Hydrogen and Polyhydroxybutyrate Producing Abilities of *Bacillus* spp. From Glucose in Two Stage System

Sanjay K. S. Patel · Mamtesh Singh ·
Vipin C. Kalia

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Abstract Metabolic activities of four *Bacillus* strains to transform glucose into hydrogen (H_2) and polyhydroxybutyrate (PHB) in two stages were investigated in this study. Under batch culture conditions, *Bacillus thuringiensis* EGU45 and *Bacillus cereus* EGU44 evolved 1.67–1.92 mol H_2 /mol glucose, respectively during the initial 3 days of incubation at 37°C. In the next 2 days, the residual glucose solutions along with *B. thuringiensis* EGU45 shaken at 200 rpm was found to produce PHB yield of 11.3% of dry cell mass. This is the first report among the non-photosynthetic microbes, where the *Bacillus* spp.—*B. thuringiensis* and *B. cereus* strains have been shown to produce H_2 and PHB in same medium under different conditions.

Keywords *Bacillus* · Dark fermentation · Hydrogen · Mixed microbial culture · Polyhydroxybutyrate

Introduction

Environmental pollution caused by wastes and rapid depletion of fossil fuels are issues of prime concern [1].

Production of hydrogen (H_2) as a clean fuel and bioplastics as alternatives to petroleum based non-biodegradable plastics are possible solutions for a cleaner and sustainable society [2–4]. Degradation of biowastes to methane (CH_4) and carbon dioxide is a multiple step process with possibilities to produce H_2 and bioplastics (from volatile fatty acids) as intermediates [1, 5]. The whole process is becoming attractive on account of higher production efficiency, stability and economic feasibility [6].

Bacillus spp. a facultative anaerobe has the capacity to adjust its metabolism and respiration depending upon the availability of oxygen (O_2) [7]. *Bacillus* grows well under aerobic conditions and can produce H_2 under anaerobic conditions [8–11]. It has reduced growth rate under anaerobic conditions and induce genes of metabolic pathways such as arginine deiminase, formate dehydrogenase and pyruvate formate-lyase [7]. The later two enzymes are involved in generating H_2 from pyruvate, a key intermediate of fermentative pathway [3]. Pyruvate is cleaved to acetyl CoA and formate [1, 12] and is linked to pH drop where Fhl system (formate dehydrogenase and pyruvate formate-lyase) is induced to counter acidification [3]. Under nutrient stress conditions acetyl CoA gets diverted from tricarboxylic acid (TCA) cycle towards polyhydroxybutyrate (PHB) biosynthetic pathway [13]. PHB production from starch was 46% with a biomass of 54 g/l under O_2 limitation and 20% with a biomass of 71 g/l without O_2 limitation [14].

Hydrogenases couple H_2 evolution with generation of membrane proton gradient. H_2 process is influenced by pH of the growth medium, which may vary from 4.5 to 9.0 [6]. Bacteria such as, *Bacillus licheniformis*, *Clostridium pasteurianum*, *Enterobacter cloacae* and *Rhodobacter sphaeroides* were found to be produce H_2 in the range of 6.5–7.5. However, their growth was adversely affected at

Sanjay K. S. Patel and Mamtesh Singh contributed equally to this paper.

S. K. S. Patel · M. Singh · V. C. Kalia (✉)
Microbial Biotechnology and Genomics, Institute of Genomics and Integrative Biology (IGIB), CSIR, Delhi University Campus, Mall Road, Delhi 110007, India
e-mail: vckalia@igib.res.in; vc_kalia@yahoo.co.in

S. K. S. Patel · M. Singh
Department of Biotechnology, University of Pune,
Pune 411007, India

pH 6.0 and below [15]. As far as the influence of pH on the activities of *Bacillus* are concerned, *B. licheniformis* could produce 70 l H₂/kg VS reduced at pH 6.0 and 74 l H₂/kg VS reduced at pH 7.0 and 8.0 [8].

Exploitation of microbes for generating energy (H₂) and PHB has been shown recently [1, 13]. Recent efforts to produce H₂ and PHB from a single organism have been limited to a few photosynthetic organisms: *Rhodospseudomonas palustris* strain 42OL, *Rhodospirillum rubrum* strains Ha and S1, and *R. sphaeroides* O.U. 001 [16–19]. Among the dark fermentative bacteria quite a few species are known to produce both the bioproducts: H₂ and PHB [2, 9]. Recently, it has been shown that the two bioproducts can be obtained also by using the same strain—*Bacillus cereus* strain EGU43 and *Bacillus thuringiensis* EGU45 [11]. Using these *Bacillus* spp. strains PHB production from biowaste and H₂ production by immobilized bacteria were reported [5, 10].

Integrative approach to couple the bioprocesses for H₂ and PHB production has been gaining importance in recent times [20–22]. Here, they have used different sets of microbes for each step and undefined microbial mixtures have been used for H₂ production process. Secondly, there has been substantial supplementation of feed with different nutrients; however, extraction of bioenergy and bioplastics from organic wastes may not need it [22]. *Bacillus* could survive quite a few distinguished living conditions because it has abilities to withstand environmental stress by forming spores, counter biofilm forming pathogenic bacteria by producing antibiotics and quorum quenching enzymes, etc. [23, 24]. In the present study, we have shown the production of both H₂ and PHB from a substrate with a single strain of *Bacillus* spp., which will help to improve the efficiency, sustainability and economics of the process.

Materials and Methods

Organisms and Growth Conditions

Bacillus strains isolated previously in our laboratory were selected for producing H₂ and PHB: *B. cereus* strains EGU3 (DQ487039), EGU43 (DQ508969) and EGU44 (DQ508970), and *B. thuringiensis* EGU45 (DQ508971) [5, 10, 11]. Their corresponding *rrs* (16S rRNA) gene sequences can be viewed at <http://www.ncbi.nlm.nih.gov/> [11]. Different bacterial strains were grown in Himedia nutrient broth [13.0 g/l distilled water: composed of (g/l): peptic digestion of animal tissue 5.0, NaCl 5.0, beef extract 1.5 and yeast extract 1.5] and incubated at 37°C at 200 rpm for 16–20 h.

Hydrogen Production

For batch-culture digestion, 250 ml of different concentrations (0.5, 1.0 and 2.0%) of glucose in (i) GM-2 medium composition g/l (yeast extract, 1.0; K₂HPO₄, 1.0 and MgSO₄·7H₂O, 0.5) and (ii) minimal medium (M-9) of composition 1x/l (Na₂HPO₄, 6.0 g; KH₂PO₄, 3.0 g; NaCl, 0.5 g; NH₄Cl, 1.0 g; MgSO₄, 1.0 mM and CaCl₂, 0.1 mM) were added in 300 ml BOD bottles [5, 10]. These were inoculated individually with different strains at the rate of 10 µg cell protein/ml of glucose solution. pH of the glucose containing medium was adjusted to 7.0. The bottles with provision for gas outlet and liquid sampling were made air tight with a glass stopper. All the bottles were then flushed with argon and incubated at 37°C. Each day, the pH of the solution was checked by opening the bottle and readjusted to 7.0 with 2 N NaOH or 2 N HCl. After replacing the glass stopper, the bottles were reflushed with argon. The evolved gases were collected by water displacement method and residual glucose was measured by DNSA method. The process of gas collection and analysis was continued until H₂ evolution ceased [10, 11]. The values presented here are based on three different experiments and two repetitions.

Polyhydroxybutyrate Production

Hydrogen production under anaerobic condition continues for 3 days. At the end of this period, the residual medium containing glucose and these volatile fatty acids and residual nutrients of the GM-2 or M-9 medium were subjected to PHB production. Glucose was estimated in the beginning and at the end of this stage and has been presented (Table 1).

For batch culture PHB production, 200 ml of medium (GM-2 or M-9) containing cultures after H₂ production stage (1st stage) were transferred to 1.0 l conical flask (pH set to 7.2 by 2.0 N NaOH or 2.0 N HCl) (2nd stage) and incubated at 37°C in shaker at 200 rpm for 24, 48, 72, 96 and 120 h. The values presented here are based on three different experiments and two repetitions.

Gas Analysis

The gas composition was determined by gas chromatography (Nucon GC5765) using a thermal conductivity detector and argon as carrier gas at flow rate of 30 ml/min. Gas collection and analyses were done daily and H₂ gas production was calculated from the head space measurement of gas composition and the total volume of biogas produced. Although no CH₄ was expected to be evolved in the absence of any added methanogens at any stage,

Table 1 Hydrogen and polyhydroxybutyrate production abilities of *Bacillus* spp. from glucose

Feed (%) glu.	Hydrogen (H ₂)			Res. glu. ^c (%)	Polyhydroxybutyrate (PHB)								
					48 h				96 h				
	Vol ^a	%	Yield ^b		DCM ^d	PHB ^e		Res. glu. ^c (%)	DCM	PHB		Res. glu. ^c (%)	
						mg/l	%			Yield ^f	mg/l		%
Medium supplement: GM-2 ^g													
<i>Bacillus cereus</i> EGU3													
0.5	125	60	0.96	22.4	890	1.1	10	nd ^h		880	nd	na ⁱ	na
1.0	170	57	0.65	22.4	955	2.6	25	3.2		930	1.6	15	nd
2.0	250	52	0.47	21.0	1025	3.4	35	8.0		1,050	2.4	25	nd
<i>B. cereus</i> EGU43													
0.5	145	62	1.12	21.6	810	3.7	30	nd		800	nd	na	na
1.0	185	63	0.71	22.8	940	3.7	35	3.2		930	2.2	20	nd
2.0	290	52	0.55	20.0	1125	5.8	65	13.0		1,020	3.4	35	nd
<i>B. cereus</i> EGU44													
0.5	250	64	1.92	23.2	940	1.6	15	nd		920	nd	na	na
1.0	270	60	1.04	23.2	930	2.2	20	4.0		935	1.6	15	nd
2.0	230	55	0.43	21.6	1050	4.3	45	10.4		1,025	1.9	20	nd
<i>B. thuringiensis</i> EGU45													
0.5	215	63	1.67	24.0	755	11.3	85	nd		750	1.3	10	na
1.0	220	62	0.85	24.0	845	7.7	65	4.4		850	1.8	15	nd
2.0	250	53	0.47	22.0	1050	7.1	75	11.0		1,175	2.1	25	nd
Medium supplement: M-9 ^g													
<i>B. cereus</i> EGU3													
0.5	100	53	0.75	16.0	950	nd	na	nd		980	nd	na	na
1.0	145	56	0.54	16.4	990	nd	na	nd		960	nd	na	nd
2.0	280	52	0.52	16.0	1025	4.9	50	1.2		975	3.1	30	nd
<i>B. cereus</i> EGU43													
0.5	105	58	0.78	16.8	855	nd	na	nd		850	nd	na	na
1.0	200	56	0.75	18.0	870	nd	na	nd		865	nd	na	na
2.0	390	66	0.73	17.8	1045	8.1	85	2.0		1,125	4.4	50	nd
<i>B. cereus</i> EGU44													
0.5	125	62	0.93	19.2	750	nd	na	nd		760	nd	na	na
1.0	170	61	0.63	18.8	935	nd	na	nd		955	nd	na	na
2.0	330	55	0.62	18.0	1025	8.8	90	2.0		985	4.6	45	nd
<i>B. thuringiensis</i> EGU45													
0.5	120	66	0.89	17.6	765	nd	na	nd		795	nd	na	na
1.0	215	62	0.80	17.2	810	5.5	45	nd		800	2.5	20	na
2.0	390	63	0.83	16.0	1190	6.7	80	1.6		1,010	6.4	65	nd

Data based on three replicates and two repetitions. Standard deviation was less than 5%

Total volume of feed: 250 ml during H₂ in 300 ml reactor and 200 ml during PHB production in 1.0 l reactor

Inoculum used was 10 mg cell protein/ml feed

^a Cumulative observed volume (ml) of H₂ in the biogas (H₂ + CO₂) over a period of 3 days

^b mol/mol glucose utilized

^c Residual glucose with respect to feed taken as 100%

^d Dry cell mass

^e Polyhydroxybutyrate

^f mg/l

^g Medium concentration (1x)

^h Not detectable

ⁱ Not applicable

however, gas samples were also analyzed for its presence daily [11].

Protein Estimation

Actively growing cell cultures were centrifuged at 6,000 rpm at 4°C for 20 min and protein content was estimated by Lowry's method [10].

PHB Analysis

Samples (200 ml) were analyzed for dry cell mass (DCM) and PHB production. Aliquots (200 ml) were centrifuged at 6,000 rpm at 4°C for 20 min. The pellet was washed with 10.0 ml saline solution (0.9% NaCl) and re-centrifuged. The pellet was dried at 85°C for 36 h and weighed to estimate DCM. About 40 mg of DCM was mixed with 2.0 ml Dichloroethane, 2.0 ml propanol-hydrochloric acid solution [Propanol : hydrochloric acid (4:1) v/v] and 0.2 ml internal standard solution (40 g benzoic acid/l propanol) in a tightly sealed 25 ml test tube. The mixture was incubated in water bath at 100°C for 2 h and then cooled to room temperature. The mixture was vortexed with 4.0 ml elix water. The DCM solution containing the esters of propanol and β -hydroxy acids from PHB hydrolysis was analyzed with GC (column- stainless steel 2 m long and 2 mm inner diameter packed with 10% Reoplex 400 with a mesh range of 80–100). Poly-3-hydroxybutyrate (Fluka Chemika, USA) was used as standard [11]. Gravimetric estimation of the polymer yield was done from DCM by extraction of the polymer using chloroform and methanol [5].

Results and Discussion

Hydrogen Production

Hydrogen production with different *Bacillus* spp. (*B. thuringiensis* strain EGU45, *B. cereus* strains EGU3, EGU43 and EGU44) were found to vary with the two media and further affected by the concentration of glucose.

In GM-2 medium the observed H_2 evolution varied from 125–250 ml at 0.5% glucose concentration (250 ml feed) and increased to 230–290 ml at 2.0% glucose concentration (Table 1). Here H_2 constituted 52–64% of the total biogas produced. However, the effective yields were higher at 0.5%—in the range of 0.96–1.92 mol H_2 /mol glucose compared to 0.43–0.55 mol H_2 /mol glucose at 2.0% glucose concentration level, which was accompanied by a lower H_2 concentration in the biogas. pH drop in the medium was from 7.0 to 3.6, 4.8 and 6.5 on day 1, 2 and 3, respectively. *Bacillus* strains were observed to grow (OD_{660}) in the range 0.8606–2.3747 with GM-2 medium

during the process of H_2 production, which were observed between 11 and 18 h of incubation in each 24 h cycle.

With the shift in growth medium (GM-2) to minimal medium (M-9), there were certain changes in H_2 production and yields. At lower glucose concentration of 0.5% w/v, observed H_2 evolution was in the range of 100–125 ml/250 ml feed in contrast to 280–390 ml H_2 at 2.0% glucose concentration (Table 1). In this case, H_2 composition was not affected much by the concentration of feed employed. In brief, H_2 yields were higher in the range of 0.75–0.93 mol/mol glucose utilized at 0.5% glucose concentration in contrast to 0.52–0.83 mol/mol glucose utilized at 2.0% glucose concentration in the feed. pH drop in the medium was from 7.0 to 4.6, 5.3 and 6.6 on day 1, 2 and 3, respectively. *Bacillus* strains were observed to grow (OD_{660}) in the range 0.8564–2.0431 with M-9 medium during the process of H_2 production. We may conclude that H_2 yields were better in GM-2 than in M-9 medium. *B. cereus* strain EGU44 can be regarded as the best among these four strains for H_2 production process, followed by *B. thuringiensis* EGU45 (Table 1).

Polyhydroxybutyrate Production

Under stress conditions, primarily those of magnesium, nitrogen, phosphorus and potassium [25], PHB production is initiated by diversion of acetyl CoA from TCA cycle due to the suppression of citrate synthase and iso-citrate dehydrogenase (key enzymes of TCA cycle) activities leading to blockage of TCA cycle. High acetyl CoA concentration and low CoA activates β -ketothiolase which converts acetyl CoA to aceto-acetyl CoA, which is then converted to 3-hydroxybutyryl CoA through the action of aceto-acetyl CoA reductase. Polymerization of these monomers is affected by polyhydroxyalkanoate (PHA) synthase [12, 13, 26]. This release of acetyl CoA during the H_2 production stage provoked us evaluate the feasibility of PHB production.

PHB production after the H_2 evolution stage was observed to be dramatically affected by the presence of O_2 as reported in literature as well [14]. Under non-shaking conditions, where O_2 becomes a limiting factor, PHB yields were found to be negligible in the range up to 1.1% of DCM, which was in the range of 220–295 mg/l at 0.5% glucose concentration and increased to 375–755 mg/l at 2% glucose. Hence, it was decided to provide proper aeration, which helped to improve PHB yields.

A comparison of DCM in GM-2 and M-9 were found to be quite similar with all the strains at a given glucose concentration under shaking conditions. DCM was in the range of 750–950 mg/l at a glucose concentration equivalent to a residual 16–19% of the initial 0.5% and 1,025–1,190 mg/l at 2.0% initial glucose concentration.

Although the average and maximum PHB production in the two media were almost similar, however GM-2 proved more effective in comparison to M-9. PHB production and yields was consistent and effective at all the glucose concentrations with GM-2, whereas it was effective largely at 2.0% glucose concentration in M-9 medium. It may be remarked here that *B. thuringiensis* EGU45 was the most effective strain with respect to PHB production and yield after 48 h of incubation. Longer incubation periods of 96 h were found to be counter productive for PHB production. The rest three strains were equally effective for PHB production through lesser than *B. thuringiensis* EGU45. The residual glucose concentrations at the end of these experiments were below the detectable limits. We had conducted experiments at higher initial concentration of up to 7.0% glucose along with the two minimal media, but we did not observe any significant gains in term of PHB production. Hence those data have not been presented here.

H₂ and PHB production have been reported largely as independent bioprocesses. However, the emphasis is being laid on integrating them [20–22]. In these studies, different sets of microbes have been used for each step. Use of undefined microbial mixtures for H₂ production process, is likely to produce high variability among the intermediates, which are to be used for the next step of PHB production. To the best of our knowledge, till date only three photosynthetic organisms have been reported with abilities to produce these two bioproducts H₂ and PHA: *R. palustris*, *R. rubrum* and *R. sphaeroides* [16–19]. Here, it is the first demonstration of producing H₂ and PHB with the same strain of non-photosynthetic bacterium, *Bacillus* in a sequential manner without any further addition of nutrients or bacteria during the second stage of PHB production.

With sucrose as feed, *Bacillus* sp. have been reported to evolve 1.53 mol/mol hexose [27]. *B. licheniformis* and *Bacillus* spp. along with other bacterial cultures have resulted in a H₂ yield in the range of 1.5–1.65 mol/mol glucose [10, 28]. PHAs may generally account up to 90% of the dry cell weight (DCW) of the microbes [29]. Among the *Bacillus* spp. the reported PHA yields vary from 11 to 69% [13]. On the basis of data on H₂ and PHB yields, we may conclude that *B. thuringiensis* EGU45 can be rated better than *B. cereus* EGU44. These two *Bacillus* strains can be exploited for producing H₂ and PHB in GM-2 medium with 0.5% glucose as carbon source. In the present study, we have been able to achieve a maximum of 1.67–1.92 mol H₂/mol glucose and a maximum PHB yield of 11.3%.

On the issue of the abilities of organism to grow aerobically but produce H₂ under anaerobic conditions, it may be highlighted that *Rhizobium* and *Bradyrhizobium* are known to fix atmospheric (molecular) nitrogen (N₂) with the help of enzyme nitrogenase and evolve H₂ by action of enzyme hydrogenase. These organisms grow well under

aerobic conditions but fix N₂ and evolve H₂ only under anaerobic conditions [30]. *Bacillus* spp. have been reported to adjust their metabolism and respiratory activities according to the prevailing environmental stress. Under O₂ limitation, genes of H₂ generation from pyruvate get induced [7]. Hydrogenases couple H₂ generation to a membrane proton gradient leading to pH drop [5]. In our present work and previous reports [11], H₂ evolution was found to follow a pattern with respect to drop in pH (higher pH drop leading to higher H₂ production). Acidification activates formate dehydrogenase and pyruvate formate-lyase system leading to H₂ generation [3:5] via formate accompanied by the release of acetyl CoA [13, 31], which may be subsequently diverted towards PHB production, depending upon environmental (nutritional) stress [4, 32].

PHB production is limited by the high cost of raw material, brittleness and its low strength and uneconomical recovery process. These problems can be tackled by using biowaste as feed, which can be expected to reduce the cost of production to the extent of 45% of the total. Co-polymers of PHA have higher strength compared to monomers. Further PHB yields of up to 69% (of DCW) are also achievable with *Bacillus* spp. It has been proposed that introduction of self-lysing genes and their expression on complete depletion of the feed material can lead to autolysis of the cell wall, which is expected ease recovery of PHB and making the process economical [13]. These processes can be optimized further by manipulating carbon and nitrogen ratio among other factors primarily during the PHA biosynthesis stage. All these steps are likely to make H₂ and PHA processes commercially viable and enhance the sustainability of the whole process.

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