Chapter 3
Materials and Methods
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3.1. Microbial cultures

Microbial isolates used in this study were collected in our laboratory from diverse environmental habitats. These isolates were selected under different physiological parameters such as pH from 2-12 with each incremental unit, salt concentration (NaCl, % - nil, 0.5, 2 and 5), sensitivity to metals i.e. (Nickel and cobalt: 1 mM) and their susceptibility to 12 different antibiotics (Ampicillin (A), Carbenicillin (Cb), Chloramphenicol (C), Gentamycin (G), Kanamycine (K), Nalidixic acid (Na), Penicillin (P), Polymyxin B (Pb), Rifampicin (R), Streptomycin (S), Tetracycline (T) and Vancomycin (Va): Contaminated food sample (Pickle waste), Marine coastal Water (Goa), MRL sludge sample (Madras Refinery Limited), IIT ETP sludge sample (Pesticide ETP, Mumbai), Pesticide ETP sludge sample (Gardha ETP, Chennai) (Porwal et al., 2008; Rani et al., 2008). These were grown and maintained on nutrient agar at pH 7 and 37 ºC. 20% glycerol stock of the cultures was maintained in -80 ºC.

3.1.1. Hydrolytic strains

Eleven bacterial strains: Bacillus sp. strains EGU85 (DQ768239), EGU367 (DQ768236), EGU444 (DQ768240) and EGU447 (DQ508976); B. sphaericus strains EGU385 (DQ487032) and EGU542 (DQ508979); B. subtilis EGU475 (DQ508977); B. thuringiensis EGU378 (DQ487033); Proteus mirabilis strains EGU30 (DQ487041) and EGU32 (DQ508964); and marine bacterium strain EGU409 (DQ487037) isolated previously in our laboratory were selected on the basis of their high hydrolytic activity and used in the study.

3.1.2. Polyhydroxyalkanoate (PHA) producing strain

Bacterial strains used in this study were isolated previously in our laboratory. A set of 6 Bacillus strains: Bacillus sp. EGU75 (EF633209); B. cereus strains EGU3 (DQ487039), EGU43 (DQ508969), EGU44 (DQ508970) and EGU520 (DQ508978); and B. thuringiensis EGU45 (DQ508971), reported to have high polyhydroxybutyrate (PHB) producing abilities were used in the study. These isolates were originally isolated from contaminated pickle, Madras Refinery Limited Effluent Treatment Plant (ETP), fermenting potato peels.
3.2. Chemicals

All the chemicals used in the study were of analytical grade and were purchased from Himedia, MERCK, BDH, SIGMA (USA), SRL and SD fine chemicals.

3.3. Gases

All the gases [N₂, H₂ and Zero air (O₂)] of high purity were procured from M/s Laser Gases, New Delhi, India.

3.4. Microbial growth medium

Inoculum was prepared in by inoculating bacterial strains into Nutrient broth (NB) medium from nutrient agar (NA) plate.

(a) Nutrient Broth (NB) Medium

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Quantity in g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Extract</td>
<td>1.5</td>
</tr>
<tr>
<td>Beef Extract</td>
<td>1.5</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.0 L</td>
</tr>
<tr>
<td>pH</td>
<td>7.0 (± 0.2)</td>
</tr>
</tbody>
</table>

(b) Growth medium (GM2)

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Quantity in g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Extract</td>
<td>1.0</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>1.0</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>10 or as per requirement</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.0 L</td>
</tr>
<tr>
<td>pH</td>
<td>7.2 (± 0.2)</td>
</tr>
</tbody>
</table>
(c) **Minimal medium (M9)**

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Quantity in g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>6.0</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>3.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>1.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.0 L</td>
</tr>
<tr>
<td>pH</td>
<td>7.2 (± 0.2)</td>
</tr>
</tbody>
</table>

After Autoclaving:

- 1 M MgSO$_4$ 1 mL/L (Working conc.: 1 mM)
- 0.1 M CaCl$_2$ 1 mL/L (Working conc.: 0.1 mM)

### 3.5. Feed materials

#### 3.5.1. Sugars solution

Glucose at concentrations of 0.5, 1, 2, 3, 5 and 7% were used as feed material for PHA production.

#### 3.5.2. Biowaste

Vegetable wastes - green Pea-shells (PS) (10% total solids; TS and 9.5% organic solids; OS) were collected from the local municipal market in Delhi, India and hydrolyzed to be used as substrate for PHA production on biowaste.

The total volume of the feed was 200 mL in 1000 mL reactor flask, unless otherwise mentioned.

### 3.6. Metabolic characterization of microbes

Bacterial strains selected as high hydrolytic bacteria were tested against different substrates (different carbohydrates as carbon source, proteins as nitrogen source and enzyme activities) to check their metabolizing abilities by KB009 Hi Carbohydrate™ Kit. 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. A single colony is picked up and inoculated in nutrient broth media. The inoculum were incubated at 37 °C for 24 h and then used for further testing. Each well of kit was inoculated with 50 μL (OD₆₆₀ = 1) of inoculum by surface inoculum method. Alternatively, the kit can also be inoculated by stabbing each individual well with a loopful of inoculum. Incubate kits at 37 °C for 24 h. Observations were recorded and analyzed.

**Carbohydrate fermentation tests:** This test is used to determine the ability of an organism to ferment various simple carbohydrates (sugars) as substrate. Fermentation is a metabolic process in which the final electron acceptor is an organic molecule. The indicator used is phenol red, which turns yellow below pH 6.8 and a darker pinkish red above pH 7.4. If the organism does not ferment the carbohydrate, the pH may remain neutral. If metabolizes the carbohydrate subsequent acid product ion will result in lowered pH and hence positive test (yellow from red).

**Esculin test:** Esculin is a glycoside composed of glucose and dihydroxycoumarin compound. On esculin hydrolysis, the original cream colour of the medium changes to black.

**Trehalose test:** Trehalose is a disaccharide composed of two glucose molecules bound by an α-1, 1-linkage. It has no reducing power. It acts as a source of energy in most organisms e.g. - bacteria, fungi, insects, plants and invertebrates. It protects organism against various stresses such as dryness, freezing and osmopressure. Anhydrobiotic organisms are able to tolerate the lack of water due to trehalose synthesis in large amounts and trehalose plays a key role in stabilizing membranes and macromolecular assemblies under extreme environmental conditions.

**Citrate utilization test:** This test is used to determine the ability of an organism using enzyme citrase, to use citrate as it’s sole source of carbon. Test is done on Simmon’s Citrate Agar containing sodium citrate as C-source and ammonium ion as the sole N-source. The pH indicator bromothymol blue will turn from green at neutral pH (6.9) to blue when a pH higher than 7.6 is reached (basic or alkaline). If the citrate is utilized, the resulting growth will produce alkaline products (pH > 7.6), changing the colour of the medium from green to blue.
ONPG test: This test is used to identify bacteria possessing the enzyme β-galactosidase. It catalyzes the breakdown of the substrate lactose (the major sugar present in milk) to galactose and glucose, which were feed into the glycolytic pathway. Ortho-nitrophynyl-β-galtoside (ONPG) is used as artificial substrate for the enzyme and in the presence of β-galtosidase, is converted to galactose and ortho-nitrophenyl (ONP). It is colourless and also at neutral or acidic pH, but in alkaline solution it is bright yellow. (Results pertaining to these experiments have been presented in Table 4.1).

3.7. Determination of whole cell protein by Lowry’s method

3.7.1. Preparation of whole bacterial cell extract for protein

The cell extract was produced as follows. 1 mL cell culture was centrifuged at 14,000 rpm for 5 min to remove supernatant. Suspended the cell pellet in 1 mL DW and added 2 mL of 2 N NaOH solution. Kept for boiling in water bath for 5 min. Solution was cooled and 2 N HCl was added to neutralize the solution. Protein concentration of the sample was estimated by using Bovine Serum Albumin (BSA) as a standard protein.

Reagents required

Normal saline: 0.9 g sodium chloride dissolved in DW make final volume 100 mL.

- 2 N NaOH: 8 g sodium hydroxide dissolved in DW makes final volume 100 mL.
- 2 N HCl: 17.5 mL conc. HCl (35% purity) dissolved in DW make final volume 100 mL.

Cells were lysed by strong alkali treatment before protein estimation as described below. 1 mL of cell culture was taken and centrifuged to remove the supernatant. Pellets were resuspended in 1 mL DW and added 2 mL 2 N NaOH, then boiled in water bath for 10 min. This solution was cooled and 2 mL of 2 N HCl was added. Normality of NaOH and HCl should be exactly same. Sample was ready for protein estimation.
3.7.2. Estimation of protein by Lowry’s method

The Lowry’s method of protein estimation depends on measuring the color quantitatively, which is obtained from the reaction of the Folin-Ciocalteu phenol reagent with the tyrosyl residues of an unknown protein. With the addition of Lowry’s reagent, which actually contains the alkaline copper tartarate to the entire sample containing the protein a cupric amino acid complex is formed. The color so formed is due to the reaction of alkaline copper with the protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The intensity of the color depends on the amount of these aromatic amino acids present. Protein absorbs strongly at 280 nm according to their content of the amino acids tyrosine and tryptophan, and this provides a sensitive form of assay. Protein also absorbs in the far UV because of the peptide bond. The addition of Folin-Ciocalteu phenol reagent results in formation of intense blue color whose absorbance can be measured by spectrophotometer at 750 nm. We can prepare a standard curve by finding the absorbance of samples containing the known amount of BSA. The concentrations of standard BSA are in the range of 20-100 µg. The amount of protein in any sample can be derived from the standard curve.

Reagents required

A. **Bovine serum albumin (Standard):** The BSA was dissolved in DW to get final concentration of 0.1 mg/mL.

B. **Folin-Ciocalteu phenol reagent:** The reagent was diluted with distilled water in 1:1 ratio.

C. **Phosphate buffer saline:** Composition/L: NaCl, 8; Na₂HPO₄, 1.4; KCl, 0.2; KH₂PO₄, 0.24.

**Table 3.1:** Composition of alkaline copper reagent (ACR)

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration (w/v)</th>
<th>Stock solution</th>
<th>Volume of stock solution to be prepared for samples (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>A</td>
<td>2% Na₂CO₃</td>
<td>0.1 N NaOH</td>
<td>50</td>
</tr>
<tr>
<td>B</td>
<td>2% S.P.T</td>
<td>DW</td>
<td>50</td>
</tr>
<tr>
<td>C</td>
<td>1% CuSO₄</td>
<td>DW</td>
<td>50</td>
</tr>
</tbody>
</table>
Table 3.2: Preparation of ACR (Alkaline copper reagent) standard

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume to be mixed (ratio)</th>
<th>Volume of to be prepared for sample (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>A</td>
<td>98</td>
<td>49</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>0.50</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>0.50</td>
</tr>
<tr>
<td>Total volume of mixture</td>
<td></td>
<td>50</td>
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</tbody>
</table>

Table 3.3: Details of Lowry’s method of protein estimation

<table>
<thead>
<tr>
<th>Stock solution (BSA)</th>
<th>10 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard working solution</td>
<td>0.1 mg/mL (10 µL from stock + 990 µL DW)</td>
</tr>
<tr>
<td>Testing range</td>
<td>10-100 µg</td>
</tr>
<tr>
<td>Sensitivity range</td>
<td>10-1200 µg/mL</td>
</tr>
</tbody>
</table>

Procedure for BSA standard preparation

- Different concentrations of BSA standard i.e. 100, 200, 400, 600, 800 and 1000 µL were taken from the working solution in separate test tubes.
- Total volume was made up to 1000 mL with DW.
- 5 mL of ACR was added to all the tubes.
- Test tubes were incubated at room temperature (RT) for 10 min.
- 500 µL of Follin’s Phenol reagent (dilution ratio is 1:1 with DW) was added to all the tubes.
- Test tubes were incubated at RT (in dark) for 30 min.
- OD was taken at 750 nm immediately.

Table 3.4: Standard for Lowry’s methods

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentration of BSA (µg)</th>
<th>Amount of working solution (µL)</th>
<th>Volume of water (µL)</th>
<th>Volume of ACR (mL)</th>
<th>Volume of Folin’s reagent (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>100</td>
<td>900</td>
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<td>500</td>
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<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>1000</td>
<td>5</td>
<td>500</td>
</tr>
</tbody>
</table>
Protocol for protein estimation of whole cell extract

- 1 mL sample of whole cell extract was taken and 5 mL ACR (98:1:1) was added.
- It was incubated at RT for 10 min.
- 500 μL of diluted Folin’s reagent (1:1 with DW) was added.
- Tubes were incubated at RT for 30 min.
- Sample was read at OD$_{750}$ nm (immediately).

3.8. Estimation of glucose

3,5-Dinitrosalicylic acid (DNSA, IUPAC name 2-hydroxy-3,5-dinitrobenzoic acid) is an aromatic compound that reacts with reducing sugars and other reducing molecules to form 3-amino-5-nitrosalicylic acid, which absorbs light strongly at 540 nm (In case of glucose).

Reagent required

A. Sodium potassium tartarate
B. 2 N sodium hydroxide (2 N NaOH)
C. Dinitro salicylic acid (DNSA)

Preparation of DNSA solution

- Prepared 20 mL of 2 N NaOH.
- Weighed 1 g DNSA and dissolved in 20 mL NaOH with the help of a magnetic stirrer for approximately 45 min.
- Weighed 30 g of sodium potassium tartarate and dissolved in 50 mL DW.
- Slowly poured sodium potassium tartarate solution in the DNSA and NaOH solution. Made the volume up to 100 mL (Note: Wait for the two to mix properly).
- Decanted the contents in a brown bottle. Filter if necessary.

Protocol

- Took at least two 20 mL test tubes (i.e. two replicates of each concentration should be tested) and took an amount of glucose stock solution in each test tube as per table given below.
- Prepared a blank, in which case added 500 μL of DW instead of sample.
- Added DW as indicated in the table above (preheated to 65 °C).
- Incubated precisely at 65 °C for 15 min in a water bath or incubator.
- Added 3 mL of DNSA.
- Kept tubes (Glucose solution + DW + DNSA) in boiling water-bath for 15 min.
- Cooled to RT. Measure the absorbance at 540 nm in a UV-VIS spectrophotometer against a suitable blank.

Table 3.5: Standard for DNSA methods

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Concentration of glucose (μmol)</th>
<th>Amount of working solution (μL)</th>
<th>Volume of DW (μL)</th>
<th>Amount of DNSA (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>100</td>
<td>1400</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
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<td>200</td>
<td>1300</td>
<td>3</td>
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<td>5</td>
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<td>500</td>
<td>1000</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>1500</td>
<td>3</td>
</tr>
</tbody>
</table>

Note: Test only 1-5 μmol concentration prepared from stock solution D, to study the linear range.

Table 3.6: Details of DNSA method of glucose estimation

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>180 g/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard working solution</td>
<td></td>
<td>1.8 mg/mL</td>
</tr>
<tr>
<td>Testing range</td>
<td></td>
<td>1-5 μmol</td>
</tr>
</tbody>
</table>

3.9. Hydrolysis of waste

3.9.1. By undefined hydrolytic culture

Green PS were used in the experiments. These were cut into small pieces (1-2 cm) before use. Pea-shell slurry (PSS), 4 L at 2% TS was inoculated with acidogens (fresh cattle dung slurry; 3% TS) (1 vol.) to slurry (9 vol.) and incubated at 37 °C for 2 days (Kalia and Joshi, 1995, Kalia et al., 1994). Slurry obtained at this stage was sieved and the filtrate (2.5 L) was centrifuged at 6000 rpm for 20 min. The centrifuged filtrate was stored at 4 °C.
3.9.2. By defined hydrolytic culture

Set of 11 high hydrolytic bacterial strains: *Bacillus* sp. strains EGU85, EGU367, EGU444 and EGU447; *B. sphaericus* strains EGU385 and EGU542; *B. subtilis EGU475*; *B. thuringiensis* EGU378; *P. mirabilis* strains EGU30 and EGU32; and a marine bacterium strain EGU409 were used for the preparation of mixed hydrolytic bacterial cultures (MHC1-MHC11). These MHCs for hydrolysis of biowaste were designed on the basis of Plackett-Burman method (Appendix I). The different isolates used for preparing the mixed cultures had 0.85-1.9×10⁶ viable cells/µg protein to achieve a concentration where different isolates were finally present in equal proportions. For batch-culture digestion, green PS were cut into small pieces (1-2 cm). PSS (250 mL) made in distilled water in 300 ml BOD bottles with PS at 2% TS was inoculated with these MHCs at the rate of 10 µg cell protein/mL feed and incubated at 37 °C for 48 h (Kumar et al., 2009; Patel et al., 2011b).

Control PSS was prepared by the same procedure described above except the PSS was not inoculated with any mixed hydrolytic culture. After the incubation of 48 h at 37 °C slurry was sieved and the filtrate was centrifuged at 6000 rpm for 20 min. The supernatant used as control PSS feed. PS were used within 1 h of their collection.

3.10. Growth curve of microbial cultures

Strains used for PHA production were grown on PSS hydrolysed with MHC1-MHC11 and compared with the growth on control PSS. Initial OD₆₆₀ for the growth curve was 0.05. Curve was plotted on the basis of incubation period (h) versus OD₆₆₀ (spectrophotometer: Lambda 35 Perkin-Elmer) taken against blank (hydrolysed PSS) with spectrophotometer.

3.11. PHB production by pure cultures on Synthetic media

PHB production by 6 *Bacillus* strains: *Bacillus* sp. EGU75; *B. cereus* strains EGU3, EGU43, EGU44 and EGU520 and *B. thuringiensis* EGU45 was monitored on growth medium; GM2 and miller medium; M9 (200 mL in 1000 mL conical flasks) supplemented with 1% glucose (w/v). The strains were inoculated into the medium at
the rate of 1μg cell protein/ml and incubated at 37 ºC at 200 rpm for 48 h. Further study on the effect of different glucose concentration to know the optimum glucose required for PHB production was tested with six Bacillus strain on GM2 media supplemented with glucose at the rate 0.5, 1, 2, 3, 5 and 7% (w/v). Different bacterial strains were grown in HiMedia nutrient broth (13 g/L distilled water) and incubated at 37 ºC at 200 rpm for 16-20 h for inoculum preparation.

Growth of different bacterial strains was monitored at 600 nm on Perkin Elmer Lambda 35 spectrophotometer. Aliquots of 200 mL were analysed for biomass (cell dry weight; cdw) and PHB after 24 and 48 h of incubation.

3.12. PHB production by pure cultures on hydrolysed biowaste

3.12.1. With undefined mixed hydrolytic culture

200 ml of the combinations of the centrifuged PSS as biowaste component (BW) and GM2 medium (M) (BW:M :: 7:3) was prepared and tested for PHB production. pH was adjusted to 7.2 with 1 N NaOH at the time of inoculation with six PHB producing Bacillus strains at the rate of 10 μg cell protein/mL of the combination (BW:M) feed. All these incubations were done at 37 ºC at 200 rpm for 96 h.

3.12.1.1. Effect of CEH concentration

Further optimization for the effect of casein enzyme hydrolysate (CEH) pancreatic digest of casein (equivalent to tryptone) as additional N source supplement on PHB production was done with four strains B. cereus strains EGU3, EGU43 and EGU44; and B. thuringiensis EGU45. CEH was added at the rate of 0.4 and 0.6 g/L feed BW:M (7:3) combination.

Aliquots of 200 mL for biomass (cdw) estimation and PHB analysis were withdrawn regularly at 24 h intervals upto 96 h of incubation.

3.12.2. With defined mixed hydrolytic culture

Samples (200 mL) of centrifuged PSS hydrolysed with MHC1-MHC11 for 2 days (adjusted to pH 7.2) were used as feed. Supernatant was inoculated with four PHB
producing strains: *B. cereus* strains EGU3, EGU43 and EGU44; and *B. thuringiensis* EGU45 at the rate of 10 μg cell protein/mL. All these incubations were done at 37 °C at 200 rpm for 48 h.

### 3.12.2.1. Effect of CEH concentration

Further optimization for the effect of CEH as additional N source supplement on PHB production from PSS (hydrolysed with MHC2 and MHC5) was done by adding it at the rate of 0.2, 0.4 and 0.6 g/L feed.

### 3.12.2.2. Effect of C source supplementation

(a) With glucose

Glucose as additional C source on PHB production from PSS (hydrolysed with MHC2 and MHC5) was studied by supplementing it at the rate of 0.1, 0.2, 0.5 and 1.0% (w/v) of the feed.

(b) With starch

Starch as carbon source was supplemented into PSS (hydrolysed with MHC2 and MHC5) at the rate of 0.1 and 1.0% (w/v) of feed at two stages, i) at the time of hydrolysis and ii) after hydrolysis at time of PHB production and tested for PHB production.

### 3.12.2.3. Effect of inoculum size

The effect of inoculum size on PHB production from PSS (hydrolysed with MHC2 and MHC5) was studied by inoculating the four *Bacillus* strains at the rate of 10, 100 and 1000 μg cell protein/mL of feed with glucose added at the rate of 0.1, 0.2, 0.5 and 1.0% (w/v) and control (no glucose addition).

### 3.12.2.4. Effect of shaking

PHB production on PSS hydrolyzed with MHC2 was tested at different shaking conditions (rpm) with *B. cereus* EGU44 inoculated at the rate 10 μg cell protein/mL. The culture was incubated at varying rpm i.e. 50, 100, 200 and 350.
Aliquots of 200 mL were processed for biomass (cdw) estimation and PHB analysis on PSS hydrolysed with MHCs after 48 h of incubation.

3.13. PHA production by mixed cultures (MCs)

3.13.1. On synthetic media

6 *Bacillus* strains: *Bacillus* sp. EGU75; *B. cereus* strains EGU3, EGU43, EGU44 and EGU520; and *B. thuringiensis* EGU45 (DQ508971), which were found to be the best PHB producing strains were used for the preparation of defined mixed cultures (consortia) (MCs) for PHA production. Different combinations of the mixed cultures (2MC1-2MC15, 3MC1-3MC20, 4MC1-4MC15, 5MC1-5MC6 and 6MC1) were prepared with varying number of strains (from 2-6) (Appendix II). The different isolates used for preparing the mixed cultures had 0.85-1.9×10^6 viable cells/µg protein to achieve a concentration where different isolates were finally present in equal proportions.

PHA production by these MCs was monitored on GM2 medium (200 mL in 1000 mL conical flasks) supplemented with 1% glucose (w/v). The strains were inoculated into the medium at the rate of 1 µg cell protein/mL and incubated at 37 ºC at 200 rpm for 48 h. Different bacterial strains were grown in HiMedia nutrient broth (13 g/L distilled water) and incubated at 37 ºC at 200 rpm for 16-20 h for inoculum preparation.

Growth of different bacterial strains was monitored at 600 nm on Perkin Elmer Lambda 35 spectrophotometer. Aliquots of 200 mL were analysed for biomass (cdw) and PHB after 48 h of incubation.

3.13.2. On hydrolyzed biowaste

Samples (200 mL) of centrifuged PSS hydrolysed with MHC2 for 2 days (adjusted to pH 7.2) and supplemented with 1% glucose (w/v) were used as feed. Supernatant was inoculated with 9 selected MCs: 6MC1, 5MC2, 5MC5, 4MC1, 4MC2, 4MC4, 3MC14, 2MC8 and 2MC11 at the rate of 10 µg cell protein/mL. All these incubations were done at 37 ºC at 200 rpm for 48 h.
3.13.2.1. Effect of Incubation period
PHA production by the selected nine MCs was monitored on PSS hydrolysed with MHC2 and supplemented with 1% glucose (w/v) at 24 h and 48 h.

3.13.2.2. Effect of mixed hydrolytic culture
PHA production with nine selected MCs on PSS hydrolysed with MHC2 and supplemented with 1% glucose (w/v) was compared with PHA production by these MCs on control PSS supplemented with 1% glucose (w/v).

Aliquots of 200 mL were analyzed for biomass (cdw) and PHB after 48 h of incubation.

3.14. Up-scaling of the PHA production
Scale-up of PSS hydrolyzed with MHC2 were done for PHA production with 5MC2, 5MC5 and B. cereus EGU44 at 0.1, 0.2, 0.4 and 1.0 L feed in the reactors of 0.5, 1, 2 and 5 L capacities, respectively, at 37 ºC at 200 rpm for 48 h.

3.15. Effect of HV precursor on PHA copolymer production
Optimization for the effect of 3HV precursor substrate supplementation into PSS hydrolysed with MHC2 on copolymer production was done with mixed culture 5MC2 and compared with that of B. cereus EGU44.

The study was performed under three different culture conditions where different concentration of HV sources (0.5, 1 and 2%) were added into hydrolyzed PSS i) at initial time (0 h) as sole C source, ii) at 0 h along with glucose (0.5%), and iii) after 24 h growth on glucose (0.5%). The culture was incubated at 37 ºC at 200 rpm upto 48 h incubation after HV precursor substrate supplementation. Aliquots of 200 mL were analyzed for cdw and PHB after 48 h incubation on HV precursor substrates.

3.16. PHB production from residual culture medium following hydrogen production: Two stage process
Hydrogen production under anaerobic condition tested for initial three days (done by lab colleague). At the end of this period, the provided residual medium containing
glucose, volatile fatty acids (VFAs) and residual nutrients of the GM2 or M9 medium were subjected to PHB production. Glucose was estimated in the beginning and at the end of this stage. For batch culture PHB production, 200 ml of medium (GM2 or M9) containing cultures after H₂ production stage (1st stage) were transferred to 1.0 L conical flask (pH set to 7.2 by 2 N NaOH or 2 N HCl) (2nd stage) and incubated at 37°C in shaker at 200 rpm for 120 h. Aliquots of 200 mL were analyzed for cdw and PHB regularly at 24 h intervals (i.e. 24, 48, 72, 96 and 120 h).

3.17. PHA analysis

Samples (200 mL) were analysed for cdw and PHA production. Aliquots (200 mL) were centrifuged at 4000 rpm for 30 min. The pellet was washed with 10-15 mL saline solution (0.9% NaCl) and re-centrifuged. The pellet was dried at 85 °C for 36 h and weighed to estimate cdw. After the propanolysis of bacterial dry cell extract, the heavier phase containing chloroform solution with the esters of propanol and β-hydroxylacids from PHA hydrolysis were analysed by gas chromatography (GC) using 10% Reoplex 400 (length 30 m × O.D. 6.35 mm × I.D. 3 mm), dimethylpolysiloxane capillary column DB-1 (30 m × 0.25 mm × 0.25 µm) and flame ionization detector (FID). Poly-3-hydroxybutyrate (Fluka Chemika, USA) and Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (Sigma-Aldrich) was used as standard.

3.17.1. PHA standard preparation

3.17.1.1. Reagents required

- Chloroform (CHCl₃) was used as solvent for all types of PHA (Dichloroethane; DCE can be used for PHB).
- Acidified n-propanol or propanol-hydrocholric acid solution (propanol : hydrochloric acid (4:1) v/v) was used for esterification
- Benzoic acid was used as internal standard (40 g benzoic acid/L propanol)

3.17.1.2. Preparation of stock solution

- 200 mg of PHB or 400 mg of P(3HB-co-3HV) powder was added in a COD cell.
ml of solvent was added into it and kept in a water bath at 100°C for 15 min till powder dissolves properly.

After the mixture was cooled down to RT 5 ml solvent was added to make up the volume upto 10 mL.

### 3.17.1.3. Preparation of standard

**Stock solutions:** PHB \(\rightarrow\) 20 mg/mL  
\[\text{P(3HB-co-3HV)} \rightarrow 40 \text{ mg/mL}\]

Dilutions from stock solution were made as shown in the table 3.7

#### Table 3.7: PHA standard preparation by propanolysis method

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Amount of stock ((\mu)L)</th>
<th>Chloroform (mL)</th>
<th>PHB concentration ((\mu)g/(\mu)L)</th>
<th>(\text{P(3HB-co-3HV)}) concentration ((\mu)g/(\mu)L)</th>
<th>Acidified Propanol (mL)</th>
<th>Benzoic acid (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blank</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>200</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>1.8</td>
<td>0.95</td>
<td>1.9</td>
<td>2</td>
<td>200</td>
</tr>
<tr>
<td>3</td>
<td>400</td>
<td>1.6</td>
<td>1.9</td>
<td>3.8</td>
<td>2</td>
<td>200</td>
</tr>
<tr>
<td>4</td>
<td>600</td>
<td>1.4</td>
<td>2.9</td>
<td>5.8</td>
<td>2</td>
<td>200</td>
</tr>
<tr>
<td>5</td>
<td>800</td>
<td>1.2</td>
<td>3.8</td>
<td>7.6</td>
<td>2</td>
<td>200</td>
</tr>
<tr>
<td>6</td>
<td>1000</td>
<td>1.0</td>
<td>4.8</td>
<td>9.6</td>
<td>2</td>
<td>200</td>
</tr>
</tbody>
</table>

### 3.17.1.4. Protocol

- Above solutions were heated at 100 °C for 2 h in thermoreaktor dry bath (MERK TR200) in sealed tubes (COD cell).
- It was cooled down to RT.
- 4 mL of elix water was added and was subjected to vortexing.
- The upper (lighter) layer was discarded and lower (denser) layer was preserved for GC analysis.
3.17.2. Propanolysis of bacterial dry cell extract

- Approx. 40 mg of dried pellet in a COD cell was taken.
- 2 mL of chloroform (or DCE) was added in it.
- 2 mL of acidified propanol was added to it.
- 200 μL of benzoic acid was added.
- Properly sealed COD cell was kept for heating at 100 °C for 2 h (in thermoreaktor dry bath).
- Tube was shaken in between for proper mixing.
- After 2 h mixture was cooled down to RT and vortexed with 4 mL of elix water.
- Upper layer was discarded and lower or denser layer was preserved for GC.
- Quantitative evaluation was done by means of quotient of peak area of hydroxy butyric acid and benzoic acid.

3.17.3. Gas chromatography (GC)

PHA was analyzed by GC (5765, Nucon Engineers) with FID mode by injecting 1 μL of sample into column. Nitrogen, hydrogen and zero air gas were used as the carrier, fuel and ignition supporter gas, respectively, at a flow rate of 30 mL/min.

Table 3.8: GC conditions

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Column</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reoplex 400</td>
</tr>
<tr>
<td>Oven temperature</td>
<td>170 °C</td>
</tr>
<tr>
<td>Injector temperature</td>
<td>230 °C</td>
</tr>
<tr>
<td>Detector temperature</td>
<td>250 °C</td>
</tr>
<tr>
<td>Oven temperature</td>
<td>170 °C</td>
</tr>
<tr>
<td>Carrier gas nitrogen</td>
<td>18 psi</td>
</tr>
<tr>
<td>N2 carrier gas cylinder outlet pressure</td>
<td>4.0 kg/cm²</td>
</tr>
<tr>
<td>Carrier A and B should be set at</td>
<td>2.0 kg/cm²</td>
</tr>
<tr>
<td>H2 gas cylinder outlet pressure</td>
<td>2.0 kg/cm²</td>
</tr>
<tr>
<td>H2 fuel meter on GC</td>
<td>0.8-1.0 kg/cm²</td>
</tr>
<tr>
<td>Zero air (O2) cylinder outlet pressure</td>
<td>2.0 kg/cm²</td>
</tr>
<tr>
<td>Zero air pressure meter on the GC</td>
<td>0.8 kg/cm²</td>
</tr>
<tr>
<td>Sensitivity (on FID)</td>
<td>100</td>
</tr>
<tr>
<td>Attenuation (on FID)</td>
<td>4</td>
</tr>
</tbody>
</table>
3.17.3.1. GC switch on sequence

- Open N₂ carrier gas cylinder (grey color) by moving the key LN in anti-clock wise direction on the top of the cylinder and set its outlet pressure at 4.0 kg/cm² on cylinder meter by turning in clockwise direction.
- Carrier inlet indicator should be 4 kg/cm² on GC pressure metre (which is located at the extreme left on the top of GC) get a flow rate of 30 mL/min.
- Carrier inlet A and B indicator on GC pressure meter (upper two located on left panel of GC) should read 2 kg/cm².
- Switch on the temperature controller module, it will display the program (prg0-9) which can be used to set different program.
- Press the digit corresponding to the programme needs to be set or run e.g. for programme no. 1 i.e prg1, press ‘1’ on temperature controller key pad on right side of control panel.
- After the controller displays the programme i.e. prg0-9 (which is being used) press ENTER (located in left side of control panel) to display the first or initial oven temperature i.e. ov1, enter the value as required using the keypad.
- Press ENTER to display the rate of heating displayed as t1 and enter the value as required.
- Press ENTER to display the retention time i.e. rt1 which is the holding time for ov1 and enter the values as required.
- Press ENTER, it will successively display oven temperature (ov2-5), rate of heating (t2-5) and holding time for previous oven temperature (rt2-5). Set the values as required according to the temperature profile.
- For isothermal processing set ov1-5 = 170 °C for Reoplex 400 column, 120 °C for DB1 column
- Values for rt1-5, t1-5 are required for thermal gradient processing only, therefore, no need to set the values for isothermal processing.
- Press ENTER to display trp that should be less than oven temperature but greater than RT. (e.g. 60)
- Press ENTER to display the injector temperature (inj) and enter the value i.e. 230 °C for Reoplex 400 or 250 °C for DB1.
- Press **ENTER** and display the detector temperature (**det**) and enter the value i.e. 250 °C for Reoplex 400 or 280 °C for DB1.
- Values for **to1-3**, **tf1-3**, **rpt** are not required for isothermal processing. Press **ENTER** till it displays **pr1** (i.e. **pr0-9**) again,
- The display order on temperature controller is as following
  
  Ov1 ➔ t-1 ➔ rt-1 ➔ ov-2 ➔ t-2 ➔ rt-2 ➔ ov-3 ➔ t-3 ➔ rt-3 ➔ ov-4 ➔ t-4 ➔ rt-4 ➔ ov-5 ➔ t-5 ➔ inj ➔ det ➔ to1 ➔ tf1 ➔ to2 ➔ tf2 ➔ to3 ➔ tf3 ➔
  
  cys ➔ rpt ➔ pr1
- Press **START** to start the heating, wait for the ready (**rdy**) signal on the temperature controller.
- Open H₂ carrier gas cylinder (red color) by moving the key LN in anti-clock wise direction on the top of the cylinder and set its outlet pressure at 2.0 kg/cm² on cylinder meter by turning in clockwise direction.
- H₂ fuel meter on GC machine should be at 0.8-1.0 kg/cm² to get a flow rate of 30 mL/min. (meter below the carrier inlet B on left panel of GC).
- Open the Zero air cylinder (blue grey colour) by moving the key LN in anti-clock wise direction on the top of the cylinder and set its outlet pressure at 2.0 kg/cm² on cylinder meter by turning in clockwise direction.
- Air pressure meter on GC machine should be at 0.8 kg/cm² level. (meter at bottom on the left side panel of GC)
- Ignite the flame by pressing and holding the ignition key on the side (A or B) on which flame is to be ignited depending on the column used. (A sound ‘pluck’ can be heard when flame get ignited)
- Switch on the FID and set polarity from polarity switch on the FID lower part (+ve or –ve depending on the column to be used).
- Switch on the computer (CPU and monitor) and data station I/F module, start the NUCHROM programme.

### 3.17.3.2. Sample injection

- Sample can be injected only when temperature controller and IF module shows ready (**rdy**) signal on the temperature controller.
• First take the micrometer syringe capacity of 5 μL.
• Wash syringe with acetone/ether/ethanol about 5 or 10 times. Acetone/ether/ethanol removes all impurities present in the syringe.
• Take a 1 μL sample (for column Reoplex 400) or 0.5 μL (for column DB-1) in the syringe (according to column).
• Inject the sample in the injector port (at the top of GC machine) and readily start the run in Nuchrome programme by clicking ‘start’.
• After completing the desired retention time stop the run by clicking ‘stop’.
• Execute the display report option, note down the data and calculate the values.

3.17.3.3. GC switch off sequence

• Close the NUCHROME programme and shut down the computer system.
• Press Reset/TCD/End Run on the temperature controller and then Set Actual to start the cooling of the temperature regulator.
• Open the door of GC oven after sometime to fasten the cooling. Wait for the temperature to come down to RT.
• Switch off FID mains and I/F module.
• Turn the pressure regulator of H₂ cylinder anticlockwise to bring the pressure down to zero in pressure meters both on cylinder and GC machine. Then, close cylinder’s main regulator with a LN key by turning it clockwise.
• Turn the pressure regulator of zero air cylinder anticlockwise to bring the pressure down to zero in pressure meters both on cylinder and GC machine. Then, close cylinder’s main regulator with a LN key by turning it clockwise.
• After the oven temperature comes down to RT, switch off the temperature regulator (the switch is at the upper right corner on back side).
• Turn the pressure regulator of N₂ cylinder anticlockwise to bring the pressure down to zero in pressure meters both on cylinder and GC machine. Then, close cylinder’s main regulator with a LN key by turning it clockwise. (Note: Don’t close the N₂ cylinder unless the temperature comes down to RT).
3.18. VFA estimation

Samples (1mL) were centrifuged at 10000 rpm for 10 min and supernatant (~200 μL) was collected in separated tubes. VFA estimation was done by GC (5765, Nucon Engineers) fitted with column Chromosorb 101 (10.1 m × 6.35 mm × 3 mm). All the GC conditions were same as for PHA analysis except the temperature profile. Temperature profile used here was: oven temperature: 180 °C, Detector temperature: 230 °C and Injector temperature: 250 °C. 1% solution of different organic acids will be used as standard sample to calculate the present amount in samples.

3.19. PHA extraction

- Culture sample was centrifuged at 4000 rpm for 30 min.
- The pellet was washed with 10-15 mL saline solution (0.9% NaCl) and re-centrifuged.
- Added chloroform 3-4 times of the PHA polymer expected from used biomass (can be calculated from GC analysis).
- Pellet was dissolved and incubated in hot chloroform for 3 h for extraction.
- Chloroform was filtered and PHA polymer was extracted by chloroform evaporation using soxhlet apparatus (Buchi Rotavapor R114) at 60 °C (around the boiling temperature of the solution used).
- After chloroform evaporation, methanol was added for PHA precipitation and purification.
- At this stage polymer can be recovered from flask using a brush or forcep (shake gently if needed) or if not possible through this can be recovered by methanol evaporation using soxhlet apparatus at 65-70 °C.
- Extracted PHA was measured gravimetrically.