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Hydrogen and polyhydroxybutyrate producing abilities of microbes from diverse habitats by dark fermentative process

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Abstract

Thirty five bacterial isolates from diverse environmental sources such as contaminated food, nitrogen rich soil, activated sludges from pesticide and oil refineries effluent treatment plants were found to belong to *Bacillus*, *Bordetella*, *Enterobacter*, *Proteus*, and *Pseudomonas* sp. on the basis of 16S rRNA gene sequence analysis. Under dark fermentative conditions, maximum hydrogen (H_2) yields (mol/mol of glucose added) were recorded to be 0.68 with *Enterobacter aerogenes* EGU16 followed by 0.63 with *Bacillus cereus* EGU43 and *Bacillus thuringiensis* EGU45. H_2 constituted 63–69% of the total biogas evolved. Out of these 35 microbes, 18 isolates had the ability to produce polyhydroxybutyrate (PHB), which varied up to 500 mg/l of medium, equivalent to a yield of 66.6%. The highest PHB yield was recorded with *B. cereus* strain EGU3. Nine strains had high hydrolytic activities (zone of hydrolysis): lipase (34–38 mm) – *Bacillus sphaericus* strains EGU385, EGU399 and EGU542; protease (56–62 mm) – *Bacillus* sp. strains EGU444, EGU447 and EGU445; amylase (23 mm) – *B. thuringiensis* EGU378, marine bacterium strain EGU409 and *Pseudomonas* sp. strain EGU448. These strains with high hydrolytic activities had relatively low H_2 producing abilities in the range of 0.26–0.42 mol/mol of glucose added and only *B. thuringiensis* strain EGU378 had the ability to produce PHB. This is the first report among the non-photosynthetic microbes, where the same organism(s) – *B. cereus* strain EGU43 and *B. thuringiensis* strain EGU45, have been shown to produce H_2 – 0.63 mol/mol of glucose added and PHB – 420–435 mg/l medium.

Keywords: *Bacillus*; Dark fermentation; Hydrogen; Polyhydroxyalkanoate; 16S rRNA gene

1. Introduction

Environmental pollution caused by inefficient waste management and burning of fuels has forced mankind to improve waste treatment methods. The exploitation of biological waste material as a renewable resource for bioenergy and bioproduct development continues to be a major challenge for biotechnology (VanWyk, 2001). The concept of effective treatment of wastewater and less costly generation of H_2 and PHA as bioenergy and bioproducts using mixed microbial population has been proposed (Angenent et al., 2004).

Due to its clean and high energy yield (122 kJ/g), H_2 is a promising candidate as an ideal fuel in the future (Levin et al., 2004). Quite a few pure cultures of microbes have

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been shown to evolve H_2. With mixed microbial community and wastes as raw material, only traces of H_2 are usually evolved due to the ubiquitous nature of H_2 consumers and interspecies H_2 transfer reactions (Ozturk et al., 1989). Different techniques have been employed to suppress methanogenic activity and enhance H_2 yield (Sparling et al., 1997; Sonakya et al., 2001; Oh et al., 2003; Morimoto et al., 2004; Valdez-Vazquez et al., 2005; VenkataMohan et al., 2007). On the other hand, much effort has been made in producing bio-plastics (Reddy et al., 2003) using less costly raw materials such as renewable resources (Madison and Huisman, 1999). So far only three photosynthetic organisms have been reported with abilities to produce these two bioproducts H_2 and PHA: *Rhodopseudomonas palustris* strain 42OL, *Rhodospirillum rubrum* strains Ha and S1 and *Rhodobacter sphaeroides* O.U. 001 (Hustede et al., 1993; Vincenzini et al., 1997; Yigit et al., 1999; Lee et al., 2002). On the contrary, there are no reports of a single organism with abilities to produce PHB and H_2, under dark fermentative conditions, which hold more promise compared to photosynthetic route (Levin et al., 2004). Prof. Yu reported production of H_2 and bio-plastics from waste water with mixed cultures (http://www.shaping-the-future.de/pdf, www/157paper.pdf). Using genomic approach, microbes such as *Burkholderia fungorum*, *Novosphingobium aromaticivorans* and *Microbulbifer degradens* with potential to produce H_2 and PHA and ability to grow on wastes have been reported (Kalia et al., 2003a,b).

In this study, the objective was to isolate non-photosynthetic organism with ability to produce PHB and H_2, and further characterize them for their hydrolytic enzymatic activities.

2. Methods

2.1. Sample collection

Samples for bacterial isolation were collected from diverse environmental sources (Table 1), for example contaminated food (pickle waste), nitrogen rich soil, fermenting potato peels and cow dung slurry and various activated sludges from effluent treatment plants treating industrial wastes such as Oil Refinery, Nitro-aromatic compounds, pesticide. All samples were brought to laboratory on ice and kept saved at −20 °C.

2.2. Isolation of bacteria

Microbial strains were isolated under different culture conditions such as: (i) pH from 2.0 to 12.0 with each incremental unit (ii) salt (NaCl, %) – nil, 0.5, 2.5, 5.0) (iii) temperature (30 °C, 37 °C) (iv) heavy metals (Co, Ni @ 1 mM concentrations). Isolation protocol followed two procedures: (i) Pretreatment and Plating and (ii) Direct plating. In Pretreatment, 1 g or 1 ml of the sample was added to 10 ml of Himedia nutrient broth (NB) 13 g in 1 l distilled

Table 1
Source and isolation conditions for different microbes used in this study

S. no.	Isolates designated as	Source	Isolated on NA plates at pH[a]	Isolated on medium with	
				Salt (NaCl) (%)	Metal and Conc. (mM)
1	EGU3	Contaminated food[b]	12 (10)	0.5	na[e]
2	EGU14	Contaminated food	5 (7)	0.5	na
3	EGU15, EGU16	Contaminated food	2 (7)	0.5	na
4	EGU17	Contaminated food	3 (7)	0.5	na
5	EGU21	Nitrogen rich soil	4 (5)	0.5	na
6	EGU30, EGU31, EGU32	Nitrogen rich soil	10 (10)	0.5	na
7	EGU34	Nitrogen rich soil	11 (10)	0.5	na
8	EGU41, EGU43, EGU44, EGU45, EGU46, EGU47, EGU85	MRL ETP sludge[c]	7 (7)	3.0	na
9	EGU48, EGU49	MRL ETP sludge	7 (7)	5.0	na
10	EGU90, EGU91	Cattle dung	7 (7)	0.5	na
11	EGU163	Nitro-aromatic ETP sludge	7 (7)	Nil	na
12	EGU367	Pesticide ETP sludge	7 (7)	Nil	na
13	EGU378, EGU385, EGU394, EGU396, EGU399	Pesticide ETP sludge	7 (7)	0.5	Ni, 1.0
14	EGU409	Pesticide ETP sludge	7 (7)	0.5	Co, 1.0
15	EGU444, EGU445, EGU447, EGU448	PP + CD[d]	2 (5)	0.5	na
16	EGU475	PP + CD	4 (5)	0.5	na
17	EGU542	PP + CD	11 (11)	0.5	na

[a] Cultures were subsequently maintained at pH values given in the parenthesis.
[b] Pickle waste.
[c] Sludge from Madras refinery limited effluent treatment plant (ETP).
[d] Acidogenic slurry of Potato peels (PP) with Cattle dung (CD).
[e] Not applicable.

water, pH −7.0, (preset at different pH; salt and heavy metal concentrations) and incubated at 30 °C and 37 °C at 200 rpm for 24 h. 100 μl/mg of these pretreated samples were added to nutrient agar (NA) (2% agar was added to NB medium) at different pH, salt and heavy metal concentrations (as mentioned above). Incubations were done at 37 °C for 24 h. In Direct plating, 1 g or 1 ml of the sample was added to 10 ml sterile distilled water. 100 μl of this sample was spread directly on NA plates at different pH, salt and heavy metals concentrations. Incubations were done at 37 °C for 24 h. (Sludge samples were incubated at 30 °C for 24–48 h). Single distinct colonies of isolates were picked and streaked on fresh NA plates. As agar was not getting solidified at pH 2.0, 3.0 and 4.0, samples from these pH were subsequently plated on pH 5.0 NA plates.

2.3. Amplification of Bacterial 16S rRNA gene

The amplification of 16S rRNA gene (~1.5 kb) was carried out from genomic DNA by PCR, using forward primer 27f, 5′-AGA GTT TGA TCA TGG CTC AG-3′, and reverse primer 1492r, 5′-TAC GGC TAC CTT GTT ACG ACT T-3′ as reported earlier (Narde et al., 2004). The reaction mixture contained 10 μl template DNA, 5 μl of reaction buffer, 2 μl of Taq DNA polymerase (3U/μl) (Amersham Biosciences USA), 5 μl of the four dNTP's (200 μM each) (Amersham Biosciences USA), 4 μl of $MgCl_2$ (3 mM), 2 μl of each primer (20 μM) and the final volume was made up to 50 μl. The thermal PCR profile was as follows: initial denaturation of 94 °C for 1 min, primer annealing at 55 °C for 1 min 30 s. and elongation step was extended to 10 min. Amplification was performed in 0.2 ml PCR softstrips (Axygen Scientific Inc. USA) in DNA thermal cycler. Aliquots of the PCR gene amplicons 2 μl were analyzed by Tris–acetate–EDTA (TAE) agarose gel electrophoresis. Positive 16 S rRNA gene amplicons were purified with QIA quick PCR purification kit (Qiagen, Hilden, Germany) as described by the manufactures. In order to minimize PCR drift, purified PCR products from three separate PCR amplifications were pooled prior to purification. DNA sequencing reactions were done with big dye termination cycle sequencing kit version 3.1 (Applied Biosystems, USA) and the products were run on an ABI 3700 machine at The Centre for Genomic Applications (TCGA), New Delhi, India. Microbial isolates were identified through BLAST analysis of the partial sequences of 16S rRNA gene and deposited in NCBI GenBank. Accession numbers of the 35 isolates are given in Table 2.

2.4. Biochemical characterization

Thirty-five strains were tested for 45 different biochemical properties like utilization of 33 different carbohydrates as carbon source, five proteins as nitrogen source and seven enzyme activities by KB009 and KB003 Hi Carbohydrate™ Kit (Himedia). The tests are based on the principle of pH change and substrate utilization. On incubation, organisms undergo metabolic changes, which are indicated by a spontaneous colour change in media. 20 μl of actively growing bacteria ($O.D_{600} = 1.0$) was poured in to each well of the kits and incubated at 37 °C for 24 to 48 h.

2.5. Determination of metabolic characteristics

Different isolates were patched individually onto selective media such as nutrient agar (as control), skim milk agar (1%), tributyrin agar (1%) and starch agar (0.2%), to identify their abilities to produce protease, lipase and amylase, respectively. All the plates were incubated at 37 °C for 2 days for protease and amylase, and up to 7 days for lipase activities, which were checked by observing for a zone of clearing around each bacterial isolate. For starch agar, the zone of clearing was observed after flooding the plates with iodine solution. Relative enzyme activity was calculated by finding the ratio of zone of clearing (mm) and size of the bacterial colony (mm). Values of zone of clearing are based on three sets of observations with a standard deviation up to 10%.

3. Analytical methods

3.1. Biological hydrogen production

Different bacterial isolates were grown in Himedia nutrient broth (13 g/l distilled water) and incubated at 37 °C at 200 rpm for 16 to 20 h. These actively growing cell cultures were centrifuged at 6000 rpm for 20 min and protein content was estimated by Lowry's method. For batch-culture digestion, 250 ml of 2% (w/v) glucose in minimal medium (Miller M-9) were added in 300 ml BOD bottles. These were inoculated individually with different bacterial strains at the rate of 20 mg protein/250 ml glucose solution. pH of the glucose containing medium was adjusted to 7.0. The OD_{600} of the inoculated medium was measured in the beginning and at the end of the experiment with spectrophotometer (Model: Lambda 35 Perkin–Elmer). 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 to maintain anaerobic conditions and incubated at 40 °C. Each day, the pH of the solution was checked by opening the bottle and readjusted to 7.0 with 2.0 N NaOH. After replacing the glass stopper, the bottles were reflushed with argon. The evolved gases were collected by water displacement method (Kalia et al., 1994). The process of gas collection and analysis was continued until H_2 evolution ceased. These experiments were replicated twice.

3.2. Gas analysis

Evolved gases (a mixture of H_2 and CO_2) were collected over water (pH 2.0) in a graduated gas holder and the vol-

Table 2
Hydrogen and polyhydroxybutyrate producing abilities of bacterial isolates

Organism (Accession no.[a])	Hydrogen (H_2)			Polyhydroxybutyrate (PHB)		
	Vol.[b] (ml)	Yield[c]	%	DCM[d] (mg/L)	Conc.[e] (mg/L)	Yield[f] (%)
Firmicutes						
Bacillus cereus strain EGU43 (DQ508969)	390	0.63	66	850	420	49.4
B. cereus strain EGU41 (DQ508967)	370	0.60	66	835	245	29.3
B. cereus strain EGU44 (DQ508970)	330	0.53	55	925	485	52.5
B. cereus strain EGU48 (DQ508974)	310	0.50	66	685	205	29.9
B. cereus strain EGU3 (DQ487039)	280	0.45	52	750	500	66.6
B. cereus strain EGU46 (DQ508972)	235	0.38	54	965	355	36.7
B. thuringiensis strain EGU45 (DQ508971)	390	0.63	63	840	435	51.8
B. thuringiensis strain EGU47 (DQ508973)	300	0.48	52	615	265	43.0
B. thuringiensis strain EGU378 (DQ487033)	160	0.26	57	515	30	5.8
B. subtilis strain EGU17 (DQ915853)	320	0.52	53	595	nd[g]	na[h]
B. subtilis strain EGU475 (DQ508977)	210	0.34	60	580	nd	na
B. subtilis strain EGU163 (DQ508966)	175	0.28	50	1225	190	15.5
B. licheniformis strain EGU90 (DQ768243)	270	0.44	60	540	15	2.7
B. licheniformis strain EGU14 (DQ768246)	100	0.16	31	560	nd	na
B. pumilus strain EGU49 (DQ508975)	280	0.45	62	570	nd	na
B. sphaericus strain EGU542 (DQ508979)	260	0.42	53	635	nd	na
B. sphaericus strain EGU385 (DQ487032)	205	0.33	51	625	nd	na
B. sphaericus strain EGU399 (DQ487036)	180	0.29	45	730	nd	na
Bacillus sp. strain EGU91 (DQ915850)	370	0.60	60	465	25	5.3
Bacillus sp. strain EGU445 (DQ915849)	260	0.42	49	425	nd	na
Bacillus sp. strain EGU85 (DQ768239)	245	0.40	52	500	150	30.0
Bacillus sp. strain EGU447 (DQ508976)	225	0.36	50	420	nd	na
Bacillus sp. strain EGU444 (DQ768240)	215	0.35	50	865	nd	na
Bacillus sp. strain EGU367 (DQ768236)	205	0.33	52	795	nd	na
Bacillus sp. strain EGU15 (DQ487038)	190	0.31	39	580	nd	na
β-Proteobacteria						
Bordetella avium strain EGU31 (DQ915851)	365	0.58	70	545	nd	na
γ-Proteobacteria						
Enterobacter aerogenes strain EGU16 (DQ768244)	420	0.68	69	885	65	7.3
Proteus mirabilis strain EGU21 (DQ768232)	360	0.58	65	650	nd	na
P. mirabilis strain EGU30 (DQ487041)	355	0.57	61	595	15	2.5
P. mirabilis strain EGU32 (DQ508964)	320	0.52	55	1030	345	33.5
P. mirabilis strain EGU34 (DQ508965)	190	0.31	41	810	255	31.5
Pseudomonas stutzeri strain EGU394 (DQ487034)	290	0.47	58	505	40	6.9
P. stutzeri strain EGU396 (DQ487035)	160	0.26	39	555	nd	na
Pseudomonas sp. strain EGU448 (DQ768241)	160	0.26	52	485	nd	na
Unclassified						
Marine bacterium strain EGU409 (DQ487037)	240	0.39	61	960	nd	na

[a] http://www.ncbi.nlm.nih.gov/.
[b] Volume of H_2 (ml).
[c] Mol. H_2/mol glucose added.
[d] Dry cell mass.
[e] PHB concentration.
[f] PHB concentration over DCM (%w/w).
[g] Not detectable.
[h] Not applicable.

umes calculated at 25 °C. The gas composition was determined using gas chromatography (GC5700, Nucon Engineers, New Delhi) at ambient temperature by standard procedure. A stainless steel molecular sieve column (1.8 m long and 2 mm inner diameter) was used for analyzing H_2, O_2 and N_2 gases, while air, CH_4, and CO_2 were analyzed by using Porapak Q column of stainless steel (1.8 m long and 2 mm inner diameter). Argon gas was used as a carrier at a flow rate of 30 ml/min. Gas standards (H_2, CH_4 and CO_2) were run before each set of gas analysis. Although no methane was expected to be evolved in the absence of any added methanogens at any stage, however, to ensure that no H_2 quenching methanogens are present ever as contaminants; CH_4 analysis was done every time gases were analyzed. Gas collection and analyses were done daily. Since a 50 ml of head space is present above the fermenting liquid, the evolved gases will get diluted, thus it is necessary to use a correction factor. H_2 gas production was

calculated from the headspace measurement of gas composition and the total volume of biogas produced, at each time interval, using the mass balance equation:

$$V = V_{0\gamma i} + \Sigma V_{i\gamma i}$$

where V is the cumulative hydrogen gas volumes at the current (i); V_0 is the volume of headspace of vials; V_i is the biogas volume discharged from the vials at the time interval (i); γi is the fraction of hydrogen gas discharged from the vials at the time interval (i) (Pan et al., 2007).

3.3. Production of poly(3-hydroxybutyrate)

Different bacterial isolates were grown and harvested as detailed above for estimating H_2 production. For batch-culture production of PHB, 400 ml of GM2 medium (Jan et al., 1996) (Yeast extract (1 g), K_2HPO_4 (1 g), $MgSO_4 \cdot 7\ H_2O$ (0.5 g), Glucose (10 g) in 1 l distilled water, pH 7.2) were added in 1 l conical flask. These were inoculated individually with different bacterial strains at the rate of 400 μg cell protein/400 ml GM2 medium and incubated at 37 °C at 200 rpm for 24 h and 48 h. These actively growing cells culture were centrifuged at 6000 rpm for 20 min. The pellet was dried at 85 °C for 36 h for determining DCM (Dry Cell Mass). About 40 mg of DCM was mixed with 2 ml Dichloroethane (DCE), 2 ml propanol–hydrochloric acid solution (propanol: hydrochloric acid 4:1 vol:vol) 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 a water bath at 100 °C for 2 h and then cooled to room temperature. The mixture was vortexed with 4 ml elix water. The DCE 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 (Riis and Mai, 1988).

4. Results and discussion

4.1. Diversity of microbial isolates

Thirty five microbial isolates were selected on the basis of various physiological parameters such as pH, salt concentration, sensitivity to metals i.e. nickel, and cobalt (Table 1) and their sensitivity to 12 different antibiotics (Supplementary Table 1). It ensured the diversity of microbes at a preliminary level. The microbial diversity was seen by the variation in their 16S rRNA gene sequences which match to the genera belonging to firmicutes – *Bacillus* and β- and γ-Proteobacteria – *Bordetella, Enterobacter, Proteus, Pseudomonas* and a marine bacterium.

4.2. Hydrogen producers

H_2 producing abilities of bacteria (Table 2) belonging to Firmicutes – *Bacillus* sp., *B. cereus*, *B. thuringiensis*, *B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. sphaericus*, β-Proteobacteria – *B. avium* and γ-Proteobacteria - *Enterobacter*, *P. mirabilis*, *P. stutzeri*, varied considerably from as low as 100 ml by *B. licheniformis* strain EGU14 to as high as 420 ml by *E. aerogenes* strain EGU16 i.e. a 4.2 fold higher yield from the same amount of feed. This amounted to a H_2 yield of 0.16–0.68 mol/mol of glucose added. Of the total biogas (a mixture of H_2 and CO_2), H_2 comprised 31–70% and CO_2 29–68%. No CH_4 was observed in any of the reactions. Most of the isolates have shown that a large proportion (>80%) of the total Biogas-H evolution takes place within 2–3 days of incubation. The process becomes slower thereafter. The evolution of H_2 was seen to follow a pattern in the drop of pH, which was 4.94 on an average at the end of every 24 h of incubation. On the subsequent days the pH was observed to fall to 5.65 and 5.75. At high partial pressure of H_2 the biochemical reactors are endergonic and inhibited by the product H_2 (Brock and Madigan, 1991). Accumulation of organic acids and a possible drop of pH contribute to biochemical inhibitor of the H_2 production (Valdez-Vazquez et al., 2005). Among the various *Bacillus* species, *B. cereus* and *B. thuringiensis* were equally competent to produce 390 ml H_2 (observed volume) equivalent to 0.63 mol H_2/mol of glucose added. However, there was a large variability in H_2 producing abilities among the strains belonging to the two *Bacillus* spp.: from 0.36 to 0.63 mol/mol of glucose added for *B. cereus* strains and from 0.26 to 0.63 mol/mol of glucose added for *B. thuringiensis* strains. The other 4 species of *Bacillus* had a relatively low H_2 yielding range: 0.16–0.45 mol/mol of glucose added. *Proteus* and *Pseudomonas* strains produced moderate to low amounts of H_2: 0.26–0.58 mol/mol of glucose added, which had 39–65% of the total biogas. High H_2 yielding strains also had higher H_2 content, invariably in the range of 60–70%. It may be remarked here that these H_2 producing capacities of the various strains were found to be negatively influenced by the growth of the cultures. It may be reasonable to conclude at this stage that the H_2 yields observed here can be improved by optimization of various parameters including initial inoculum size, glucose concentration and culture conditions such as immobilization of H_2 producers (Kumar et al., 1995).

4.3. PHB producers

Out of the 35 strains, represented by 6 genera (13 species), PHB production was observed in 8 different species (Table 2). *B. cereus* (6 isolates) and *B. thuringiensis* (3 isolates), produced PHB in the range of 205–500 mg/l and 30 to 435 mg/l of medium supplemented with glucose, respectively. Among the isolates from *B. licheniformis*, *B. subtilis*, *Bacillus* sp., *P. mirabilis* and *P. stutzeri*, there were some which produced PHB. Here it varied from moderate quantities of 345 mg/l to low quantities of 15 mg/l. Incidentally, none of the members of *B. pumilus*, *B. sphaericus*, *B. avium* and a marine bacterium were observed to produce any PHB. A very interesting observation was a positive correla-

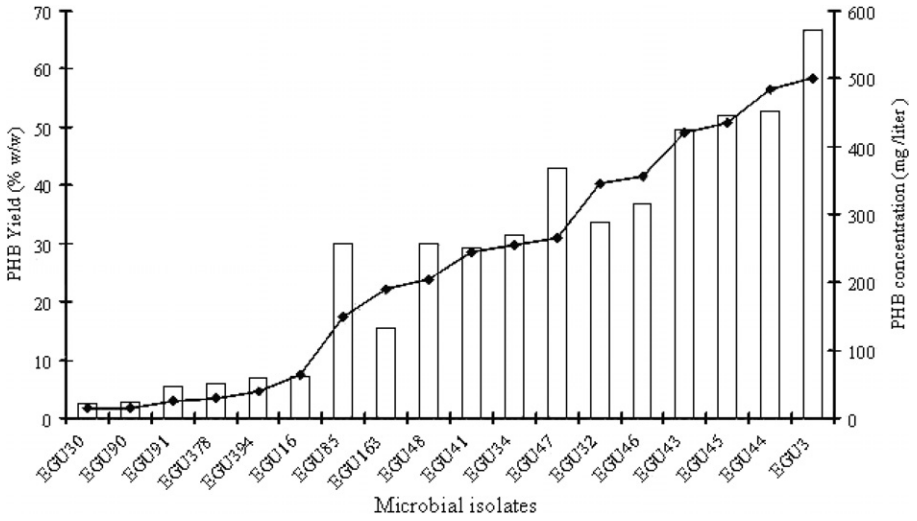

Fig. 1. Correlation of PHB yield (%w/w) and concentration (mg/l) in different microbial isolates. Isolates: EGU30, *P. mirabilis*; EGU90, *B. licheniformis*; EGU91, *Bacillus* sp.; EGU378, *B. thuringiensis*; EGU394, *P. stutzeri*; EGU16, *E. aerogenes*; EGU85, *Bacillus* sp.; EGU163, *B. subtilis*; EGU48, *B. cereus*; EGU41, *B. cereus*; EGU34, *P. mirabilis*; EGU47, *B. thuringiensis*; EGU32, *P. mirabilis*; EGU46, *B. cereus*; EGU43, *B. cereus*; EGU45, *B. thuringiensis*; EGU44, *B. cereus*; EGU3, *B. cereus*.

tion between the concentration of PHB and the relative yield (w/w) (Fig. 1).

A comparison of H_2 yielding and PHB producing capabilities of various isolates reveals very interesting set of information, such that *E. aerogenes* in spite of being the highest H_2 yielding isolate, did not figure among the highest PHB producers. *B. cereus* strains EGU43, EGU44 and EGU49 can be considered among the best strains as far as these two bioproducts are concerned i.e. 280–390 ml H_2 and 420–500 mg/l PHB (a yield of 49.4–66.6%). A similar response was also recorded with *B. thuringiensis* strains EGU45 and EGU47.

4.4. Hydrolytic enzyme activities of microbial isolates

In order to exploit the H_2 and PHB producing abilities of different microbes, it is desirable to produce them from cheap raw materials such as biological wastes. It is economical to use those microbes which also solubilize the various constituents of the biowastes. So, the strains were checked for amylase, lipase and protease activities. The proteolytic activities of certain strains of *Bacillus* sp., *B. avium* and *P. mirabilis* were quite high, with abilities to produce a zone of hydrolysis in the range of 56–62 mm. *Bacillus* strains EGU444, EGU445 and EGU447 were among the highest protease producers. On the other hand *P. mirabilis* strains EGU21, EGU30, EGU32 and EGU34 had a zone of hydrolysis in the range of 58 to 70 mm and also had a high relative protease activity of 9–21. Similarly, *B. cereus* strains EGU48, *B. thuringiensis* strains EGU47 and EGU378, *B. subtilis* strain EGU475, *B. sphaericus* strains EGU385, EGU399, EGU542, *P. stutzeri* strain EGU396 and marine bacterium strain EGU409 had a zone of hydrolysis in the range of 30–38 mm with a relative lipase activity up to 2.7. The abilities of these microbial isolates to metabolize starch were quite variable i.e. 4 to 23 mm and the relative amylase activities varied from 1 to 5. Maximum amylase activities of 23 mm were recorded with *B. thuringiensis* strain EGU378, *Pseudomonas* sp. strain EGU448 and marine bacterium strain EGU409.

4.5. Metabolic characteristics

Out of 35 strains, only 7 strains – 4 belonging to *Bacillus* and 3 belonging to *Proteus* had good metabolic characteristics (such as the ability to metabolize a wide range of sugars, etc.) (Table 3). *Proteus* strains EGU30, EGU32 and EGU34 show positive response (change in colour) on arabinose, ribose, xylose, dextrose, trehalose and glycerol, whereas *P. mirabilis* strain EGU32 could also utilize fructose, glucose and saccharose. Out of the 4 *Bacillus* strains, EGU85 could metabolize a large number of sugars but could not grow on citrate and malonate. The other 3 *Bacillus* strains EGU342, EGU385 and EGU399 showed a behavior which was complementary to that of EGU85. The 7 strains were able to utilize different nitrogen sources. A consortium of these strains may result in efficient solubilization of a wide range of complex organic matter components. The ability of *Proteus* strains to hydrolyze esculin (a glycoside) into glucose and esculetin, which posses multiple pharmacological activities can also be exploited further.

5. Conclusions

Biological treatment of waste needs microbes with abilities to produce H_2, CH_4 and PHAs (Kalia and Joshi, 1995; Angenent et al., 2004). A review of literature has revealed that only three photosynthetic organisms – *Rhodopseudomonas*, *Rhodospirillum* and *Rhodobacter* have abilities to

Table 3
Biochemical characterization of microbes tested for hydrolytic, hydrogen producing and polyhydroxybutyrate producing abilities

Biochemical characteristics	*Proteus* strains			*Bacillus* strains			
	EGU 32	EGU 34	EGU 30	EGU 85	EGU 385	EGU 399	EGU 542
L-Arabinose	w[a]	+[b]	–[c]	–	–	–	–
Ribose	+	+	+	+	–	–	–
Xylose	+	+	+	–	–	w	–
Dextrose	+	+	+	+	–	–	–
Fructose	+	–	–	+	w	w	–
Glucose	+	–	–	+	–	–	–
Trehalose	+	+	+	+	–	–	–
Saccharose	+	–	–	+	–	–	–
Maltose	–	w	–	+	–	–	–
Galactose	+	+	–	–	–	w	–
Melibiose	–	–	+	–	–	–	–
Sucrose	+	w	–	+	–	–	–
Mannose	–	–	–	+	–	–	–
Cellobiose	+	+	–	–	–	–	–
Glycerol	+	+	+	+	–	–	–
Citrate utilization	–	+	–	–	+	+	+
Malonate	–	–	–	–	+	+	+
ONPG β-galactosidase	w	+	+	–	–	–	–
Sodium gluconate	+	+	+	–	–	–	–
Lysine decarboxylase	+	w	+	–	+	+	+
Ornithine decarboxylase	+	+	+	–	+	+	+
Phenylalanine deaminase	+	+	+	–	–	–	–
Arginine utilization	–	+	+	–	+	+	+
Urease	+	+	+	–	+	+	+
Alkaline phosphatase	+	+	+	+	+	+	+
Nitrate reduction	+	+	–	+	–	–	+
Esculin	+	+	+	–	–	–	–

[a] Weak response.
[b] Positive response.
[c] Negative response.

produce H_2 and PHB (Hustede et al., 1993; Vincenzini et al., 1997; Yigit et al., 1999; Lee et al., 2002). In our study, *B. cereus* strain EGU43 and *B. thuringiensis* strain EGU45 have been found to evolve 0.63 mol of H_2/mol of glucose added and produce PHB in the range of 420–435 mg/l of medium, albeit under different conditions. Yet another *B. cereus* strain EGU3 had moderate H_2 producing ability (0.45 mol/mol of glucose added) and 500 mg PHB/l of medium. It is interesting to note that *Bacillus* sp. have been independently shown to produce H_2 and PHB (Kalia et al., 2000; Labuzek and Radecka, 2001; Sonakya et al., 2001; Yilmaz et al., 2005; Kotay and Das, 2007). Here, we have reported for the first time, the same *Bacillus* strain with both the activities under non-photosynthetic conditions. It has been realized that dark fermentative H_2 production is more economically feasible than photosynthetic H_2 production (Levin et al., 2007).

6. Future prospects

Our study has perhaps provided *Bacillus* strains with a conducive environment, where they were the only microbes, which did not have to compete with others. The use of pure cultures will circumvent the problem of interspecies H_2 transfers since no methanogens will be involved during H_2 evolution phase. Further optimization of H_2 producing conditions such as immobilization of microbes (Kumar et al., 1995) will improve the overall H_2 conversion efficiencies from glucose, presently observed to be 15.57% in this study, based on the assumption that a maximum of 4 mol of H_2/mol of glucose added (Logan et al., 2002). Finally, PHA production enables to further improve the process economics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2007.11.011.

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