Dark-Fermentative Biological Hydrogen Production from Mixed Biowastes Using Defined Mixed Cultures

Sanjay K. S. Patel^{1,2} · Jung-Kul Lee² · Vipin C. Kalia¹

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Abstract Biological hydrogen (H₂) production from the biowastes is widely recognized as a suitable alternative approach to utilize low cost feed instead of costly individual sugars. In the present investigation, pure and mixed biowastes were fermented by defined sets of mixed cultures for hydrolysis and H₂ production. Under batch conditions, up to 65, 67 and 70 L H₂/kg total solids (2%, TS) were evolved from apple pomace (AP), onion peels (OP) and potato peels (PP) using a combination of hydrolytic mixed culture (MHC5) and mixed microbial cultures (MMC4 or MMC6), respectively. Among the different combinations of mixed biowastes including AP, OP, PP and pea-shells, the combination of OP and PP exhibited maximum H₂ production of 73 and 84 L/kg TS with MMC4 and MMC6, respectively. This study suggested that H₂ production can be effectively regulated by using defined sets of mixed cultures for hydrolysis and H₂ production from pure and mixed biowastes as feed even under unsterile conditions.

Keywords *Bacillus* · Biowaste · Biomass hydrolysis · Dark-fermentation · Hydrogen · Mixed microbial culture

Introduction

Fossil fuels as energy source are marred by their availability in limited quantities and the environmental pollution caused by their combustion. Another major cause of environmental pollution is the generation of unmanageable quantities of biological wastes. Under uncontrolled conditions, biowastes undergo slow fermentation and release green-house gases such as methane (CH₄) and carbon dioxide (CO₂). The need is for getting a clean fuel preferably from a renewable source [1]. In this race for searching an energy rich pollution free fuel, hydrogen (H₂) stands out to be a strong contender [2]. Chemical and electrical methods are being used to produce H₂. Biological H₂ production process is in the incipient stages and quite uneconomical at present. Biological H₂ production (BHP) has a great potential to succeed as a major source of H₂ due to the following two factors: (1) can be produced at ambient conditions, and (2) can utilize biowaste as feed, i.e. reduced input costs [3–5]. These incentives sufficient to drive people to work on improving the efficiency of BHP. A few efforts in this direction, have revealed that: (1) microbes can work individually and in combination, (2) pure sugars and biowastes can be used as primary feed in various combinations, (3) dark and photosynthetic H₂ production processes can operate in sequential manner, with synergistic effects on H₂ yield [6, 7]. In spite of these potential benefits, H₂ yields from biowastes have been quite low in the range of 4–5 mol H₂/mol hexose and may exceptionally go up to 7–8 mol H₂/mol hexose [6, 7]. The major limiting factors in using biowaste as feed are: (1) their complex biochemical composition, (2) poor bacterial metabolic rate to produce H₂, (3) variations in quantity and quality of biowaste from lot to lot, (4) the presence of contaminating bacteria, which may accompany the

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✉ Sanjay K. S. Patel
sanjaykspatel@gmail.com; sanjaykspatel@yahoo.co.in

¹ Microbial Biotechnology and Genomics, CSIR–Institute of Genomics and Integrative Biology (IGIB), Delhi University Campus, Mall Road, Delhi 110007, India

² Department of Chemical Engineering, Konkuk University, 1 Hwayang-Dong, Gwangjin-Gu, Seoul 143-701, Korea

biowaste [2]. To overcome these limitations, the following proposals have been tried: (1) use of defined mixed microbial cultures, (2) use of mixed biowastes, and (3) pretreatment (hydrolysis) of biowastes with defined microbial mixtures having high hydrolytic enzyme activities [8–13].

In this study, we have employed defined sets of: (1) hydrolytic bacterial mixtures, (2) high H₂-producing bacteria, (3) biowastes in combinations—apple pomace (AP), onion peels (OP), potato peels (PP) and pea-shells (PS), under unsterile conditions. This integrative approach has worked effectively in enhancing H₂ yields.

Materials and Methods

Organisms and Growth Conditions

Bacterial strains used for the preparation of MHCs (MHC1–MHC11) included: (a) *Bacillus* sp. strains (EGU85, EGU367, EGU444 and EGU447), *B. sphaericus* strains (EGU385 and EGU542), *B. subtilis* (EGU475), *B. thuringiensis* EGU378; *Proteus mirabilis* strains (EGU30 and EGU32) and *Bacillus* sp. (EGU409), (Table S1); and MMC: (b) MMC4—*B. cereus* EGU43, *Bacillus pumilus* HPC464, *Bacillus* sp. HPC459, *B. thuringiensis* EGU45, *Enterobacter aerogenes* EGU16 and *P. mirabilis* EGU21; and MMC6—*B. cereus* EGU41, *B. megaterium* (HPC686), *B. pumilus* HPC464, *E. aerogenes* EGU16 and *P. mirabilis* strains (EGU21 and EGU30) [9, 10, 14]. All strains were grown in nutrient broth (13 g/L) at 37 °C and 200 rpm for 16–20 h to prepare the inocula of the MHC1–MHC11, MMC4 and MMC6 as described previously [10].

Hydrolysis of Individual and Mixed Biowaste

Biowastes including, AP, OP, PP and PS were obtained from local market of Delhi (India). These individual wastes were mixed in combinations of two, three and four, in equal proportions. For batch-culture digestion, 2% total solids (TS) of pure or mixed biowaste were used. 250 mL of slurry (unsterile conditions) was prepared with tap water in 300 mL BOD bottles and inoculated with MHC1–MHC11 [13]. Here, each MHC was prepared by mixing different strains in equal proportions to a final protein concentration of 10 mg/mL [10]. The resulting slurry was incubated at 37 °C for 2 days [8, 10, 13].

Biohydrogen Production

Batch culture H₂ production was evaluated using MMC4 and MMC6 from 250 mL of different hydrolyzed slurries of individual or mixed biowastes. The final pH was

adjusted to 7.0 [10]. Each bottle was flushed with argon to create anaerobic condition and incubated at 37 °C. The evolved biogas was measured through water displacement method [9]. Daily, pH of the each bottle was adjusted to 7.0 using 2N solution of HCl or NaOH. Since, H₂ production was evaluated under unsterile condition, therefore the role of endogenous microorganisms (as contaminants) in H₂ production was also examined. As only 5–10 mL of H₂ production was recorded from 250 mL of individual or mixed biowaste as feed (2%, TS), it was treated as insignificant. Thereafter, the influence of biowaste composition on the H₂ production was evaluated using MMC4 and MMC6.

Analytical Methods

Gas Analysis

The gas composition was determined using gas chromatography (Nucon GC5765, India) equipped with a thermal conductivity detector using argon as the carrier as described earlier [9]. The produced biogas was analyzed daily till H₂ production ceased [14].

TS Analysis

The TS of the biowaste was measured by heating a sample at 110 °C for 24 h, as described previously [8, 13].

Results

H₂ Production from Biowaste

Apple Pomace

In our previous studies, the influence of hydrolytic and H₂ producing cultures on H₂ production was established with PS as a biowaste [10]. In this work, the MHCs (MHC1–MHC11) resulted in the H₂ production in the ranges of 85–210 mL from the 2% TS of AP slurry as a feed (Table 1). The evolved biogas contained 33.5–61.3% H₂, with a H₂ yield of 17–42 L/kg of TS fed. Without MHC, the inherent microflora (as contaminants) evolved 5 mL of H₂ from 250 mL of AP slurry and hence can be treated as insignificant. The combination of MHCs with MMC4 and MMC6 produced H₂ in the ranges of 95–325 and 90–300 mL with the yield of 19–65 and 18–60 L H₂/kg of TS fed, respectively. Here, H₂ constituted 39.7–63.6 and 39.4–61.9% of total biogas evolved from 250 mL of AP slurry, respectively. These combinations of MHCs with MMC4 and MMC6 enhanced H₂ production up to 115 and 90 mL compared to their respective individual hydrolytic

Table 1 Hydrogen producing potential of mixed microbial cultures from pre-hydrolysed biowaste^a: apple pomace

Mixed hydrolytic culture	Hydrogen (H ₂)								
	Control			MMC4			MMC6		
	Vol ^b	%	Yield ^c	Vol	%	Yield	Vol	%	Yield
MHC1	130	58.3	26	185	62.4	37	140	57.2	28
MHC2	205	61.3	41	290	63.6	58	265	61.8	53
MHC3	135	53.8	27	140	58.2	28	155	55.4	31
MHC4	120	55.3	24	155	51.7	31	130	51.2	26
MHC5	210	60.5	42	325	62.5	65	300	61.9	60
MHC6	105	38.9	21	120	39.7	24	90	39.4	18
MHC7	120	41.7	24	95	40.1	19	135	43.2	27
MHC8	185	49.2	37	225	56.5	45	205	58.7	41
MHC9	155	51.4	31	180	54.8	36	140	55.8	28
MHC10	160	53.3	32	135	48.2	27	175	54.4	35
MHC11	85	33.5	17	105	47.6	21	120	51.0	24

Values are based on three sets of experiments and standard deviation was less than 10%

^a Total volume of feed: 250 mL (2%, TS) hydrolysed with MHC1–MHC11 (2 days)

^b Observed volume (mL) of H₂ in the biogas (H₂ + CO₂)

^c L/kg TS fed

cultures. Interestingly, the combination of MHC5 with both MMC6 and MMC4 resulted in the maximum H₂ yield of 60 and 65 L H₂/kg of TS fed, respectively, i.e. 1.43- and 1.55-fold enhancement (Fig. 1). Overall, the MHC5 + MMC4

combination was very efficient for H₂ production from AP slurry with a maximum of 65 L H₂/kg TS fed.

Onion Peels

With 2% TS of OP slurry (250 mL), MHCs (MHC1–MHC11) evolved H₂ production in the ranges of 85–150 mL with yield equivalents to 17–30 L H₂/kg of TS feed (Table 2). H₂ contents were observed to be 33.7–54.6% of total biogas. The inherent microflora without MHC evolved 10 mL of H₂ under similar conditions, which could be treated as insignificant. Out of various combinations of MMC4 and MMC6 with MHC1–MHC11, MHC5 proved to be the most effective, with H₂ yields of 56 and 67 L H₂/kg of TS fed, respectively. Here, a significant enhancement of 1.87- and 2.23-fold was recorded by MMC4 and MMC6 in association with MHC5, respectively, in contrast to that observed with MHC5 alone. Overall, the MHC5 + MMC6 combination was the best for the maximum H₂ production of 67 L H₂/kg of TS feed, which was 2.57- and 2.31-fold compared to MMC4 and MMC6 alone (Fig. 1).

Potato Peels

The different MHCs cultures (MHC1–MHC11) evolved 95–165 mL of H₂ from 250 ml slurry (2% TS, PP) (Table 3). Here, H₂ constituted 43.4–55.4% of total biogas with yields of 19–33 L H₂/kg of TS fed. PP slurry without MHC evolved 5 mL of H₂ due to inherent microflora. H₂ production from PP inoculated with individual MMC4 and

Fig. 1 Hydrogen production potential of mixed microbial cultures (MMC) from apple pomace (AP), onion peels (OP) and potato peels (PP): **a** MMC4 and **b** MMC6

Table 2 Hydrogen producing potential of mixed microbial cultures from pre-hydrolysed biowaste^a: onion peels

Mixed hydrolytic culture	Hydrogen (H ₂)								
	Control			MMC4			MMC6		
	Vol ^b	%	Yield ^c	Vol	%	Yield	Vol	%	Yield
MHC1	120	48.1	24	205	51.2	41	165	58.2	33
MHC2	145	45.4	29	230	58.8	46	210	61.4	42
MHC3	95	33.7	19	125	50.4	25	145	55.2	29
MHC4	105	41.8	21	140	48.6	28	120	47.3	24
MHC5	150	54.6	30	280	59.5	56	335	63.0	67
MHC6	90	35.2	18	145	53.6	29	80	38.4	16
MHC7	100	38.9	20	85	38.5	17	95	41.1	19
MHC8	130	44.5	26	180	44.7	36	175	48.7	35
MHC9	85	46.3	17	105	48.2	21	90	46.2	18
MHC10	105	41.2	21	135	45.4	27	85	43.8	17
MHC11	110	43.6	22	100	39.9	20	105	47.5	21

Legends as shown in Table 1

Table 3 Hydrogen producing potential of mixed microbial cultures from pre-hydrolysed biowaste^a: potato peels

Mixed hydrolytic culture	Hydrogen (H ₂)								
	Control			MMC4			MMC6		
	Vol ^b	%	Yield ^c	Vol	%	Yield	Vol	%	Yield
MHC1	115	48.8	23	130	53.7	26	160	55.6	32
MHC2	160	51.2	32	310	60.4	62	295	63.2	59
MHC3	105	43.6	21	145	55.8	29	165	61.6	33
MHC4	140	48.3	28	160	58.4	32	145	54.7	29
MHC5	165	55.4	33	320	61.9	64	350	62.9	70
MHC6	110	49.3	22	180	59.6	36	120	51.3	24
MHC7	125	51.2	25	105	38.3	21	100	42.8	20
MHC8	95	43.4	19	120	44.5	24	105	44.6	21
MHC9	130	47.9	26	145	50.2	29	165	47.3	33
MHC10	125	51.3	25	160	52.7	32	130	51.0	26
MHC11	115	50.6	23	95	41.2	19	100	43.2	20

Legends as shown in Table 1

MMC6 was 33 and 35 L/kg TS fed (Fig. 1). MHCs (MHC1–MHC11) in combination with MMC4 and MMC6 resulted in H₂ production in the ranges 19–64 and 20–70 L H₂/kg of TS feed, respectively. Combinations of MHC5 along with MMC4 or MMC6 turned out to be the most effective in enhancing H₂ production from PP. In these cases, 64 and 70 L H₂/kg TS fed were 1.94- and 2.12-fold higher than H₂ production by the control.

A summary of the various MHC and MMC combinations revealed that the H₂ producing capacities of MMC4 and MMC6 were the best in combination with MHC5: (1) AP: 325 and 300 L/kg TS fed; (2) OP: 280 and 335 L/kg TS fed; and (3) PP: 320 and 350 L/kg TS fed, respectively.

H₂ Production from Mixed Biowastes

The composition of feed has direct influence on the H₂ producing abilities of the bacteria [13]. The combinations of AP, OP, PP and PS (with biowaste mixed in 1:1 ratio) on hydrolysis with MHC5, revealed that H₂ yields were in the range of: (1) 53–73 L/kg TS fed with MMC4, and (2) 58–84 L/kg TS fed with MMC6 (Table 4). These H₂ yield were 35–75 L more than the theoretically expected values of PP + OP combinations. Thus, combinations of PP + OP resulted in synergic effect, 1.22-fold higher H₂ yield compared to their individual capacities.

Table 4 Hydrogen producing potential of mixed microbial culture (MMC4) from pre-hydrolysed mixed biowaste^a

Mixed biowastes				Hydrogen (H ₂)							
Apple pomace	Potato peels	Onion peels	Pea shells	MMC4				MMC6			
				Vol ^b		%	Yield ^d	Vol ^b		%	Yield
				Exp. ^c	Obs.			Exp.	Obs.		
+	+	–	– ^f	305	320	58.7	64	320	330	60.4	66
+	–	+	–	325	305	61.3	61	325	345	63.3	69
+	–	–	+	280	310	62.0	62	265	290	58.8	58
–	+	+	–	300	365	63.5	73	345	420	64.2	84
–	+	–	+	255	230	55.9	46	285	295	58.6	59
–	–	+	+	275	300	58.7	60	290	305	56.3	61
+	+	+	–	310	305	61.8	61	330	360	62.5	72
+	+	–	+	275	295	58.9	59	290	335	61.2	67
+	–	+	+	290	320	59.1	64	290	300	57.8	60
–	+	+	+	275	265	55.4	53	305	345	62.0	69
+	+	+	+	290	345	61.0	69	305	320	59.7	64

Values are based on three sets of experiments. Standard deviation was less than 10%

^a Total volume of feed in equal ratio: 250 mL (2%, TS) hydrolysed with MHC5 (2 days)

^b Observed volume (mL) of H₂ in the biogas (H₂ + CO₂)

^c Expected volume of H₂, based on the average of H₂ producing capacities of mixed microbial culture from individual biowaste under similar experimental conditions

^d L/kg TS fed

^e Present

^f Absent

Discussion

Most bioprocesses, which are driven by a single bacterium are associated with the fear of failure. In these case, the risk of contaminating bacteria outgrowing the inoculum is always high. It may lead to complete process failure and economic losses. So either we need to sterilize the biowaste or use mixture of well-defined bacterial cultures. Eco-biotechnological approaches based on mixture of well-defined bacteria, which may get easily acclimatized to the conditions prevailing in the bioreactor are likely to succeed [15–17].

In addition to robust bacteria, the use of biowastes as feed is an attractive proposal for improving the efficiency of BHP. Compared to pure sugars as substrates for H₂ production, biowastes need to be metabolized into simpler and soluble intermediates. Keeping in view, that the degradable components of fruits, vegetables and foods are largely carbohydrates, proteins and fats, we focused on isolating bacteria with high amylase, protease and lipase activities [18]. Screening a few thousand bacteria allowed us to identify a few, whose hydrolytic enzymatic activities were operative over a wide pH range of 5–9 [17]. Among the 11 hydrolytic bacterial combinations, MHC2 and MHC5 composed largely of bacteria belonging to *Bacillus*

and *Proteus* species were the most effective in enhancing H₂ and polyhydroxyalkanoate (PHA) production from PS, mixed vegetable wastes, OP, PP [8, 10, 12, 13, 16]. Co-cultures of *Bacillus*, *Enterobacter*, *Klebsiella* and *Citrobacter* have also proved effective in obtaining H₂ yield of 3 mol/mol glucose [9, 11, 19]. Based on the positive responses recorded in earlier studies, and the work done here with MHC1–MHC11 along with MMC4 and MMC6, it was realized that MHC5 could hydrolyse AP, OP, PP and enabled defined mixed H₂-producers (MMC4 and MMC6) to enhance H₂ production process by 1.5 to 2.2-fold with a final yield of up to 70 L/kg TS fed. Since, we cannot expect wastes to be well segregated into distinct groups, efforts to test MHC and MMCs on mixed biowastes were found to be helpful. Mixture of PP and OP turned out to be the best combination, where there was a synergistic effect on H₂ production process leading to 84 L H₂/kg TS fed. The overall economy of this process can be also improved through its integration with production of CH₄ or PHA [12, 20–26].

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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