Chapter 4
Results
Microbial diversity of fermenting biowastes
4.1 Segregation of isolates

Around 500 isolates were picked from nutrient agar plates at pH 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11. In order to reduce the overlapping of isolates, each of the 500 isolates was subjected to 12 different antibiotics (Table 4.1). On this basis of high similarity (antibiotic response) among them, only one representative of each group was selected and 117 isolates were processed further.

Henceforth, One hundred and seventeen strains were isolated from fermentating biowaste (Potato peels incubated with cow dung slurry) (Appendix 1). In order to ensure that isolates are different from each other, their antibiotic sensitivity responses were subjected to statistical analysis (materials and methods). The strains were distinguishable on the basis of all the 12 different antibiotics as in Table 4.1. The response of the various isolates to antibiotics reveals that they differ in their tolerances abilities. The basic objective was to distinguish the strains on the basis of their response to antibiotics and not to establish their antibiotic tolerance abilities or calculating minimal inhibitory concentration. Strains were distinguished from each other on the basis of response to 12 different antibiotics at the chosen concentrations. This method was applied to characterize each of the strain and the pattern of distribution into different groups reveals the extent to which the microbes are sensitive to each of the antibiotic. Very few strains are resistant to ampicillin and gentamycin whereas some strains are highly sensitive to carbenicillin (Car), nalidixic acid (Nal), penicillin G (Pen), polymyxin B (Pol) and streptomycin (Str). Invariably, there is a mixed response, which varies from highly resistant to highly sensitive in the cases of chloramphenicol (Chl), rifampicin (Rif), tetracycline (Tet) and vancomycin (Van). Most of the strains are moderately sensitive to these antibiotics. The results of distribution also help us to sort out and segregate the strains by comparing strains with each other.
Table 4.1: Antibiotic sensitivity test of microbial isolates

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Table contains the antibiotic sensitivity test results for microbial isolates at different pH levels. The table includes S.No., isolate number, and the zone of inhibition (mm) for each isolate at pH 2, 3, 4, and 5.
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**Strains isolated at pH 6**

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| 46    | EGU491        | 19 14 11 21 21 10 16 10 18 17 17 16 |
| 47    | EGU492        | 19 18 22 26 26 16 18 10 16 15 18 16 |
| 48    | EGU493        | 28 26 17 20 13 9 23 10 22 15 15 17 |
| 49    | EGU494        | 12 12 19 17 16 18 10 9 17 14 19 13 |
| 50    | EGU495        | 16 17 14 20 21 0 13 0 20 16 16 15 |
| 51    | EGU496        | 19 12 17 17 19 21 12 0 18 13 19 13 |
| 52    | EGU498        | 25 30 19 17 14 0 27 11 15 16 16 12 |
| 53    | EGU499        | 19 13 11 20 21 11 13 10 19 17 23 16 |
| 54    | EGU500        | 18 16 14 22 22 12 13 10 17 18 21 20 |

**Strains isolated at pH 7**

| 55    | EGU191        | 0 20 0 12 9 0 0 11 10 12 0 0 |
| 56    | EGU192        | 12 10 12 24 22 10 11 10 18 12 15 17 |
| 57    | EGU193        | 0 17 0 13 7 0 0 10 8 10 0 0 |
| 58    | EGU194        | 20 14 23 22 22 15 10 9 18 20 15 16 |
| 59    | EGU195        | 0 21 0 19 7 0 0 10 10 12 0 0 |
| 60    | EGU196        | 18 17 19 24 21 13 16 9 18 21 26 22 |
| 61    | EGU197        | 18 11 21 21 19 15 7 7 18 21 13 13 |
| 62    | EGU198        | 16 13 17 22 21 11 11 10 18 17 16 18 |
| 63    | EGU199        | 30 32 20 29 20 0 36 11 0 18 19 19 |
| 64    | EGU200        | 34 35 18 21 21 0 36 10 22 16 17 17 |
| 65    | EGU201        | 42 32 31 25 20 0 0 12 35 22 26 23 |
| 66    | EGU202        | 0 25 0 11 0 0 0 10 7 11 0 0 |
| 67    | EGU203        | 0 22 0 14 0 0 0 10 10 11 0 0 |
| 68    | EGU204        | 15 11 20 21 19 16 8 9 12 22 14 15 |
| 69    | EGU205        | 0 0 0 13 0 0 0 8 10 10 0 0 |
| 70    | EGU206        | 25 16 10 23 28 12 0 12 13 25 12 0 |
| 71    | EGU207        | 28 29 26 25 27 20 30 10 20 21 25 20 |
| 72    | EGU208        | 21 25 30 26 28 16 25 11 16 18 26 17 |
| 73    | EGU209        | 22 24 26 25 26 17 24 10 17 17 25 15 |
| 74    | EGU210        | 23 30 33 22 29 20 33 12 22 20 22 23 |
| 75    | EGU211        | 23 15 8 23 30 17 0 13 15 0 10 0 |
| 76    | EGU212        | 23 24 26 24 24 18 22 10 15 19 21 15 |
| 77    | EGU213        | 12 11 17 20 18 17 12 9 12 21 16 15 |
| 78    | EGU214        | 16 14 17 22 23 12 16 11 20 20 13 17 |
| 79    | EGU215        | 21 15 0 21 20 12 21 11 20 20 11 18 |

**Strains isolated at pH 8**

| 80    | EGU501        | 28 0 8 16 7 0 0 26 0 11 17 25 23 |
| 81    | EGU502        | 28 21 22 30 25 0 18 11 25 28 22 22 |
| 82    | EGU503        | 30 30 18 17 20 20 25 14 13 18 10 18 |
| 83    | EGU504        | 28 32 17 20 0 0 20 0 13 16 10 14 |

73
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<th>S.No.</th>
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Strains isolated at pH 9

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Strains isolated at pH 11

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a: Ampicillin (Amp), Carbenicillin (Car), Chloramphenicol (Chl), Gentamycin (Gen), Kanamycin (Kan), Nalidixic acid (Nal), Penicillin G (Peni), Polymyxin B (Poly), Rifampicin (Rif), Streptomycin (Str), Tetracyclin (Tet) and Vancomycin (Van).
Table 4.2a: Isolates were segregated based on the zones of inhibition (mm) for each antibiotic

<table>
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<th>Group</th>
<th>Amp 25 mcg</th>
<th>Car 100 mcg</th>
<th>Chl 10 mcg</th>
<th>Gen 30 mcg</th>
<th>Kan 10 mcg</th>
<th>Nal 30 mcg</th>
<th>Pen 10 units</th>
<th>Poly 100 units</th>
<th>Rif 15 mcg</th>
<th>Str 10 mcg</th>
<th>Tet 10 mcg</th>
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Groups I to IV for each antibiotic were made by taking into consideration the total range of response of a particular antibiotic.

Table 4.2b: Frequency of occurrence of different isolates (as per Table 4.2a) segregated on the basis of sensitivity towards various antibiotics

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<th>Chl 10 mcg</th>
<th>Gen 10 mcg</th>
<th>Kan 30 mcg</th>
<th>Nal 30 mcg</th>
<th>Pen 10 units</th>
<th>Poly 100 units</th>
<th>Rif 15 mcg</th>
<th>Str 10 mcg</th>
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Response of strains isolated at pH 2

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<th>Nal 30 mcg</th>
<th>Pen 10 units</th>
<th>Poly 100 units</th>
<th>Rif 15 mcg</th>
<th>Str 10 mcg</th>
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Response of strains isolated at pH 3

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<th>Pen 10 units</th>
<th>Poly 100 units</th>
<th>Rif 15 mcg</th>
<th>Str 10 mcg</th>
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Response of strains isolated at pH 4

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<th>Rif 15 mcg</th>
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Strains were grouped according to the range of response for each antibiotic (Table 4.2a). The susceptibility patterns of various strains revealed that response of strains isolated at same pH showed a common behaviour. For strains isolated at pH 2, Kanamycin, Penicillin and Tetracycline were more potent in action and majority of strains showed large zones of inhibition with these antibiotics with more than 9 strains falling in group IV. For strains isolated at pH 3, most of the strains were sensitive to Ampicillin, Chloramphenicol and Kanamycin. Response of strains isolated at pH 4 was very different. They were resistant to almost all
antibiotics except Chloramphenicol and Tetracycline. For strains isolated at pH 5, Ampicillin, Chloramphenicol and Kanamycin could restrict the growth of most strains. Strains isolated at pH 7 were susceptible to Ampicillin, Chloramphenicol and Kanamycin (which fall in Group IV). Strains isolated at pH 6 and pH 9 were particularly resistant to almost all antibiotics with a majority of strains and pattern of distribution was restricted to Group I and II. Ampicillin and Carbenicillin were potent against most strains isolated at pH 8. Rifampicin could inhibit growth of strains isolated at pH 10 and most of the strains were susceptible to rest eleven antibiotics. Strains isolated at pH 11 were susceptible to Tetracycline, Rifampicin, Penicillin and Kanamycin.
Hydrolyzing abilities of microbial isolates
4.2 Metabolic characteristics and biodegradative abilities of microbial isolates

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 (Appendix 2). Relative enzyme activity was calculated by finding the ratio of zone of hydrolysis (mm) and size of the bacterial colony (mm).

Among the various methods of waste management, anaerobic digestion process has matured in the last few decades as an effective means for waste (water) treatment. Anaerobic treatment is mainly considered as an effective pretreatment; however the process efficiency can be enhanced by harvesting value added products such as hydrogen (H₂), polyhydroxyalkanoates (PHA), volatile fatty acids such as acetic acid, butyric acid and ultimately methane (CH₄) (Kalia et al., 1995, 1997).

On the basis of Zone of Hydrolysis (ZOH) for protease, 9 strains were chosen which had ZOH of more than 30 mm (Table 4.3a). The strain with the highest ZOH was EGU443 followed by EGU465 with ZOH for lipase of 24 mm each and Relative Amylase Activity (RAA) and Relative Lipase Activity (RLA) of more than 2.4. EGU460 not only had a high value of ZOH for protease as 35 mm but also had Relative Protease Activity (RPA) of 3.5, which was remarkably good. EGU541 and EGU496 both had ZOH of protease > 30 and RPA > 2 but are negative for amylase activity. EGU507 and EGU454 share 3 common values as both had ZOH for protease as 30 mm, ZOH for lipase as 17 and RLA as 1.4.

Table 4.3a: Bacterial isolates with high protease activity

<table>
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<th>Isolate number</th>
<th>Protease ZOH (mm)</th>
<th>RPAb</th>
<th>Amylase ZOH (mm)</th>
<th>RAAc</th>
<th>Lipase ZOH (mm)</th>
<th>RLAd</th>
<th>Ureaseg</th>
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<td>24</td>
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[^a]: Protease activity (Zone of hydrolysis) for the isolates were observed to be in the range of 6 to 47 mm. In this Table 4.3a, high protease activity i.e. zone of hydrolysis in the range of 30 to 47 mm have been presented. Values
for rest of the isolates are presented in Appendix 2; b: Relative Protease Activity (Zone of hydrolysis/colony size); c: Relative Amylase Activity (Zone of hydrolysis/colony size); d: Relative Lipase Activity (Zone of hydrolysis/colony size); e: No zone; f: Not applicable; g: a colour change of pink indicates positive urease test.

Ten strains having RPA > 3 have been listed here (Table 4.3b). EGU460 is the strain, which is common between both the categories of protease activity (ZOH > 30 and RPA > 3). EGU464 and EGU192 had RPA of 4.3 and 4 respectively but average amylase and lipase activities. EGU481 had RPA of 3.4 and ZOH for lipase as 20 mm. EGU197 had RPA for protease as 3 and also common among one more category i.e. ZOH for amylase > 15. Definitely, this strain is good for both protease and amylase activities. Rest all strains viz EGU439, EGU468 and EGU523 also had RPA=3.

**Table 4.3b: Bacterial isolates with high relative protease activity (RPA<sup>a</sup>)**

<table>
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<tr>
<th>Isolate number</th>
<th>Enzyme activities</th>
<th>Protease ZOH&lt;sup&gt;b&lt;/sup&gt; (mm)</th>
<th>RPA</th>
<th>Amylase ZOH&lt;sup&gt;c&lt;/sup&gt; (mm)</th>
<th>RAA&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Lipase ZOH&lt;sup&gt;d&lt;/sup&gt; (mm)</th>
<th>RLA&lt;sup&gt;d&lt;/sup&gt;</th>
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<td>nz</td>
<td>na&lt;sup&gt;f&lt;/sup&gt;</td>
<td>-</td>
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</tbody>
</table>

a: Relative Protease Activity (RPA) (Zone of hydrolysis/colony size) for the isolates were observed to be in the range of 1.1 to 4.3. In this Table 4.3b, those strains with high RPA i.e. in the range of 3 to 4.3 have been presented. Values for rest of the isolates are presented in Appendix 2; b: Zone of hydrolysis; c: Relative Amylase Activity (Zone of hydrolysis/colony size); d: Relative Lipase Activity (Zone of hydrolysis/colony size); e: No zone; f: Not applicable; g: a colour change of pink indicates positive urease test.

Nine strains belonged to this category and had ZOH for amylase more than 15 (Table 4.3c). The strain showing the highest zone was EGU208 having ZOH of 35 mm followed by EGU207. Both these strains also have RAA of 1.2. EGU194 had ZOH for amylase of 23 mm and RAA of 2.3 but had no lipase activity. EGU459 has RAA of 2.3 and RLA of 2.4 with ZOH for amylase as 19 mm. EGU449 and EGU197 had ZOH for amylase of > 15 and RAA > 2.5 so it falls in both the categories of high ZOH and high RAA. Although EGU478 had ZOH for amylase as just 15 mm but
ZOH for protease was more than 30 mm. So it can prove to be a good candidate for both amylase and protease.
Table 4.3c: Bacterial isolates with high amylase activity

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Enzyme activities</th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Protease ZOHb (mm)</td>
<td>RPAc</td>
<td>Amylase ZOH (mm)</td>
<td>RAAe</td>
<td>Lipase ZOH (mm)</td>
<td>RLa d</td>
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<td>15</td>
<td>1.8</td>
<td>10</td>
<td>1.2</td>
</tr>
</tbody>
</table>

a: Amylase activity (Zone of hydrolysis) for the isolates were observed to be in the range of 4.5 to 35 mm. In this Table 4.3c, high amylase activity i.e. zone of hydrolysis in the range of 15 to 35 mm have been presented. Values for rest of the isolates are presented in Appendix 2; b: Relative Protease Activity (Zone of hydrolysis/colony size); c: Relative Amylase Activity (Zone of hydrolysis/colony size); d: Relative Lipase Activity (Zone of hydrolysis/colony size); e: No zone; f: Not applicable; g: a colour change of pink indicates positive urease test.

Twelve strains were found to have RAA of more than 2.5 (Table 4.3d). Among these, EGU477 had the highest value of 3.6. The same strain happened to have ZOH for lipase as 27 mm which is quite high. Both EGU215 and EGU451 had RAA of 3 and ZOH for amylase as 12 each. EGU197, EGU212 and EGU449 had RAA of 2.6. Rest 5 strains had RAA as 2.5. Among these, EGU466, EGU486 and EGU489 had RPA values of more than 2.2, which indicate that they can be useful for both amylase and protease.

Table 4.3d: Bacterial isolates with high relative protease activity (RAA^a)

<table>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
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<td>Protease ZOHb (mm)</td>
<td>RPAc</td>
<td>Amylase ZOH (mm)</td>
<td>RAAe</td>
<td>Lipase ZOH (mm)</td>
<td>RLa d</td>
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</tr>
</tbody>
</table>

a: Relative Amylase Activity (RAA) (Zone of hydrolysis/colony size) for the isolates were observed to be in the range of 1.0 to 3.6. In this Table 4.3d, those strains with high RAA i.e. in the range of 2.5 to 3.6 have been presented. Values for rest of the isolates are presented in Appendix 2; b: Zone of hydrolysis; c: Relative Protease Activity (Zone of hydrolysis/colony size); d: Relative Lipase Activity (Zone of hydrolysis/colony size); e: a colour change of pink indicates positive urease test.
Ten strains were included in the category of ZOH for lipase more than 23 mm (Table 4.3e). EGU495 had the highest ZOH for lipase i.e. But showed no protease and amylase activities. EGU494 had ZOH for lipase of 28 mm and ZOH for protease of 21 mm. EGU477 had RAA of 3.6 which is quite high and ZOH for lipase as 27 mm. EGU493 also had ZOH for amylase > 25 mm but no other enzyme activity. Both EGU519 and EGU509 had ZOH for lipase of 25 mm but no amylase activity.

**Table 4.3e: Bacterial isolates with high lipase activity**

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Protease ZOH (mm)</th>
<th>RPA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Amylase ZOH (mm)</th>
<th>RAA&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Lipase ZOH (mm)</th>
<th>RLA&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Urease&lt;sup&gt;g&lt;/sup&gt;</th>
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</tbody>
</table>

a: Lipase activity (Zone of hydrolysis) for the isolates were observed to be in the range of 10 to 35 mm. In this Table 4.3e, high lipase activity i.e. zone of hydrolysis in the range of 25 to 35 mm have been presented. Values for rest of the isolates are presented in Appendix 2; b: Relative Protease Activity (Zone of hydrolysis/colony size); c: Relative Amylase Activity (Zone of hydrolysis/colony size); d: Relative Lipase Activity (Zone of hydrolysis/colony size); e: No zone; f: Not applicable; g: a colour change of pink indicates positive urease test.

Nine strains were grouped under the category of RLA> 2.3 (Table 4.3f). Many strains belonging to this group were also common with rest 5 categories. Both EGU443 and EGU465 had RLA of 2.3, ZOH for protease as 47 mm and 38 mm and RAA of 2.4 and 2.5. Thus these 2 strains were good for all the 3 enzyme activities. Similarly, EGU459 and EGU466 had relative activities of more than 2.2 for all enzymes.
Table 4.3f: Bacterial isolates with high relative Lipase activity (RLA<sub>a</sub>)

<table>
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<tr>
<th>Isolate number</th>
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<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
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<td></td>
<td>Protease ZOH&lt;sub&gt;b&lt;/sub&gt; (mm)</td>
<td>RPA&lt;sub&gt;c&lt;/sub&gt;</td>
<td>Amylase ZOH&lt;sub&gt;d&lt;/sub&gt; (mm)</td>
<td>RAA&lt;sub&gt;d&lt;/sub&gt;</td>
<td>Lipase ZOH&lt;sub&gt;d&lt;/sub&gt; (mm)</td>
<td>RLA</td>
<td>Urease&lt;sub&gt;e&lt;/sub&gt;</td>
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</table>

|               |                          |   |   |   |   |   |   |

<sup>a</sup>: Relative Lipase Activity (RLA) (Zone of hydrolysis/colony size) for the isolates were observed to be in the range of 1.0 to 2.4. In this Table 4.3f, those strains with high RLA i.e. in the range of 2.3 and 2.4 have been presented. Values for rest of the isolates are presented in Appendix 2; <sup>b</sup>: Zone of hydrolysis; <sup>c</sup>: Relative Protease Activity (Zone of hydrolysis/colony size); <sup>d</sup>: Relative Amylase Activity (Zone of hydrolysis/colony size); <sup>e</sup>: a colour change of pink indicates positive urease test.

Strains were sorted out on the basis of 6 different criteria and arranged in descending order based on high zones of hydrolysis and relative enzyme activities. Further these strains were recategorized into a group which had high values for atleast two enzyme activities. The net result of such an arrangement was fishing out of 8 best strains (Table 4.4) as summarized below. EGU443 and EGU465 were among the best strains with zones of hydrolysis for protease activity as 47 and 38 mm respectively and with RAA and RLA both above 2.4. Both of these can be considered satisfactory strains for hydrolyzing abilities for all the 3 enzymes. EGU541 had above average values for ZOH for protease and lipase i.e. 32 and 27 mm respectively, although it was showing no activity for the enzyme amylase. EGU449 had good ZOH for protease and RPA and RAA above 2.4. It can also be considered an overall good strain for all 3 enzymes. EGU459 and EGU466 had relative enzyme activities for protease, amylase and lipase above 2.2 with ZOH in the range of 10 to 23 mm. Both the strains henceforth have good degradative abilities.

Table 4.4: Selected strains with high overall hydrolytic activities

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Enzyme activities</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
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<td></td>
<td>Protease ZOH&lt;sub&gt;a&lt;/sub&gt; (mm)</td>
<td>RPA&lt;sub&gt;b&lt;/sub&gt;</td>
<td>Amylase ZOH&lt;sub&gt;d&lt;/sub&gt; (mm)</td>
<td>RAA&lt;sub&gt;d&lt;/sub&gt;</td>
<td>Lipase ZOH&lt;sub&gt;d&lt;/sub&gt; (mm)</td>
<td>RLA&lt;sub&gt;c&lt;/sub&gt;</td>
<td>Urease&lt;sub&gt;e&lt;/sub&gt;</td>
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</table>

<sup>a</sup>: Zone of hydrolysis (mm); <sup>b</sup>: Relative Protease Activity (Zone of hydrolysis/colony size); <sup>c</sup>: Relative Lipase Activity (Zone of hydrolysis/colony size); <sup>d</sup>: Relative Amylase Activity (Zone of hydrolysis/colony size); <sup>e</sup>: No zone; <sup>f</sup>: Not applicable; <sup>g</sup>: a colour change of pink indicates positive urease test.
Polyhydroxyalkanoates producing abilities of microbial isolates
Microbes isolated from diverse environmental sources such as contaminated food, nitrogen rich soil, tannery wastewaters, soils from high altitude, activated sludge from effluent treatment plants dealing with pesticide and oil refineries were found to belong to *Bacillus*, *Bordetella*, *Anoxybacillus*, *Myroides*, *Alcaligenes*, *Marinobacter*, *Halomonas*, *Proteus* and *Pseudomonas* sp. on the basis of 16S rRNA gene sequence analysis.

### 4.3 Optimization of inoculum size

The inoculum size is one of the important factors for efficient degradation of substrates in fermentation process. Two-stage cultivation is widely used to produce PHAs because nutrient limiting conditions are favorable for the efficient synthesis of PHAs. The first stage is therefore principally for cell biomass production. In this study cells were cultivated for a period of 24 h in the first stage of cultivation and then harvested and transferred to the second stage cultivation (48h). However, not much is known about the physiological state of the bacterial cells. To investigate this matter, different inoculum concentration were used to check the optimum inoculum size for efficient bioconversions. Therefore, a range of 0.25 to 20 µg/ml (w/v) inoculum concentration (protein) was tested for 3 bacterial strains EGU1, EGU5 and EGU 17 (Table 4.5). As per the observations, inoculum size of 1 µg/ml for all the three tested strains was the best for the tested parameters (O.D., DCM and PHB). There was constant doubling of DCM (Dry Cell Mass) as we move from 0.25 µg/ml to 05µg/ml to 1 µg/ml and thereafter a decline in DCM was observed. Although O.D. remained nearly constant after 1µg/ml but there was a significant decline in PHB values thereafter.

Table 4.5: Optimization of inoculum size for polyhydroxybutyrate production

<table>
<thead>
<tr>
<th>Inoculum Size (µg/ml)</th>
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<th>EGU17</th>
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a: Optical Density; b: Dry Cell Mass (mg/L), c: Polyhydroxybutyrate (mg/L); d: %w/w

### 4.4 Correlation of polyhydroxybutyrate yield and concentration

A very interesting observation was made when a curve was plotted between the concentration of PHB and the relative yield (w/w). 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 (Porwal et al., 2008). On the other hand, at high concentration of PHB (mg/liter) yields were in the range of 49.4 to 66.6 % (Fig. 4.1). Further, the plot of PHB yield and PHB concentration gives a straight line with a positive slope, which indicates a positive correlation between the two.

More than hundred strains were screened for polyhydroxybutyrate production. Sixty were found to produce varying amounts of PHB. Thirteen strains could produce PHB in the range of 320 to 565 mg/L with the corresponding yields of 40 to 87% (Table 4.6). Maximum number of isolates with a wide range of PHB producing abilities belonged to the genus *Bacillus*. Thirty-eight *Bacillus* strains were screened out of which 6 strains outperformed others by producing PHB in the range of 320 to 565 mg/L of PHB. *Myroides odoratus* EGU882 recorded the PHB production of 502 mg/L at 48h interval. On the other hand *M. odoratus* EGU885 had a much lower value of 45 mg/L of PHB. Among the three *Anoxybacillus* strains, *Anoxybacillus hidirlerensis* touched PHB production level of 565 mg/L. The other 2 strains had PHB production level of 66 to 85 mg/L with the yield of up to 20% only. Among the 14 *Bacillus* sp. strains, *Bacillus* sp. EGU75 was the highest producer with a value of 460 mg/L and corresponding yield of 87% which was also the highest among all the strains tested during screening for PHB production. Pattern of PHB production of thirteen *B. cereus* strains was quite different. The range of PHB productions was from 40 to 500 mg/L. The best strain which had considerably high value at both 24h and 48h intervals was *B. cereus* EGU5 with PHB production value of 250 and 280 mg/L respectively and also a yield of 58 to 59%. Among six *B. licheniformis* strains EGU13 produced 260 mg/L of PHB with a yield of 25%. *B. pumilis* EGU6 is among the best PHB producer with 485 mg/L of PHB and 77% of yield. In the group of β- proteobacteria, *Bordetella* sp. EGU497 and *Alcaligenes faecalis* EGU 38 produced PHB in the range of 360 to 530 mg/L and corresponding yield of 23 to 61%. Among gamma-proteobacteria, *Alcanivorax* sp. EGU619 is the best producer with the PHB production value of 345 mg/L (51%w/w) and 465 mg/L (63%w/w) at 24h and 48h intervals. Among *Pseudomonas* strains, *P. stutzeri* EGU837 could produce up to 170 mg/L PHB. Between 2 strains of *Marinobacter*, EGU893 produced 235 mg/L of PHB with and an yield of 47%.
Table 4.6: Polyhydroxybutyrate producing abilities of bacterial isolates

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**β- Proteobacteria**

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**γ- Proteobacteria**

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Unclassified

52 Marine bacterium clone EGU409 (DQ487037) | 505 | 80 | 16 | 540 | 105 | 19 |

Values are based on two sets of experiments observed after 48 h of incubation. Standard deviation was less than 5%.\(^a\) http://www.ncbi.nlm.nih.gov/; \(^b\) Dry cell mass; \(^c\) Polyhydroxybutyrate; \(^d\) Yield, % w/w. \(^e\) Not detectable; \(^f\) Not applicable.

4.5 Biochemical characterization of selected polyhydroxybutyrate producers

Some of the selected strains were tested for different biochemical properties like utilization of thirty three different carbohydrates as carbon source, five proteins as nitrogen source and seven enzyme activities (Alkaline phosphatase, Ribose, Dextrose, Fructose, Glucose, trehalose, Sachharose, Mannose, Glycerol, Nitrate reduction, Citrate Utilization, arginine utilization, Urease, Malonate, Lysine decarboxylase, Ornithine decarboxylase, Galactose, Xylose, Melibiose, Cellobiose, ONPG β-galactosidase, Sodium Gluconate, Phenylalanine deaminase, L-arabinose, Esculin). Forty-eight different tests were performed by inoculating 24h old bacterial culture into wells of the kits containing specific medium to test the biochemical profile of organisms. On incubation microbes undergo metabolic changes, which are indicated by a spontaneous colour change of the media. Following description of tests helped us to interpret the results based on which biochemical characterization was done.
**Esculin test:** Esculin is a glycoside composed of glucose and dihydroxy coumorin compound. On esculin hydrolysis, the original cream colour of the medium changes to black.

**Trehalose test:** It is a disaccharide composed of two glucose molecules bound by an $\alpha-1, 1-$ 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.

**Carbohydrate fermentation tests:** This test is used to determine the ability of an organism to ferment various simple carbohydrates (sugars). 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 the organism does not ferment the sugar and also utilizes the peptone, accumulation of the ammonia as a degradation product will raise the pH. If the microbe metabolizes the carbohydrate subsequent acid product ion will result in lowered pH and hence positive test (yellow from red).

**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.

**Decarboxylase test:** This test is used to detect the ability of an organism to decarboxylate an amino acid. Decarboxylation is a reaction, which removes the carboxyl group (COOH) of an amino acid, producing an amine and CO$_2$. The amino acid is added to the test medium, along with the pH indicator bromocresol purple. The decarboxylation of the amino acid by the decarboxylase enzyme then results in alkaline end products. These in turn will cause the pH indicator to turn purple.
(positive). Lysine and ornithine are commonly used amino acids for test. Arginine and other amino acids are tested in different chemical reaction dehydroxylation.

**Methyl red test:** It is used to identify bacteria that produce stable acid end products by means of mixed acid fermentation of glucose. Bacteria those are able to perform mixed-acid fermentation of glucose and produce large amounts of stable acids. The pH indicator methyl red is used in this test. If the pH is less than 4.4, the indicator will turn red. A red colour is read as positive, a yellow colour (pH greater than 6.0) is negative, and an orange colour indicating a pH between the two will usually require further incubation.

**Voges-Proskauer (VP) test:** This test is used to identify organism able to produce acetoin from the degradation of glucose during 2, 3 butane diol fermentation. In some fermentative organism, the chief end products of glucose metabolize are acetoin and 2,3 butane diol. After incubation Barritt’s Reagent A (α- naphthol) and Barritt’s Reagent B (potassium hydroxide) are added to the sample. Formation of red colour will indicate a positive reaction. No color change or a copper colour indicates negative results.

**Nitrate reduction test:** This test detects the ability of an organism to reduce nitrate (NO₃) to nitrite (NO₂) or some other nitrogenous compounds, such as molecular nitrogen (N₂) using the enzyme nitrite reductase. Nitrate (NO₃) may be reduced to several different compounds, either by anaerobic respiration or by denitrification. This test is used to detect whether or not the reduction has taken place. The nitrate medium contains potassium nitrate as the substrate. If the organism reduces the nitrate to nitrite, the nitrite will react with added reagents sulphanillic acid and α-napththalamine to produce a red colour. If no colour is produced, this can indicate either of two reactions: (i) the nitrate was not reduced (ii) the nitrate was reduced even further to compounds other than nitrite.

**Phenyalnine deamination:** This test is used to identify bacteria possessing the enzyme phenylanine deaminase. This medium contains the amino acid phenylalanine. The enzyme will remove the amine group and release it as free ammonia (NH₃). This leaves phenyl pyruvic acid, which can be detected by adding an oxidizing reagent such as ferric chloride to the incubated tube. If the acid is present, a green colour can be detected.
**ONPG test:** This test is used to identify bacteria possessing the enzyme β-galactosidase. β-galactosidase catalyzes the breakdown of the substrate lactose (the major sugar present in milk) to 2 products, galactose and glucose, compounds which readily feed into the glycolytic pathway. ONPG is used as artificial substrate for the enzyme. Ortho-nitrophynyl- β-galactoside (ONPG) in the presence of β-galactosidase, is converted to galactose and ortho- nitrophenyl (ONP). ONPG is colourless. ONP is also colourless at neutral or acidic pH, but in alkaline solution it is bright yellow.

**Urease test:** It is used to detect the presence of enzyme urease. It breaks the carbon-nitrogen bond of amides to form CO₂, NH₃ and H₂O. When urea is broken down, ammonia is released and pH of the medium increases (becomes more basic). This pH change is detected by a pH indicator that turns pink in a basic environment. A pink medium indicates a positive test for urease.

An analysis of the various metabolic activities of strains against 48 different substrates reveals these strains have varying capacities to ferment glucose, maltose, Fructose, Dextrose, Sucrose, Mannose, α-methyl-D-Mannose, Cellobiose, D-Arabinose, Arabinose and ONPG Decarboxylase activity and to degrade Nitrate, Arginine, Xylose and Sorbitol (Table 4.7). EGU30 had the highest metabolic abilities with maximum number of positive results for 48 different substrates. It had the ability to degrade 4 out of 9 monosachharides (Glucose, Dextrose, Ribose and Xylose) and 3 out of 12 disachharides (Trehalose, galactose and Melibiose). Among sugar alcohols, it could degrade glycerol which is good commercial value also. It could metabolize all tested amino acids (Lysine, Ornithine and Phenylalanine). Also, it could produce H₂S, Urease, Citrase, ONPG beta galactosidase and Alkaline Phosphatase. It could also utilize Malonate and Sodium Gluconate. EGU378 could utilize Arginine and Indole. Among sugars, it could survive on sucrose, mannose, L-Arabinose, D-Arabinose, Sucrose, Maltose and Trehalose. EGU163 could utilize Arginine and Citrate and also produce Lysine decarboxylase and Urease. It was VP positive strain and could utilize Glucose, Fructose, L-Arabinose, Lactose and Saccharose as source of carbon. EGU409 could utilize Citrate and Esculin. It is VP positive. Among the sugars, it could utilize Mannose, L- Arabinose, Sucrose, Trehalose and Raffinose. EGU394 could utilize Glucose, Xylose, Melibiose. It could produce Alkaline Phosphatase and utilize Malonate.
Table 4.7: Biochemical characterization of microbial isolates

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Isolate number</th>
<th>Substrate</th>
<th>EGU30</th>
<th>EGU378</th>
<th>EGU163</th>
<th>EGU409</th>
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*a: Weak response  b: Positive response  c: Negative response*
Metabolism of biowastes by microbial isolates into polyhydroxyalkanoates
4.6 Efficacy of PHB production from *Bacillus* strains

In the first phase of testing with synthetic media (GM2) of known composition was undertaken and the 6 best strains were chosen for further studies. As per the plan of work, the percentage of synthetic media was then replaced sequentially by acidogenic slurry of pea shells. Further work was carried after the selection of 6 *Bacillus* strains and these were tested for their efficiency to produce PHB from waste biomass (2 day old acidogenic slurry of pea shells). Since waste biomass is a mixture of complex carbohydrate sources, firstly, all the strains were tested for their ability to utilize different sugars for their growth and PHB production. This work was biphasic in the sense that firstly the organisms were grown on minimal media supplemented with individual sugars and growth curves were plotted taking O.D. at 600nm as the measure of growth. Following this, PHB production media (GM 2) with individual sugars was used to further test for PHB production of each strain.

The 6 strains presented here are among the high PHB producers selected out of 100 strains screened for this characteristic. PHB production was monitored for all the 6 strains, on GM2 medium (400 ml in 1000 ml conical flasks) supplemented separately at the rate of 1% w/v with 5 different sugars (glucose or fructose or maltose or sucrose or lactose), and incubated at 37 ºC at 200 rpm.

The growth of 6 *Bacillus* strains varied considerably on GM2 medium supplemented with different sugars. The growth of all the strains was high on glucose, fructose and maltose, moderate on sucrose and relatively poor on lactose (Fig. 4.2). *B. cereus* EGU520 grew well on all the sugar supplemented medium. *B. cereus* EGU3 could utilize glucose and fructose better than others whereas *B. thuringiensis* EGU45 and *Bacillus* sp. EGU75 grew well on maltose and sucrose. The growth of all the strains was poor on lactose supplementation. Since *Bacillus* strains are not known to grow well on lactose as carbon source, hence a comparison of growth of all the 6 strains on minimal medium containing lactose or sucrose or glucose revealed very poor growth (Observed maximum O.D.₆₀₀ :
0.1538) on lactose. Hence, the little growth observed on lactose supplemented GM2 medium was perhaps due to other carbon sources present in yeast extract (one of the components of GM2). The growth on minimal medium with glucose or sucrose was in the normal range (Observed maximum O.D.₆₀₀: 2.2125 and 1.7646 respectively). Hence, we may conclude that these strains have the ability to utilize multiple sugars, as carbonaceous source.

PHB producing abilities of different *Bacillus* strains varied from 190 mg/L to 485 mg/L on glucose supplemented GM2 medium. PHB constituted 31 to 62% of the total DCM (Table 4.8). It may be remarked here at this stage that PHB production was recorded up to 48 h. *B. cereus* EGU44 and *B. thuringiensis* EGU45 could be rated among the high PHB producers. With the change of sugar from glucose to fructose, there was a dramatic change in the PHB producing abilities of all the strains. For *Bacillus* sp. EGU75, there was a marked enhancement in its PHB production from 190 mg/L with glucose to 385 mg/L with fructose i.e. an improvement of two fold. It also led to a higher yield of PHB, which comprised of 75% of the DCM. All other strains showed a decline in PHB production to the extent of 44 to 97%. The maximum loss was recorded with *B. cereus* EGU3, where the PHB production was practically negligible. On switching on to maltose as sugar supplement, the abilities of all the 6 *Bacillus* strains to produce PHB showed further decline, so much so that only 8 to 43% of the PHB could be realized in comparison to that with glucose. The only exception was *B. cereus* EGU43, which showed only 36% loss with a final PHB production of 220 mg/L and a yield of 48% w/w. A similar pattern of decline in PHB production and yield was seen with all the strains on sucrose as sugar supplement. Incidentally, *B. cereus* EGU44 and *B. cereus* EGU520 showed a similar response on fructose and sucrose. Although these strains have abilities to grow and convert sugars to PHB with different efficiencies, however glucose turned out to be the best sugar supplement followed by fructose. PHB could not be detected on GM2 medium containing lactose, where growth itself was very poor.
Table 4.8: Effect of sugar on polyhydroxybutyrate production by different Bacillus strains

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<thead>
<tr>
<th>Medium* with sugar</th>
<th>Cell mass and PHBb</th>
<th>B. thuringiensis EGU45</th>
<th>B. cereus EGU44</th>
<th>B. cereus EGU520</th>
<th>B. cereus EGU43</th>
<th>B. cereus EGU3</th>
<th>Bacillus sp. EGU75</th>
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<td>Glucose</td>
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<td>DCMc</td>
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<td>565</td>
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<td>485</td>
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Values are based on two sets of experiments observed after 48 h of incubation. Standard deviation was less than 5%.  a: GM2 medium supplemented with sugar at the rate of 1% w/v; b: Polyhydroxybutyrate, mg/L; c: Dry cell mass, mg/L; d: % w/w; e: Not detectable; f: Not applicable.

4.6.1 Effect of different sugars on polyhydroxybutyrate production

The results of this study indicate important effects of simple sugars as carbon sources on growth and accumulation of PHB by different Bacillus strains. Such correlations of carbon sources and PHB accumulation vary considerably with the organism being used. Incidentally, maltose has not proved to be a good carbon source with different Bacillus species except B. thuringiensis EGU45 and B. cereus EGU43 being reported here. However, maltose has been metabolized to 0.68 to 2.3 g PHB/L by A. vinelandii (Page, 1992). Bacillus strains being reported here provide us with an opportunity to develop a consortium with abilities to utilize a wide range of carbon sources for PHB production. A consortium of strains (EGU3, EGU43 EGU44 and EGU45) was also tested on sugar-supplemented media for each of the sugars (Table 4.9). It was inferred that for each of the sugars the increase in DCM retrieval and PHB production was much higher than the strains that were used in isolation. Yield of up to 88% was achieved through this method, which was not got, previously by any of the strains alone.

Four Bacillus strains were used for mixed culture cultivation and PHB production on GM2 supplemented with 5 different sugars. As shown in the results (Table 4.9), PHB
production by mixed culture was quite successful on glucose. The maximum PHB production was attained when the mixed culture was used @ 1% w/v each as compared to values obtained when each strain was used individually at the same inoculum level. (Table 4.9). PHB production was in the range of 325 to 485 mg/L with EGU3, EGU43, EGU44 and EGU45 whereas the value touched 990 mg/L when all the strains were cultured together. This is a clear indication that the strains worked synergistically to produce PHB rather than competing for the substrate. For PHB production in the presence of fructose, a similar trend was observed wherein PHB production values rose from 10 to 205 mg/L (with individual strains) to 955 mg/L in mixed culture. The rise in yield was also proportional and showed a similar trend of increase from 40% to 73%. The results of PHB production from maltose to sucrose were not so good when obtained with individual strains (70 to 220 mg/L on maltose and 70 to 170 mg/L on sucrose) but there was a substantial rise in PHB production when mixed culture was used in the presence of these sugars (955 mg/L on maltose and 620 mg/L on sucrose). Lactose still remained a poor substrate for PHB production, which coincided with the previous results of absolutely no production as Bacillus strains lack the enzyme lactase.

Table 4.9: Polyhydroxybutyrate production by use of mixed culture

<table>
<thead>
<tr>
<th>Medium(^b) with sugar</th>
<th>Cell mass and PHB(^c)</th>
<th>Incubation period (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DCM(^d)</td>
<td>PHB</td>
</tr>
<tr>
<td>Glucose</td>
<td>1015</td>
<td>515</td>
</tr>
<tr>
<td>Fructose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
<td>955</td>
<td>565</td>
</tr>
<tr>
<td>Sucrose</td>
<td>520</td>
<td>54</td>
</tr>
<tr>
<td>Lactose</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are based on two sets of experiments observed after 48 h of incubation. Standard deviation was less than 5%. \(a\): B. cereus EGU3, B. cereus EGU43, B. cereus EGU44 and B. thuringiensis EGU45; \(b\): GM2 medium supplemented with sugar at the rate of 1% w/v; \(c\): Polyhydroxybutyrate, mg/L; \(d\): Dry cell mass, mg/L; \(e\): % w/w; \(f\): Not detectable; \(g\): Not applicable.
Figure 4.2: Growth patterns of *Bacillus* strains on medium (GM2) supplemented with different sugars. a: Glucose; b: Fructose; c: Maltose; d: Lactose; e: Sucrose inoculated with: *Bacillus* sp. EGU75 (□); *B. cereus* EGU3 (♦); *B. cereus* EGU43 (●); *B. cereus* EGU44 (▲); *B. cereus* EGU520 (▲) and *B. thuringiensis* EGU45 (◊)

4.6.2 Polyhydroxybutyrate production by *Bacillus* strains from biowaste

In the last phase of testing, of the 6 chosen organisms, pea shell slurry in combination with GM2 media was used for production of PHB. Various parameters were changed (inoculum size, supplementation with external additional source of nitrogen etc) and their respective effects on PHB production were recorded.
Further optimization for the effect of CEH on PHB production from BW:M (1:1) was done by adding it at the rate of 0.2, 0.4 and 0.8 g/L and compared with control (no supplementation). BW:M (1:1) supplemented with 0.4g/L CEH was further tested for the effect of inoculum size on PHB production by adding microbes at the rate of 10, 100 and 1000 mg cell protein/L of feed.

Production of PHB by 6 Bacillus strains from combinations of BW:M in different ratios, with increasing contribution of biowaste is presented in Table 4.10. PHB production from BW:M in 3:7 ratio was in the range of 140 to 650 mg/L, which constituted 12 to 47 % w/w of the DCM. In spite of reducing the contribution of medium (containing glucose) by 30% in the feed, we could observe that Bacillus strains are able to utilize biowaste to the same extent in the case of B. cereus EGU44, B. thuringiensis EGU45 and B. cereus EGU520. A 1.4 to 3.4 fold increase in PHB production with B. cereus EGU3 and Bacillus sp. EGU75 on BW:M compared to that on GM2, respectively indicate that pea-shell waste is proving to be a good feed material. B. cereus EGU43 was the only strain which showed a dramatic loss of 59% in PHB production on replacing 30% of the medium with biowaste.

Since the overall objective is to maximize the contribution of biowaste in the feed, we checked the abilities of the six Bacillus strains on higher BW:M ratio of 1:1 and 7:3. Increasing the contribution of waste from 30% to 50% i.e. BW:M :: 1:1 resulted in 13 to 24% increase in PHB production with B. cereus EGU520, B. cereus EGU43 and B. thuringiensis EGU45. Incidentally in these 3 cases, PHB yields were only marginally affected and continued to be in the range 13 to 25% w/w, indicating thereby that the waste to PHB conversion efficiency of these Bacillus strains was maintained. On the other hand, with the other 3 Bacillus strains, the increase in biowaste contribution in the feed, the decline in PHB production was in the range of 31 to 71%. It appears that there has been a metabolic shift from PHB pathway to other metabolism such that PHB yields reduced from 25 to 47% (w/w) at BW:M :: 3:7 to 8 to 18% at BW:M :: 1:1 with Bacillus sp. EGU75, B. cereus EGU44 and B. cereus EGU3 (Table 4.10). Further increase in biowaste contribution to BW:M :: 7:3 continued to show a decline in PHB production by B. cereus EGU44, B. thuringiensis EGU45 and B. cereus EGU520. The yields decreased to a very low level of 9 to 18%, equivalent to 170 to 190 mg PHB/L. With the other 3 Bacillus strains, the PHB production was almost similar to that recorded at BW:M :: 1:1 level. It may be
concluded here that the ability of *Bacillus* strains to utilize biowaste for PHB production varies: *Bacillus* sp. EGU75, *B. cereus* EGU44 and *B. cereus* EGU3 were most efficient on BW:M :: 3:7 level; *B. thuringiensis* EGU45 and *B. cereus* EGU520 found BW:M :: 1:1 as conducive and *B. cereus* EGU43 proved to be the most efficient on GM2 medium supplemented with glucose.

### Table 4.10: Production of polyhydroxybutyrate from biowaste by *Bacillus* strains

| Biowaste<sup>a</sup>; Medium<sup>b</sup> (BW:M) | Cell mass and PHB<sup>d</sup> | Bacillus sp. | Bacillus sp. |
|---|---|---|---|---|
| | | *B. thuringiensis* EGU45 | *B. cereus* EGU44 | *B. cereus* EGU520 | *B. cereus* EGU43 | *B. cereus* EGU3 | *Bacillus* sp. EGU75 |
| 3:7 | DCM<sup>e</sup> | 1855 | 1040 | 1910 | 1165 | 1820 | 1855 |
| | PHB | 390 | 490 | 325 | 140 | 405 | 650 |
| | Yield | 21 | 47 | 17 | 12 | 25 | 35 |
| 1:1 | DCM | 2040 | 1890 | 2175 | 1270 | 1435 | 2375 |
| | PHB | 510 | 340 | 370 | 165 | 215 | 190 |
| | Yield | 25 | 18 | 17 | 13 | 15 | 8 |
| 7:3 | DCM | 1945 | 1000 | 1055 | 1100 | 1875 | 2055 |
| | PHB | 175 | 170 | 190 | 165 | 225 | 185 |
| | Yield | 9 | 17 | 18 | 15 | 12 | 9 |
| 3:7 | DCM | 1675 | 1355 | 1100 | 2060 | 1445 | 1850 |
| | PHB | 905 | 285 | 430 | 1485 | 955 | 870 |
| | Yield | 54 | 21 | 39 | 72 | 66 | 47 |
| 1:1 | DCM | 2190 | 1450 | 1990 | 2665 | 1525 | 3205 |
| | PHB | 1205 | 945 | 855 | 800 | 930 | 705 |
| | Yield | 55 | 65 | 43 | 30 | 61 | 22 |
| 7:3 | DCM | 1830 | 1500 | 1040 | 1400 | 1415 | 1160 |
| | PHB | 165 | 75 | 250 | 350 | 680 | 255 |
| | Yield | 9 | 5 | 24 | 25 | 48 | 22 |

Values are based on two sets of experiments observed after 72 h of incubation. Standard deviation was less than 5%. a: Pea shell slurry; b: GM2 Medium; c: Casein enzyme hydrolysate at the rate of 0.2 g/L; d: Polyhydroxybutyrate, mg/L; e: Dry cell mass, mg/L; f: % w/w.

### 4.6.3 Effect of supplemental nitrogen on polyhydroxybutyrate production from biowaste

Polyhydroxybutyrate biosynthesis is greatly affected by high Carbon (C): Nitrogen (N) limitation and improvements in its production has been seen by supplementation of a small amounts of complex nitrogen sources such as yeast extract, tryptone, nutrient broth, peptone, casein and soybean hydrolysate. In view of these informations, the effect of nitrogen supplementation on PHB production from biowaste was initially checked at the rate of 0.02% w/v of CEH. It was found to greatly enhance the PHB production and yields to various extents (Table 4.11). At
30% level of biowaste in the feed (BW:M :: 3:7), a 25 to 81% increase in PHB production accompanied by a net enhancement of 12 to 60% in PHB yield compared to the non CEH- supplemented BW:M (3:7) as feed was recorded. The only exception was *B. cereus* EGU44, where the PHB producing abilities declined from 490 mg/L to 285 mg/L on CEH supplementation. It was also accompanied by decrease in PHB yield from 47% (w/w) to 21% (w/w) (Table 4.11). *B. cereus* EGU43 resulted in maximum PHB production of 1485 mg/L and a yield of 72% (w/w) on CEH supplemented BW:M :: 3:7 as feed. It’s interesting to mention that this strain was not able to produce good PHB yield on BW:M combination without CEH supplementation.

At higher contribution of biowaste in feed, supplementation with CEH proved effective up to BW:M :: 1:1 level. The trend of improvement in PHB production could not be sustained at BW:M :: 7:3 level in spite of CEH supplementation. It may be remarked that at 70% biowaste contribution in the feed, CEH supplementation led to higher yields in most of the cases in comparison to non-supplemented BW:M combinations.

Table 4.11: Effect of supplemental nitrogen on microbial production of polyhydroxybutyrate from biowaste

<table>
<thead>
<tr>
<th>Biowaste(^a): Medium(^b) with CEH(^c)</th>
<th>Cell mass and PHB(^d)</th>
<th>Bacillus sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>B. cereus</em> EGU44</td>
</tr>
<tr>
<td>Control</td>
<td>DCM(^e)</td>
<td>1890</td>
</tr>
<tr>
<td></td>
<td>PHB</td>
<td>340</td>
</tr>
<tr>
<td></td>
<td>Yield(^f)</td>
<td>18</td>
</tr>
<tr>
<td>0.2</td>
<td>DCM</td>
<td>1455</td>
</tr>
<tr>
<td></td>
<td>PHB</td>
<td>945</td>
</tr>
<tr>
<td></td>
<td>Yield</td>
<td>65</td>
</tr>
<tr>
<td>0.4</td>
<td>DCM</td>
<td>2700</td>
</tr>
<tr>
<td></td>
<td>PHB</td>
<td>1945</td>
</tr>
<tr>
<td></td>
<td>Yield</td>
<td>72</td>
</tr>
<tr>
<td>0.8</td>
<td>DCM</td>
<td>1520</td>
</tr>
<tr>
<td></td>
<td>PHB</td>
<td>745</td>
</tr>
<tr>
<td></td>
<td>Yield</td>
<td>49</td>
</tr>
</tbody>
</table>

Values are based on two sets of experiments observed after 72 h of incubation. Standard deviation was less than 5%. \(a\): Pea shell slurry; \(b\): GM2 Medium; \(c\): Casein enzyme hydrolysate (g/l); \(d\): Polyhydroxybutyrate, mg/L; \(e\): Dry cell mass, mg/L; \(f\): %w/w.
Figure 4.3: Effect of casein enzyme hydrolysate (CEH) supplementation on polyhydroxybutyrate production from biowaste inoculated with (a) Bacillus cereus EGU44; (b) B. thuringiensis EGU45. CEH: Nil (♦); 0.2 g/L (●); 0.4g/L (▲); 0.8g/L (●)

An overall analysis of different combinations of BW:M, with CEH supplementation reveals that different Bacillus strains respond differently to these conditions. B. cereus EGU43 proved best at BW:M :: 3:7 combination; 1485 mg PHB/L, 72% w/w; B. thuringiensis EGU45; B. cereus EGU44 and B. cereus EGU3 gave best yields at BW:M :: 1:1 combinations (930 to 1205 mg PHB/L; 55 to 65 % w/w). Since the overall objective is to maximize the contribution of biowaste in the feed, the following combinations of biowaste (BW:M with CEH supplementation at 1:1 level) and bacteria (B. cereus EGU44 and B. thuringiensis EGU45) were selected for further optimization.
The effect of concentration of CEH supplementation as nitrogen supplement seems to be very clear-cut on PHB production by *Bacillus* strains (Table 4.12). A comparison of CEH supplementation at 0.2, 0.4 and 0.8 g/L (Fig. 4.3) shows that there is a marked increase in PHB production in comparison to control with maximum yields appearing after 72 h of incubation. Maximum enhancement in PHB production was observed with 0.4 g/L (0.04% w/v) supplementation, where the PHB production achieved was 1945 to 2075 mg/L and a yield of 71 to 72% w/w. These enhancements were 2.7 to 4.1 fold compared to control and 1.7 to 2.0 fold compared to 0.2 g/L CEH. A further addition of CEH at 0.8 g/L proved detrimental to PHB production since C: N ratio might have been dramatically reduced. The final PHB yield of 630 mg/L and 745 mg/L at 0.8 g/L CEH supplementation were however, still better than the control.

**4.6.4 Effect of inoculum size on polyhydroxybutyrate production from biowaste**

The major factors that influence the PHB production are the environmental stresses being faced by the microbes. These include limitations of N, P, Mg and O. On the other hand, the issue can be viewed from the point of view that if the feed:microbe ratio is also changed such that the feed becomes limiting, we can expect another physiological stress. In our study we have employed an inoculum size variation from 0.1% to 100% v/v.

The initial observation on the effect of inoculum size on PHB production was recorded on GM2 medium, where 1 mg cell protein/L was found to be equally effective to 10 mg cell protein/L. However, with biowaste in the medium a preliminary assay had shown that 10 mg cell protein/L is better than 1 mg cell protein/L. An increase in inoculum size from 10 mg cell protein/L to 100 mg cell protein/L of feed has helped to improve the PHB production to 3010 to 3370 mg/L i.e. an improvement of 32 to 42% (Fig. 4.4 and Table 4.12). A further increase in inoculum size to 1000 mg cell protein/L equivalent to 100% v/v did not lead to any significant change (Table 4.12). It perhaps could not create a physiological stress favourable for rapid diversion of metabolism towards PHB biosynthesis, it may be concluded that although the PHB production has improved, however, the PHB metabolic efficiency in terms of yield has not been maintained at a high level of 71 to 72% (w/w) observed at low microbial populations. The effort for 10 times higher inoculum size i.e. from 10 mg cell protein/L to 100 mg cell protein/L is not compensated by a corresponding increase in PHB production. It reflects that there is a potential for converting waste into PHB but we may have to look for some other parameters also to further improve the yields.
Table 4.12: Effect of inoculum size on polyhydroxybutyrate production from biowaste

<table>
<thead>
<tr>
<th>Inoculum(^a) in Biowaste(^b): Medium(^c)</th>
<th>Cell mass and PHB(^d)</th>
<th>Bacillus sp.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DCM(^e)</td>
<td>PHB</td>
<td>B. cereus EGU44</td>
</tr>
<tr>
<td>10</td>
<td>2700</td>
<td>1945</td>
<td>2920</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>8220</td>
<td>3370</td>
<td>7340</td>
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<tr>
<td></td>
<td>41</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>7225</td>
<td>3035</td>
<td>6085</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

Values obtained are based on two sets of experiments observed after 72 h of incubation. Standard deviation was less than 5%.  
\(^a\): mg cell protein/L; \(^b\): Pea shell slurry; \(^c\): GM2 Medium; \(^d\): Polyhydroxybutyrate, mg/L; \(^e\): Dry cell mass, mg/L; \(^f\): % w/w.

Figure 4.4: Effect of inoculum size (mg cell protein/L feed: 10 ( ♦); 100 ( ■); 1000 ( ▲)) on polyhydroxybutyrate production from biowaste. (a) *Bacillus cereus* EGU44; (b) *B. thuringiensis* EGU45
4.7 Sugar utilization and polyhydroxybutyrate production by unique microbial strains

The uniqueness of the strains tested in the last phase of experimentation lies in the fact that these strains have not been reported for PHB production so far. Production of PHB varied from strain to strain on glucose supplemented GM2 medium. Glucose was replaced by different sugars one by one and PHB production was recorded at 48h interval as summarized in Table 4.13. *Alcanivorax* sp. EGU619, *M. aqueolei* EGU893 and *M. odoratus* EGU882 produced PHB in the range of 315 to 460 mg/L whereas both the strains of *Anoxybacillus* sp. EGU145 and EGU146 and *Planomicrobium* sp. EGU782 produced a lesser amount i.e. 40 to 85 mg PHB/L. A reversal of PHB production abilities was seen when fructose was used as the sole source of carbon in the medium. The first three strains suffered a decline of 55 to 80% in PHB production whereas nearly 2.5 fold increase in the next three. The response to change of sugar to maltose from glucose was mixed. No PHB could be obtained from *M. odoratus* EGU882. In comparison to PHB from glucose, there was a general decline observed for that on maltose in rest of the strains (except *Anoxybacillus* sp. EGU145 which registered 3 fold increase in the value). In *Alcanivorax* sp. EGU619 and *M. aqueolei* EGU893, 71% and 17% decline in the PHB production value was observed respectively whereas with *Anoxybacillus* sp. EGU146 and *Planomicrobium* sp. EGU782, only half the original value could be obtained. When glucose was replaced by sucrose, the first three strains recorded a fall of 1.1 to 2.8 fold in PHB production, and a very poor response was obtained by two strains of *Anoxybacillus* sp. EGU145 and EGU146 and *Planomicrobium* sp. EGU782. On media supplemented with lactose there was an overall improvement in PHB production by the strains, *M. odoratus* EGU882 (1.6 times), *Anoxybacillus* sp. EGU145 (1.5 times) and EGU 146 (2.2 times) and *Planomicrobium* sp. EGU782 (7 times). A fall of 57% and 31% was recorded in *Alcanivorax* sp. EGU619 and *M. aqueolei* EGU893 respectively as compared to the values with glucose as the carbon source.
Table 4.13: Effect of sugars on polyhydroxybutyrate production by unique strains

<table>
<thead>
<tr>
<th>Mediuma with Sugar</th>
<th>Cell mass and PHBb</th>
<th>Alcanivorax sp. EGU619</th>
<th>Marinobacter aquoele EGU893</th>
<th>Myroides odoratus EGU882</th>
<th>Anoxybacillus sp. EGU145</th>
<th>Anoxybacillus sp. EGU146</th>
<th>Planomicrobium sp. EGU782</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Incubation (48h)</td>
<td>DCMc</td>
<td>PHB</td>
<td>Yield</td>
<td>DCMf</td>
<td>PHB</td>
<td>Yield</td>
</tr>
<tr>
<td></td>
<td></td>
<td>530</td>
<td>500</td>
<td>815</td>
<td>500</td>
<td>410</td>
<td>355</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td>PHB</td>
<td>130</td>
<td>105</td>
<td>60</td>
<td>225</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yield</td>
<td>37</td>
<td>14</td>
<td>13</td>
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</tr>
<tr>
<td>Maltose</td>
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<td>DCM</td>
<td>420</td>
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<td>665</td>
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<td>450</td>
</tr>
<tr>
<td></td>
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<td>PHB</td>
<td>130</td>
<td>195</td>
<td>ndf</td>
<td>280</td>
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<td></td>
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<td>31</td>
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<td>Sucrose</td>
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<td>100</td>
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<td></td>
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<td>Yield</td>
<td>35</td>
<td>36</td>
<td>32</td>
<td>31</td>
<td>36</td>
</tr>
<tr>
<td>Lactose</td>
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<td>780</td>
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<td>935</td>
<td>395</td>
<td>570</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PHB</td>
<td>195</td>
<td>160</td>
<td>505</td>
<td>135</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yield</td>
<td>25</td>
<td>27</td>
<td>54</td>
<td>34</td>
<td>28</td>
</tr>
</tbody>
</table>

Values are based on two sets of experiments. Standard deviation was less than 5%. a: GM2 medium supplemented with sugar at the rate of 1% w/v; b: Polyhydroxybutyrate, mg/L; c: Dry cell mass, mg/L; d: % w/w; e: Not detectable; f: Not applicable.

4.7.1 Polyhydroxybutyrate production by unique microbial strains from biowaste

The optimization of ratio of amount of BW:M combinations (1:1), addition of supplemental nitrogen source i.e. casein enzyme hydrolysate (at the rate of 0.4g/L) and inoculum size (10 mg cell protein/L) was done previously. All six strains were tested on these conditions and PHB production was recorded at 4 different time intervals (Table 4.14). A common trend of steady improvement in PHB production was seen with increasing time (Fig. 4.5) with all the strains. *Alcanivorax* sp. EGU619 showed an increase of 60% (from 315 mg PHB/L at 24h to 805 mg PHB/L in 48h). At 72h interval, the PHB concentration remained constant and further increased to a final value of 1015 mg PHB/L. In case of *M. aqueolei* EGU893, 62% rise was observed (from 280 mg PHB/L in 24h to 755 mg PHB/L in 48h). Another significant improvement was seen at 96h interval where the % increase was 45% from the previous value. Both the strains of *Anoxybacillus* sp. EGU145 and EGU146 had very low PHB production values at 24h interval after which there was a trend of improvement up to 96h with the final values of 975 and 815 mg PHB/L respectively. *Planomicrobium* sp. EGU782 gave an almost straight line with a regular increase at
every 24h interval with a final value of 945 mg PHB/L at 96h. All the 6 strains gave
the highest value (in the range of 815 mg PHB/L to 1145 mg PHB/L) at 96h.
Although, in some strains the trend of improvement was seen as a straight line (Figure
4.5) with a positive slope and rest had maintenance of nearly same value if not a
decline in PHB production.

Table 4.14: Production of polyhydroxybutyrate from biowaste\textsuperscript{a} by unique strains

<table>
<thead>
<tr>
<th>Incubation Period (h)</th>
<th>Cell mass and PHB\textsuperscript{b}</th>
<th>Alcanivorax sp. EGU619</th>
<th>Marinobacter aquoelei EGU893</th>
<th>Myroides odoratus EGU882</th>
<th>Anoxybacillus sp. EGU145</th>
<th>Anoxybacillus sp. EGU146</th>
<th>Planomicrobium sp. EGU782</th>
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Values are based on two sets of experiments. Standard deviation was less than 5%. a: Biowaste (Pea shell slurry):GM2 Medium :: 1:1 supplemented with 0.4g/L casein enzyme hydrolysate; b: Polyhydroxybutyrate, mg/L; c: Dry cell mass, mg/L; d: % w/w.

Figure 4.5: Polyhydroxybutyrate production from biowaste by novel bacterial strains. Anoxybacillus sp EGU145 (\textbullet), Anoxybacillus sp.EGU146 (\textbullet), Alcanivorax sp. EGU619 (\textdagger), Planomicrobium sp. EGU782 (\textdagger), Myroides odoratus EGU882 (\textdagger) and Marinobacter aquoeoli EGU893 (\textdagger)}