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Original article

Hydrogen Fermentation Potential of *Escherichia coli* XL1 blue using various carbon sources

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Abstract:

The biohydrogen production potential of *E. coli* XL1 blue was investigated with pure substrate (glucose), complex natural feedstock (Acid (HCl) +microwave assisted and enzymatic (Viscozyme mediated) hydrolysate) of Deoiled jatropha waste (DJW) in order to check the efficiency of the strain towards fermentative hydrogen production. The results show that the strain has a conversion efficiency of sugar degradation of more than 75% in all cases. The hydrogen formation from DJW hydrolysate revealed that the strain could work efficiently in complex substrates also. Applied immobilization technique effectively enhanced the production rates. The peak hydrogen production rate (HPR), specific hydrogen production rate (SHPR) and hydrogen yield (HY) were obtained as 3.18 ± 0.2 L H_2/L-d, 7.95 ± 0.5 L H_2/g DCW-d and 153.3 ± 3.3 mL H_2/ g substrate $_{added}$ when substrate (glucose) concentration 5 g/L, incubation temperature 37 ^{0}C and biomass concentration was set to 0.4 g DCW/L (immobilized cells). These values indicated that process efficiency is almost doubled compared with free cells. The energy evaluation report states that the bioenergy produced from this process calculated as peak energy generation rate (EGR) and energy yield are 37.3 ±0.2 kJ/L-d and 1.8 ±0.6 kJ/g Substrate $_{added}$.

1. INTRODUCTION

Hydrogen is revealed as a future potential energy carrier by various researchers in the world due to the advantages like high energy content, non-pollutant nature and utilization of wide varieties of substrates for the production methods, especially in biological way. Biotechnological way of hydrogen production is received more interest of all the hydrogen production methods (chemical and thermo chemical means) is mainly because of the easy operational procedures and high production rates which promises towards scale up and commercialization process (Das and Veziroglu, 2001). Even though the fermentative hydrogen production is an attractive process, the process efficiency in terms of yield and immature technological process are the bottlenecks of the commercialization process. Some pilot scale plants were established using the pure cultures. The advantages of pure cultures over the mixed cultures are the relatively high yield, although the chances of contamination and difficulties in maintenance. Thus the facultative anaerobes are attaining much more attention and great interest towards biological production because of their easy operation and possibilities of metabolic engineering applications. For example (Khanna et al., 2011) have demonstrated the *Enterobacter cloacae* IIT-BT08 in various studies as a feasible hydrogen producer due to the alterations done in the genes responsible for hydrogen production.

E. coli is showed as an attractive hydrogen producer by many authors either immobilized or genetically engineered in recent studies (Ishikawa et al., 2006; Ghosh and Hallenbeck, 2010). Here we demonstrate the feasibility and potential of the *E. coli* XL1 blue which has been used by a group of researchers, recently and proved as a hydrogen producer (Bakonyi et al., 2011). Easy maintenance and fast growing nature of this bacterium gained a great attention among scientists. There are few strains for example *E. coli* DJT 32 and some other strains by modifying formate hydrogen lyase gene usually (Ishikawa et al., 2006; Yoshida et al., 2005), are metabolically engineered to produce more amount of hydrogen. However employing real time wastewaters for the hydrogen production would be an economically viable and sustainable. Thus, in this study exploration of hydrogen fermentation potential of *E. coli* XL1 blue was demonstrated as a hydrogen producer and the feasibility checked initially with pure (glucose) substrate .However pure substrates are cost effective, thus use of real waste could attain more interest in this field. For this purpose the potential of this strain also checked with a real waste Deoiled Jatropha waste (DJW) acid and enzyme hydrolysate) in terms of fermentative hydrogen production.

2. MATERIALS AND METHODS

2.1 Bacterial source

E. coli XL1 blue strain was received from University of Pannonia, Veszprem Hungary as a part of the student exchange programme with Feng Chia University, Taiwan. The inoculum preparation and maintenance were followed as mentioned accordingly (Bakonyi et al., 2011).

2.2 DJW hydrolysate and pure substrate

The DJW hydrolysate used in this study was prepared in such a way that DJW is first treated with acid (HCl) and then kept in microwave oven purchased from local supermarket (model , Taiwan) for 15 minutes at 200MV. Then the supernatant was used in the fermentation reactions. Enzyme hydrolysate is prepared as the solid portion remained in the acid treatment is treated with 1% enzyme (Viscozyme purchased from sigma Aldrich) at 50 ^{0}C for 30 minutes in a boiling water bath. Glucose is of commercial grade was used.

2.3 Immobilization of E. coli XL1 blue

The cell suspension was made by preparing the cell concentration of 0.4 g/L dry cell weight and centrifuged at 14,000 rpm for 10 minutes. The cells were washed thrice with sterile 0.9 NaCl saline solution to remove the residual nutrients from the cell suspension. The prepared cell suspension was mixed with a mild constant stirring in a sodium alginate stock solution (2.5 % w/v) to make a final alginate concentration of 2% (w/v). The alginate-cell mixture was extruded into sterile cold calcium chloride solution (0.2 M) for cell entrapment in alginate beads. The beads formed (2–3 mm) were further hardened by stirring the beads in a fresh solution of calcium chloride (0.2 M) for two more hours. Finally, the beads were washed thrice with sterile distilled water and dried, later stored in refrigerator until further use.

2.4 Hydrogen fermentation

The batch fermentation was performed in 125 ml vials. The working volume was set to 60 ml with the mixture of 40 ml of the substrate (glucose) at a concentration of 5 g/L in case of DJW hydrolysate the supernatant was added, 5 mL pH adjustment solution (1 N NaOH or HCl), and inoculum (*E. coli* XL1 blue) 10 ml and rest was distilled water. The strict anaerobic condition was established by argon gas purging for 8-10 minutes. Immobilization experiments were carried out with the similar conditions except the addition of immobilized beads instead of free cells. The initial cultivation pH of 6.5 was set prior to fermentation. Then the vials were placed in a reciprocal air-bath shaker at 150 rpm with the temperature control of 37 ^{0}C.

The volume and composition of gas were analyzed in a periodic interval. Fermentation was carried out until the observation of no gas production. All reagents used were analytical grade and the distilled water was used and others were specified. The vials used were shown in Fig.1.

2.5 Liquid and gas analysis

The pH was measured using a pH meter. The reducing sugar (RS) analysis was done following the DNS method. The biogas which is a mixture of hydrogen and CO_2 were analyzed using glass syringe and the contents measured as mentioned in the earlier studies (Chen et al., 2002).

2.6 Gompertz equation

Gompertz equation (Eq. 1) was used to get the kinetic parameters such as hydrogen production potential (P), maximum hydrogen production rate (R_m) and lag phase time (λ) under each experimental condition. The software details are Sigma plot software 10.0 (Systat Software Inc., USA).

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

Where, H (t) represents the cumulative hydrogen production (mL); P is the hydrogen production potential (mL); Rm is the maximum hydrogen production rate (mL/h); e is 2.71828; λ is

the lag phase time (h) and t is the cultivation time (h). Hydrogen production rate (HPR L H_2/L-d) was defined as R_m value divided by the reactor volume (0.06 L) and multiplied with a day (24 h). Hydrogen yield (HY) was calculated by the cumulative hydrogen production (ml) divided by the substrate concentration added (g /L for glucose and g VS added for DJW hydrolysates).

3. RESULTS AND DISCUSSION

3.1 Hydrogen production profile of E. coli XL1 blue regards to free and immobilized cells

Biohydrogen production is attaining great interest towards the sustainable future and solution to the energy demand due to the wide variety of feedstock and organisms present in nature. Here a demonstrative study of hydrogen fermentation of a strain *E. coli* XL1 blue with regards to pure substrate (glucose) and real time application of this study in terms of usage of a complex substrate (DJW hydrolysate**,** acid and enzyme mediated) is elucidated. Hydrogen production and biogas formation values were given in table 1. The Hydrogen production rate (HPR), Specific hydrogen production rate (SHPR) and hydrogen yield of glucose was 1.59±0.3 L H_2/L-d, 3.97±0.3 L H_2/g DCW-d and 152.2±1.9 mL H_2/ g substrate $_{added}$. The efficiency of this strain towards a complex substrate Deoiled jatropha waste (DJW) hydrolysate, since the solid waste can't be used directly for the fermentation process, pretreatment methods such as acid (HCl) + microwave assisted and enzyme (Viscozyme) mediated also reported. The acid hydrolysate and Enzyme hydrolysate are mentioned as AH, EH hereafter. The results show that HPR, SHPR and HY values are ranged for AH as 0.79±0.1 L H_2/L-d, 1.97±0.3 L H_2/g DCW-d and 30.0±0.7 mL H_2/ g VS $_{added}$, for EH as 1.48±0.2 L H_2/L-d, 4.93±0.6 L H_2/g DCW-d and 39.2±0.8 mL H_2/ g VS $_{added}$ respectively. These values revealed that this strain could efficiently work in complex substrates also. The exploration of new feedstock could be an interesting study in near future.

Table 1 Hydrogen production performance

Substrate used	Inoculum used	Total biogas (mL)	Cumulative H_2 (mL)	HPR (L H_2/L-d)	SHPR (LH_2/g DCW-d)	HY (mL H_2/g Substrate $_{added}$)
Glucose	Free cells	72.7±1.2	45.7±0.5	1.59±0.5	3.97±0.3	152.2±1.9
	Immobilized	71.7±0.5	46.0±1.0	3.18±0.2	7.95±0.5	153.3±3.3
Acid hydrolysate	Free cells	76.3±2.3	48.7±1.2	0.79±0.1	1.97±0.3	30.0±0.7*
	Immobilized	79.3±3.1	49.6±1.5	0.81±0.3	2.02±0.7	31.0±0.5*
Enzyme hydrolysate	Free cells	108.0±1.7	66.7±1.1	1.48±0.2	4.93±0.6	39.2±0.8*
	Immobilized	123.7±0.6	73.3±0.6	2.95±0.3	7.38±0.3	43.1±0.4*

*calculated based on g VS $_{added}$

Hydrogen production in immobilized cells is enlightened in table 1. The main purpose of immobilization is to make the cells reuse and some authors reported that immobilization could acts as anaerobic condition for the bacteria, thus increases the hydrogen production (Ishikawa et al., 2006).Thus the enhanced hydrogen production is seen in immobilized cells. The production rates were improved almost double than the free cells; however the yield was not much improved. The AH and EH are comprised of mainly cellulose and hemicellulose since the substrate (DJW) is a lignocellulosic waste. These form of sugar is not utilizable to this strain, this ultimately turn in to the lower yield and production rated comparatively with pure substrate.

The trend of hydrogen generation is shown in (fig 2). It is clearly seen that pure substrate (glucose) is easily metabolized by *E. coli* since the hydrogen evolution time is very short and ended up nearly 50 hours. However the formation of hydrogen from AH and EH was prolonged to nearly 150 hours which is approximately three times longer than the pure substrates. The peak HPR, SHPR and HY were achieved from the glucose feedstock and the values as 3.18±0.2 L H_2/L-d, 7.95±0.5 L H_2/g DCW-d and 153.3±3.3 mL H_2/ g substrate $_{added}$ respectively.

3.2 Water quality analysis

The final pH, ORP and sugar degradation values are reported in table 2. The pH reduction values indicating that the pathway was mediated by formation of acid which is the intermediate step of the anaerobic fermentation. The OPR values were ranged from 314±10 to 391±21, which favored the anaerobic condition. Sugar degradation is calculated based on the sugar consumption from the initial level provided. The results show that the better degradation occurred with the pure substrate. In fact glucose is having the 100% conversion, since easy utilization by the organisms biologically. However the complex substrate was shown less conversion efficiency than the pure substrate. This might be due to the complex (cellulosic and hemi cellulosic) nature of the substrate.

3.3 Evaluation of energy production

The details of the total energy production values are given in Table 3. The peak total energy production rate and yield were obtained as 37.3±0.2 KJ/L-d and 1.8 ±0.6 KJ/g substrate $_{added}$. This kind of bioenergy is much cleaner comparatively with the fossil fuel derived energy. The heating value parameter of hydrogen (285.8 J/mmol) was considered as a factor for the energy evaluation. Since it is the basic study towards the potential of the above mentioned organism, further studies related to its optimal conditions such as substrate concentration, temperature and pH could generate more energy.

Figure.1 Prepared immobilized cells (left) batch vials used (right)

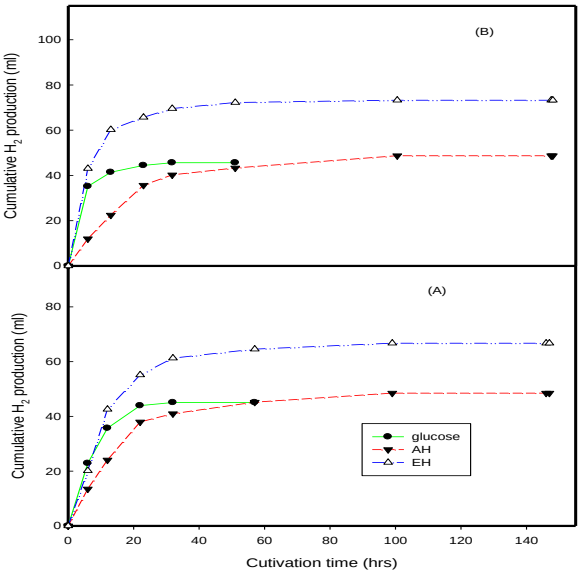

Figure.2 Time profile of hydrogen formation (AH-Acid hydrolysate, EH-Enzyme hydrolysate), A) free cells, B) Immobilized cells

Table 2 Water quality analysis

Substrate	Inoculum used	Final pH	Final ORP (-mV)	Sugar degradation (%)
Glucose	Free cells	5.0±0.3	317±23	87.3±2.4
	Immobilized	5.8±0.2	314±14	88.2±3.6
Acid hydrolysate	Free cells	5.6±0.2	391±20	77.3±1.2*
	Immobilized	5.6±0.1	380±9	78.7±2.1*
Enzyme hydrolysate	Free cells	5.9±0.1	389±10	82.0±1.0*
	Immobilized	5.7±0.1	390±10	84.3±1.5*

*based on RS (reducing sugar)

Table 3 Energy evaluation

Substrate	HPR (mmol/L-d)	EPR (kJ/L-d)	HY (mmol/ g substrate $_{added}$)	EY (KJ/g substrate $_{added}$)
Glucose	130.5±0.6	37.3±0.2	6.3±0.2	1.8±0.6
AH	33.7±0.5	9.6±0.1	1.6±0.3	0.4±0.1
EH	120.9±0.5	34.5±0.2	1.9±0.1	0.5±0.3

AH-Acid hydrolysate, EH-Enzyme hydrolysate, *heating value parameter: H_2 285.8 J/mmol, EPR-energy production rate, EY-energy yield

4. Strategies to improve the energy formation via enhancement of hydrogen production

The yield of the biological process is always lower when compared with the production rates especially in case of dark fermentative hydrogen production whilst using pure cultures. The main reason for this can be enlightened as the substrate provided/ converted is boosting the growth of organisms rather than the formation of hydrogen (Hallenbeck et al., 2009).There are various approaches which deals this issue. One of the promising approaches is the metabolic engineering of these wild type organisms. Since this technique could block the genes which are involve in the metabolic activity that results in the unwanted products (such as lactate in hydrogen fermentation) and different aspects of genetic engineering were proposed so far, however the proper understanding of metabolic pathways is essential (Oh et al., 2011). The second option could be the exploration of wide varieties of organisms (isolation from various places such as hot spring- since extreme thermopiles bearing stable hydrogenase enzymes which could enhance the yield) and substrates rather than pure substrates (such as solid and lignocellulosic waste, they are rich in sugar content). These strategies could enhance the energy yield of the biological process.

5. Conclusions

This study made an attempt in terms of bioenergy production via Hydrogenic fermentation of a facultative anaerobic organism *E. coli* XL1 blue. The results show that this strain could efficiently work on pure and complex substrates. The production rates and yields were compared. The energy produced also evaluated and provides basic information about

this process towards scale up for further energy production enhancement, ultimately the strategies to be handled as the solution of the bottlenecks which are related with this process also discussed. Further studies related to optimization and scale up of this process could efficiently enhance the ways target as a solution of the energy related issues.

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