O = 0.96 C_2H_3O_2^- + 0.09 C_3H_5O_2^- + 0.40 C_4H_7O_2^- + 0.41 C_2H_5OH + \mathbf{0.98 H_2} + 1.36 CO_2 + 2.25 H^+$

The H₂ yield presents as bold value

NaCl was introduced to the ASBR, the effluent TSS and VSS increased due to sludge break down. Amin et al., (2014) demonstrated that the sedimentation problem coincides with the biological wastewater treatment of saline wastewater [5].

As seen in Fig. 10, the 20 and 30 g/L of NaCl, lower concentrations of TSS and VSS were observed. At these NaCl concentrations, the biohydrogen production decreases and induces lower levels of sludge resuspension.

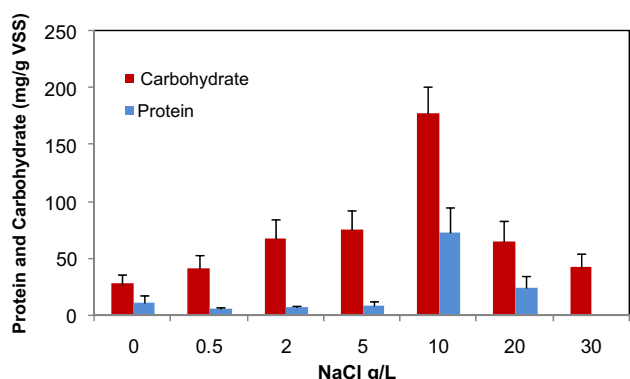
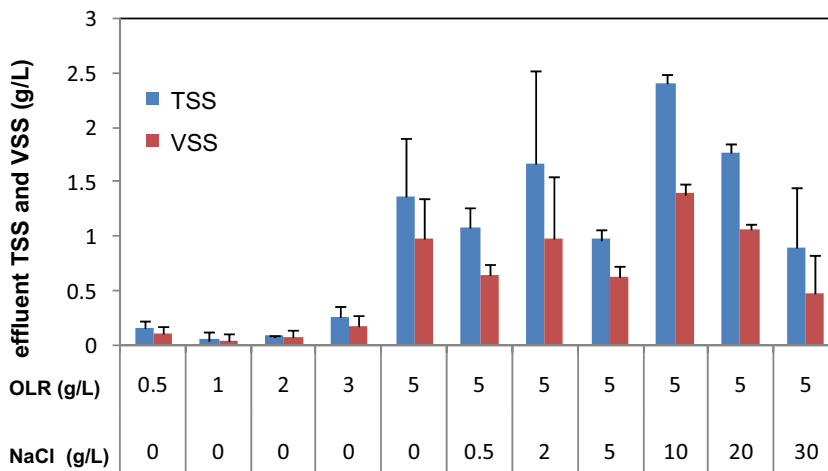


Fig. 9 Associated protein and carbohydrate with EPS during ASBR operation (OLR: 5 g glucose/L.d) with different NaCl concentrations

Conclusion

In this study, the biohydrogen production as function of effect of OLR and NaCl concentration by a lab scale ASBR was investigated. The biohydrogen production was highly OLR and NaCl dependent and enhanced with increasing OLR from 0.5 to 5 g glucose/L.d and decreased by NaCl concentrations from 0.5 to 30 g/L. Operation of ASBR at high concentrations of NaCl shifted the metabolic pathway from acidogenic toward solventogenic. The highest and lowest biohydrogen yields were 1.1 ± 0.2 and 0.3 ± 0.1 mol H₂/mol glucose and obtained at 0.5 and 30 g/L NaCl, respectively. Based on the inhibition model, a NaCl concentration of 0.5 g/L showed a

Fig. 10 TSS and VSS in ASBR effluent as a function of OLR and NaCl concentration



stimulating effect on biohydrogen production, and the critical NaCl concentration was 119.9 g/L.

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Authors' contributions Bijan Bina supervised the study, Ensiyeh Taheri was the main investigator, collected the data, Mohammad Mehdi Amin and Hamidreza Pourzamani were advisor the study, Ensiyeh Taheri, Ali Fatehizadeh, and Henri Spanjers drafted the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interest The authors declare that they have no competing interest.

Abbreviations C , Added NaCl concentration (g/L); C_{crit} , Critical NaCl concentration by H_2 production ceases (g/L); $C_{1,50}$, NaCl concentration with repress 50% of relative activity (g/L); k_c , Specific growth rate (1/h); K_s , Apparent half velocity constant for the substrate (g/L); n , Inhibition degree; $r(H_2)$, H_2 production rate (mL H_2 /g VSS/h); $r(H_2)_{max}$, Maximum H_2 production rate (mL H_2 /g VSS/h); S , Substrate concentration (g/L); SHPR, Specific biohydrogen production rate (L H_2 /g VSS.d); ν , Specific substrate degradation rate (g/gVSS.h); ν_m , Maximum specific substrate degradation rate (g/gVSS.h); X , Microbial concentration (gVSS/L); X_0 , Initial microbial concentration (g VSS/L); X_{max} , Maximum microbial concentration (g/L)

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