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ORIGINAL RESEARCH ARTICLE

Co-digestion of Biowastes to Enhance Biological Hydrogen Process by Defined Mixed Bacterial Cultures

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Abstract Co-digestion of biowastes for hydrogen (H_2) production using defined mixed cultures can overcome the high risk of failure due to contamination and imbalanced nutrient status. H_2 production from biowastes—pea-shells, potato peels (PP), onion peels (OP) and apple pomace, either individually or in various combinations was evaluated by hydrolyzing with defined hydrolytic mixed bacterial culture (MHC5) and subjecting the hydrolysate to mixture of defined H_2 producers (MMC6). Co-digestion of OP and PP hydrolysate supplemented at H_2 production stage with GM-2 and M-9 media resulted in 95 and 102 l H_2/kg of Total solids (TS), respectively compared to 84 l H_2/kg of TS in control. Upscaling the process by digesting 4.0 l slurry (16-fold) resulted in 88.5 and 95 l H_2/kg of TS, respectively compared to 72 l H_2/kg of TS in control. Thus, H_2 production by co-digestion of biowastes could be improved through the supplementation with very dilute medium (0.1 ×) and selection of suitable biowastes under unsterile conditions. The overall efficiency can be further enhanced by integrating it with bioprocesses for biopolymers such as polyhydroxyalkanoates and or biofuels like methane production.

Keywords Biowaste · *Bacillus* · Hydrogen · Onion peels · Potato peels · Mixed microbial culture

Introduction

The production of biofuels as an alternative to fossil fuels has received substantial attention in the recent few decades [1–5]. Various strategies of microbial production of biofuels, including hydrogen (H_2), methane (CH_4), and bioalcohols have been demonstrated [6–9]. The production of H_2 has been recommended as a clean fuel due its two major characteristics: (1) non-polluting nature—water being the end-product of combustion, and (2) high energy efficiency (122 kJ/g) [10–12]. The production of H_2 through a biological process under ambient physiological conditions seems to be an attractive approach over other approaches such as chemical, thermal, nuclear and wind energy sources [10]. Different microbial strains have been established as consistent H_2 producers especially *Bacillus* and *Enterobacter* [2, 13, 14]. These culture dependent approaches have been supported by genomic approach and have proved effective in identifying novel H_2 producers [15, 16]. Broadly, biological H_2 production through dark-fermentative process is more efficient than photo-fermentative process. Here, the major limiting factors is the requirement of regular supply of light [2, 10].

A variety of sugars, including glucose, fructose and sucrose have been used for producing H_2, by microbes which can easily metabolize them [2, 13, 17, 18]. In order to circumvent the high cost of sugars, the use of biowaste as low-cost feed for large-scale H_2 production appears

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economically feasible [19–24]. However, the complex and imbalanced nutrient status in the biowaste and presence of undesired inherent microbes accompanying the biowaste need special attention. To overcome these limitations, three approaches can be employed (1) hydrolysis of biomass through different methods, including physical, chemical, enzymatic and microbial, (2) co-digestion of biowastes and supplementation of nutrients, and (3) use of defined mixed microbial cultures at each stage of fermentation [21–27]. The microbial hydrolysis of biomass seems effective pre-treatment for producing H_2 even under unsterile conditions [21, 26]. In the present study, the influence of media supplementation on production of H_2 was examined by defined mixed bacterial cultures for hydrolysis of feed and as H_2 produces using biowastes, including pea-shells (PS), potato peels (PP), onion peels (OP), and apple pomace (AP), using unsterile conditions.

Materials and Methods

Bacteria and Culture Conditions

Strains selected in present study were reported in our previous works [16, 26]. The hydrolytic mixed culture (designated as MHC5, [26]) consists of *Proteus mirabilis* strains (EGU30 and EGU32), *Bacillus sphaericus* strains (EGU385 and EGU542) and *Bacillus* sp. strains (EGU444 and EGU447). The H_2 producing microbial mixed culture (designated as, MMC6 [26]) consists of *Enterobacter aerogenes* EGU16, *Bacillus cereus* EGU41, *P. mirabilis* strains (EGU21 and EGU30), *Bacillus megaterium* HPC686 and *B. pumilus* HPC464. Bacteria were grown in nutrient broth to prepare inocula of hydrolytic and H_2 producing cultures as reported previously [26].

Biowaste Hydrolysis

Biowastes—PS, OP, PP, and AP were used as feed [2% of total solids (TS)]. In brief, 250 ml of slurry was made using distilled-water in reagent bottles (300 ml). The resulting slurry was hydrolyzed using MHC5 by incubating for two days at 37 °C [21, 28]. Similarly, co-digestion of biowastes was carried out by mixing them in combinations of 2–4 [22].

H_2 Production

The biowaste slurries hydrolyzed by MHC5 were used as feed-stock. Production of H_2 was carried out by digesting biowaste hydrolysates with MMC6 under batch culture conditions. After, adjusting pH of the slurries to 7.0, argon was flushed to maintain anaerobic conditions. The reactor bottles were incubated at 37 °C. The daily production of resulting biogas was calculated by water-displacement procedure [13].

Effect of Medium Supplementation

The influence of medium supplementation on H_2 producing abilities of MMC6 were checked by adding 0.1 × of M-9 or GM-2 at the following stages: (1) hydrolysis and (2) H_2 production from individual and mixed biowastes.

Effect of Feed Concentration

The influence of feed concentration on H_2 production was assessed by mixing biowastes (OP and PP) at 1, 2, 3, 5 and 7% TS.

Up-Scaling of H_2 Production

The batch culture up-scaling of H_2 production was tested using 0.75, 1.5 and 4.0 l of mixed biowaste (OP and PP) as a feed in the reactors of 1, 2 and 5 l capacities, respectively.

Analytical Methods

Gas Analysis

The evolved biogas gas contents were measured by gas chromatography system (Nucon GC5765, India) as reported previously [13, 29].

Results

Microbial fermentation of biowastes is limited by their initial hydrolysis into simpler forms and their biotransformation into useful bioproducts including H_2. Well-defined mixture of bacteria with ability to produce hydrolytic enzymes were used to provide feed for H_2 producing bacteria.

Production of H_2 from Biowaste

Hydrolysis of different biowastes for 2 days with MHC5 followed by incubation with MMC6 resulted in H_2 production of 225–350 ml/250 ml of slurry (Table 1). Biogas produced had a H_2 content of 58.9–63.0%, such that the H_2 yield (l/kg of TS) ranged from 45 with PS, to 70 with PP. It showed that the bacterial culture had a very high potential to produce H_2, and it is greatly influenced by the type feed material. H_2 yield from PP was 56% higher than that recorded with PS as feed. In view of the increase in H_2

Table 1 Hydrogen production by defined bacteria from biowastes

Feed[a]	Hydrogen (H_2)														
	Control			Supplementation of medium (0.1x)											
				At hydrolysis stage						At H_2 production stage					
				M-9			GM-2			M-9			GM-2		
	Vol[b]	%	Yield[c]	Vol	%	Yield	Vol	%	Yield	Vol	%	Yield	Vol	%	Yield
Pea-shells	225	58.9	45	180	43.2	36	145	41.9	29	310	61.7	62	285	56.1	57
Apple pomace	300	61.9	60	285	57.2	57	245	44.8	49	415	65.4	83	405	59.7	81
Onion peels	335	63.0	67	265	49.7	53	270	46.5	54	425	63.8	85	430	61.3	86
Potato peels	350	62.9	70	305	51.2	61	285	51.3	57	460	64.2	92	435	60.5	87

Values are mean of three experiments and SD was < 10%

[a]Total volume of feed: 250 ml (2%, TS) hydrolysed with MHC5 (2 days) followed with H_2 production by MMC6 (up to 5 days)

[b]Observed volume (ml) of H_2 in the biogas ($H_2 + CO_2$)

[c]l/kg of TS fed

yields on switching the feed, it was envisaged that supplementation of nutrients may help to further enhance the H_2 producing ability of the bacterial cultures. A comparative study was carried out by adding nutrients at two stages during biowaste fermentation process: (1) hydrolysis, and (2) H_2 production. Addition of nutrients as M-9 and GM-2 media at the hydrolytic stage, lead to H_2 yields of 36–61 l/kg of TS depending up on the biowaste. However, these yields were 5–21% lower than their respective controls (Table 1). Addition of GM-2 to different biowastes being subjected to hydrolytic bacterial culture resulted in further decline of 18–36% in H_2 yields in all the cases. In each of these cases, maximum loss in H_2 was recorded with PS as feed.

In the next set of experiments, the two media were added at the H_2 production stage. Here, addition of M-9, led to a H_2 yield of 62–92 l/kg of TS. These yields were 27–38% higher than their controls (Table 1). An interesting feature of this fermentation process was that the H_2 yields were influenced positively by the biowaste in the same order (PP > OP > AP > PS) as in the control. The increase in H_2 yield was observed to be due to better H_2 metabolism i.e. biogas had higher H_2 content. Subsequent experiments, where GM-2 media was added at the H_2 production stage, a 24–35% increase in H_2 yield with respect to the controls was recorded. These H_2 yields of 57–87 l/kg of TS were almost similar to those recorded with M-9 supplementation. Hence, the overall improvement in H_2 production was recorded on addition of M-9 to AP or PS and GM-2 to AP (Table 1).

H_2 Production by Co-digestion of Biowastes

One of the major issues, on the usage of municipal market biowastes is their availability in mixed form. Hence, it becomes important to analyze, which wastes should be co-digested. The four biowastes were co-digested in different combinations (Table 2). Mixing all the four kinds of biowastes in equal proportion resulted in 320 ml of H_2, which was 5% higher than the expected value of 305 ml/250 ml slurry. In the next stage, various combinations of three biowastes resulted in 5–11% improvement over their expected values. In the cases, where AP or OP were missing from the combinations of three biowastes, a 15% enhancement in H_2 yields was observed, it indicated that these wastes are not compatible with other biowastes. In the third group, co-digestion of two biowastes at a time resulted in 2–23% increase in H_2 yields over expected values. In these cases, co-digestion of OP and PP proved to be among the most effective, with an enhancement in H_2 from 340 to 420 ml/250 ml slurry. It implies that the presence of PS is more deleterious than addition of AP to the co-digestion of OP and PP (Table 2).

Supplementation of biowaste hydrolysate with medium M-9, resulted in: (1) 10% loss on co-digesting the four biowastes, (2) 1–3% loss on co-digesting three biowastes and 6% enhancement on combining OP, PP and PS, and (3) 7–16% enhancement in most of the co-digestions involving only two biowastes. Here, the maximum benefit in H_2 yield was recorded in the co-digestion of OP and PP with M-9, a trend that is similar to one seen in control. Overall, the maximum H_2 production of 102 l/kg of TS was recorded by using OP and PP combination, which was hydrolysed by MHC5 and fermented with MMC6 as mixed H_2 producers. Supplementation of different combinations of biowastes

Table 2 Hydrogen production by defined mixed microbial culture from co-digestion of biowastes

Biowastes[a]				Hydrogen (H_2)											
				Control				Medium (0.1 ×)							
								M-9				GM-2			
Apple pomace	Onion peels	Potato peels	Pea shells	Vol[b]		%	Yield[d]	Vol		%	Yield	Vol		%	Yield
				Exp[c]	Obs[b]			Exp	Obs			Exp	Obs		
+[e]	+	+	+	305	320	59.7	64	400	365	63.2	73	390	345	58.7	69
+	+	+	−[f]	325	360	62.5	72	435	430	61.9	86	425	395	63.0	79
+	+	−	+	285	300	57.8	60	385	375	63.6	75	375	365	61.2	73
+	−	+	+	290	335	61.2	67	400	390	64.8	78	375	310	59.3	62
−	+	+	+	300	345	62.0	69	400	425	64.7	85	385	405	59.2	81
+	+	−	−	315	345	63.3	69	420	460	65.3	92	415	410	57.7	82
+	−	+	−	325	330	60.4	66	440	405	62.7	81	415	420	61.8	84
+	−	−	+	265	290	58.8	58	365	390	61.4	78	340	370	58.3	74
−	+	+	−	340	420	64.2	84	440	510	66.7	102	430	475	63.2	95
−	+	−	+	280	305	56.3	61	365	415	64.2	83	355	360	60.6	72
−	−	+	+	290	295	58.6	59	385	385	61.8	79	355	310	58.4	62

Values are mean of three experiments and SD was < 10%

[a]Feed in equal ratio: 250 ml (2%, TS) hydrolysed with MHC5 (2 days) followed with H_2 production by MMC6 (up to 5 days)

[b]Observed volume (ml) of H_2 in the biogas ($H_2 + CO_2$)

[c]Expected volume of H_2, based on the average of H_2 producing capacities of mixed microbial culture MMC6 from individual biowaste

[d]l/kg of TS fed

[e]Present

[f]Absent

with GM-2 medium was observed to be quite similar to that observed with the addition of M-9 medium. With GM-2, observed H_2 yield was 11% higher than the expected values. Here, the maximum yield of 95 l H_2/kg of TS was recorded with the co-digestion of OP and PP.

Effect of Feed Concentration on H_2 Production

To improve the efficiency of the bioprocess, it is desirable to use higher concentration of feed in the reactor. Based on the experiments described above, different feed concentration (1–7% TS) were tested only for co-digestion of OP and PP (Table 3). With increase in the feed concentration from 1 to 7% TS, the volumetric H_2 production was observed to increase from 220 to 1075 ml, equivalent to 61.4–88.0 l/kg of TS. Here, H_2 constituted 54.4–64.2% of total biogas produced. Further, the supplementation of M-9 and GM-2 showed higher volumetric production in the ranges of 290–1590 and 275–1410 ml H_2/250 ml of slurries, respectively. Here, H_2 yields (l/kg of TS) were equivalent to 90.9–116 with M-9 and 80.6-110 with GM-2 supplements. The maximum yields of 116 and 110 l/kg of TS were observed with supplementation of M-9 and GM-2 as compared with control 88 l/kg of TS, respectively. It may be remarked that increasing the TS from 1 to 7% resulted in only marginal reduction in H_2 yields: 30%, 22% and 27% in the case of control, M-9 and GM-2, respectively.

Up-Scaling

Up-scaling of H_2 evolution by MMC6 from co-digestion of biowastes (OP and PP) has been presented in Table 4. In control, the volumetric production H_2 increased from 0.42 to 5.75 l with an increase in the working volume from 0.25 to 4.0 l. Here, the yield was observed in the range of 71.9–84.0 l H_2/kg of TS with H_2 contents of 52.8–64.2% of total evolved biogas. These results suggest that the up-scaling of H_2 production from biowaste is quite stable, with an over variation of 14% on upscaling to 4.0 l. Volumetric production of H_2 from biowastes improved to 7.6 and 7.08 l on supplementation with M-9 and GM-2 medium, respectively. In contrast, to 14% variation recorded in the case of control, the variation in H_2 yield was only 7% in the cases where biowastes supplemented with nutrient media were used. Overall, these results demonstrate that 16-fold

Table 3 Effect of biowaste concentration on hydrogen production by defined mixed bacteria

Feed[a] (%)	Hydrogen (H_2)								
	Control			Medium (0.1 ×)					
				M-9			GM-2		
	Vol[b]	%	Yield[c]	Vol	%	Yield	Vol	%	Yield
1	220	61.0	88.0	290	65.4	116	275	59.5	110
2	420	64.2	84.0	510	66.7	102	475	63.2	95.0
3	580	63.3	77.3	750	61.5	100	710	61.7	94.7
5	905	58.7	72.4	1230	60.2	98.4	1165	58.7	93.2
7	1075	54.4	61.4	1590	58.9	90.9	1410	55.6	80.6

Values are mean of three experiments and SD was < 10%

[a]Feed (OP and PP): 250 ml (2%, TS) hydrolysed with MHC5 (2 days) followed with H_2 production by MMC6 (up to 5 days)

[b]Observed volume (ml) of H_2 in the biogas (H_2 + CO_2)

[c]l/kg of TS fed

Table 4 Up-scaling of hydrogen production from biowaste

Feed[a]	Hydrogen (H_2)								
	Control			Medium (0.1 ×)					
				M-9			GM-2		
	Vol[b]	%	Yield[c]	Vol	%	Yield	Vol	%	Yield
0.25	0.420	64.2	84.0	0.510	66.7	102	0.475	63.2	95.0
0.75	1.135	59.4	75.7	1.495	61.7	99.7	1.375	57.3	90.0
1.50	2.175	55.5	72.5	2.735	63.2	91.2	2.510	61.5	83.7
4.00	5.750	52.8	71.9	7.600	59.4	95.0	7.080	57.9	88.5

Values are mean of three experiments and SD was < 10%

[a]Feed (OP and PP): 250 ml (2%, TS) hydrolysed with MHC5 (2 days) followed with H_2 production by MMC6 (up to 5 days)

[b]Observed volume (ml) of H_2 in the biogas (H_2 + CO_2)

[c]l/kg of TS fed

up-scaling in the working volume of feed was quite consistent.

Discussion

Production of H_2 from the pure sugars and biowaste as a primary feed is widely evaluated using pure and mixed cultures [2, 5, 13, 26]. Since, biowaste are highly complex in nature, they need pretreatment for their effective metabolization to produce at higher H_2 yield [22, 26]. Microbial activity seems to be a viable and cost-effective approach to improve biowaste hydrolysis [21, 22]. In our previous studies, we have effectively demonstrated the use of defined sets of MHCs and MMCs combinations to improve the H_2 production from biowaste as feed [26]. Another major limitation which has not been paid much attention is the imbalanced nutrient status of the biowastes. In this study, we have checked the influence of media (M-9 and GM-2) by using them as supplements at two stages of overall fermentation: (1) at hydrolytic, and (2) at H_2 production. Secondly, we opted for co-digestion of biowastes to achieve the desired nutritional status.

Using combination of MHC5 and MMC6, H_2 production from biowastes—AP, OP, PP and PS (2% TS) was shown to be 60, 67, 70 and 45 l/kg of TS respectively. Here, significant variation in the H_2 yield might be associated with the variation in the composition of these biowastes. Interestingly, the supplementation of both M-9 and GM-2 media at H_2 production stage showed positive influence on H_2 production. An enhancement up to 1.3 to 1.4-fold in H_2 yield was recorded using media. In contrast, the supplementation of media at hydrolysis stage did not prove beneficial. Among the various combinations of co-digestions evaluated, the combination of PP and OP resulted in the production of 84 l H_2/kg of TS, which was more than

the yield of 67 and 70 l H_2/kg of TS, recorded with these wastes individually. Further, supplementation of media to different co-digestions of biowastes, once again provided to be most effective in the case of OP and PP combination. Here, the H_2 yield was found to get enhanced to 95 l/kg of TS with GM2 and to 102 l/kg of TS with M-9.

Another very interesting feature, which was observed in co-digestion and supplementation was the potential to further improve the H_2 production process. It was found that lower feed concentration of 1% TS (OP + PP) supplemented with M-9 and GM-2 media could enhance the H_2 yield (l/kg of TS) from 102 to 116 and from 95 to 110, respectively. Since, the process efficiency can be improved by increasing the loading rate, we found that there was a marginal decline in H_2 yield on increasing the TS concentration up to 7%. Thus, a 7-fold improvement in reactor size can be achieved at the cost of 22–27% loss in H_2 yield. However, at 7% TS level, there was a 1.48-fold enhancement in H_2 yield with M-9 in comparison to control.

For all bioprocesses, the ultimate goal is to produce the bioproduct on a large scale [26]. We thus evaluated the efficiency of the process by upscaling the process by 16 times. On up-scaling of process at a working volume of 4.0 l, it exhibited high volumetric production up to 7600 ml of H_2 using M-9 medium. Here, 1.3-fold improvement in H_2 production was recorded as compared with control. Overall, these results suggest that supplementation of medium at H_2 production stage is very effective to enhance the production yield using biowastes under unsterile conditions. Here, H_2 production was significantly higher than the previously reported yield of 60.2 l H_2/kg of feed with enzymatically pre-treated oil palm [4]. Similarly, undefined mixed culture had shown lower H_2 production yield 55.3 l/kg of volatile solids using mixed biowaste consisting of macro-algae (*Laminaria digitata*) and micro-algae (*Arthrospira platensis*) [6]. However, the H_2 production has been shown to be stabilized using defined mixed cultures [2, 5, 11, 26]. Further, robustness of defined mixed culture can be significantly improved by designing selective microbes with unique feature such as hydrolytic, H_2 production, quorum sensing mediated biofilm formation and anti-microbial properties to improve yield under unsterilized conditions [1, 10, 16, 30, 31]. Further economic improvement in this process can be achieved through its integration with processes leading to the production of CH_4, PHA or biomethanol, through biorefinery approach [5, 28, 32–38].

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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