Chapter 3

Screening of isolated cultures for the production of polyhydroxyalkanoates (PHA)
## CONTENTS

### 3.1 INTRODUCTION

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1.1 Classification of Polymers</td>
<td>92</td>
</tr>
<tr>
<td>3.1.2 Growth of Plastics and its implications on the Society</td>
<td>95</td>
</tr>
<tr>
<td>3.1.3 “White Pollution”- Hazards of Plastics</td>
<td>96</td>
</tr>
<tr>
<td>3.1.4 Plastic Waste Management</td>
<td>97</td>
</tr>
<tr>
<td>3.1.5 ‘Reduce, Reuse, Recycle and Recovery of energy</td>
<td>98</td>
</tr>
<tr>
<td>3.1.6 Biodegradable Alternatives</td>
<td>99</td>
</tr>
<tr>
<td>3.1.7 Plant Based Polymers</td>
<td>99</td>
</tr>
<tr>
<td>3.1.8 Animal Based</td>
<td>101</td>
</tr>
<tr>
<td>3.1.9 Microbial Based</td>
<td>101</td>
</tr>
<tr>
<td>3.1.10 Challenges for Bioplastics</td>
<td>103</td>
</tr>
<tr>
<td>3.1.11 Potential benefits of the biopolymer to society and the environment</td>
<td>106</td>
</tr>
<tr>
<td>3.1.12 Disposal</td>
<td>107</td>
</tr>
</tbody>
</table>

### 3.2 REVIEW OF LITERATURE

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2.1 General Properties of Polymers</td>
<td>109</td>
</tr>
<tr>
<td>3.2.2 Melting point</td>
<td>110</td>
</tr>
<tr>
<td>3.2.3 Glass transition temperature</td>
<td>110</td>
</tr>
<tr>
<td>3.2.4 Crystallinity</td>
<td>110</td>
</tr>
<tr>
<td>3.2.5 Tensile strength</td>
<td>110</td>
</tr>
<tr>
<td>3.2.6 Biodegradability</td>
<td>111</td>
</tr>
<tr>
<td>3.2.7 Polyhydroxyalkanoates (PHA)</td>
<td>111</td>
</tr>
<tr>
<td>3.2.8 Polyhydroxyalkanoates in Nature</td>
<td>112</td>
</tr>
<tr>
<td>3.2.9 Chemistry of the PHAs</td>
<td>113</td>
</tr>
<tr>
<td>3.2.10 Physical Properties of PHAs</td>
<td>115</td>
</tr>
<tr>
<td>3.2.11 The Biology of PHA</td>
<td>116</td>
</tr>
<tr>
<td>3.2.12 Biosynthesis of PHA</td>
<td>117</td>
</tr>
<tr>
<td>3.2.13 PHA biosynthesis in Eucaryotic Cells</td>
<td>119</td>
</tr>
<tr>
<td>3.2.14 Microbial synthesis of PHA</td>
<td>121</td>
</tr>
<tr>
<td>3.2.15 Significance of Microbial Synthesis of PHAs</td>
<td>124</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>3.2.16 Recovery of PHA</td>
<td>124</td>
</tr>
<tr>
<td>3.2.17 Structure of PHA Granules</td>
<td>125</td>
</tr>
<tr>
<td>3.2.18 Quantitative Analysis using Gas Chromatography</td>
<td>128</td>
</tr>
<tr>
<td>3.2.19 Other types of PHA</td>
<td>129</td>
</tr>
<tr>
<td>3.2.20 Other Properties of PHAs</td>
<td>129</td>
</tr>
<tr>
<td>3.2.21 The enzymology of PHA production and organisation of the PHA biosynthetic genes</td>
<td>130</td>
</tr>
<tr>
<td>3.2.22 Uses and applications of PHAs</td>
<td>132</td>
</tr>
<tr>
<td>3.2.23 Biodegradability of PHA</td>
<td>134</td>
</tr>
<tr>
<td>3.2.24 Biodegradation in the environment</td>
<td>135</td>
</tr>
<tr>
<td>3.2.25 National Status of PHA Research</td>
<td>136</td>
</tr>
<tr>
<td>3.3 MATERIALS AND METHODS</td>
<td>138</td>
</tr>
<tr>
<td>3.3.1 Isolation of bacterial strains from the Rotating biological contactor (RBC)</td>
<td>138</td>
</tr>
<tr>
<td>3.3.2 PHA production and extraction in shake flask cultures</td>
<td>138</td>
</tr>
<tr>
<td>3.3.3 Nile blue A staining Staining techniques</td>
<td>139</td>
</tr>
<tr>
<td>3.3.4 Extraction of PHA by osmotic cell lysis methodology</td>
<td>140</td>
</tr>
<tr>
<td>3.3.5 Extraction of PHA</td>
<td>140</td>
</tr>
<tr>
<td>3.3.6 Quantification procedures</td>
<td>141</td>
</tr>
<tr>
<td>3.3.7 PHA identification using Gas Chromatography–Mass Spectrometry (GCMS)</td>
<td>142</td>
</tr>
<tr>
<td>3.3.8 Confocal imaging of inclusion bodies of <em>Enterobacter sp.</em></td>
<td>142</td>
</tr>
<tr>
<td>3.3.9 PHA inclusions detected by electron microscopy</td>
<td>143</td>
</tr>
<tr>
<td>3.3.10 Characterization and identification of bacteria</td>
<td>143</td>
</tr>
<tr>
<td>3.3.11 Multiple sequence alignment and phylogenetic tree construction</td>
<td>145</td>
</tr>
<tr>
<td>3.3.12 Production of PHA – Overall view</td>
<td>145</td>
</tr>
<tr>
<td>3.4 RESULTS AND DISCUSSIONS</td>
<td>147</td>
</tr>
<tr>
<td>3.4.1 Screening of potential PHA producer</td>
<td>147</td>
</tr>
<tr>
<td>3.4.2 Screening of PHA granules using Nile Blue A staining method and TEM</td>
<td>148</td>
</tr>
<tr>
<td>3.4.3 <em>Enterobacter sp</em>– DNA isolation and Gel picture</td>
<td>153</td>
</tr>
<tr>
<td>3.4.4 Observation of PHA granules</td>
<td>153</td>
</tr>
<tr>
<td>3.4.5 Determination of biomass</td>
<td>154</td>
</tr>
<tr>
<td>3.4.6 PHA extraction and quantification</td>
<td>154</td>
</tr>
</tbody>
</table>
3.4.7 Identification analysis of PHA samples using confocal microscope and Gas Chromatography–Mass Spectrometry (GC-MS) 155

3.5 CONCLUSION 160
Introduction
3.1 INTRODUCTION

Plastic materials which have made entry in every sphere of human life are now causing serious environmental problems due to their non biodegradability. The intrinsic qualities of durability and resistance to degradation, over the last two decades, have been increasingly regarded as a source of environmental and waste management problem emanating from plastic materials (Porier et al., 1995). One option to solve this problem is to produce truly biodegradable polymers, which may be used in the same applications as the existing synthetic polymers. These materials, however, must be processible, impervious to water and retain their integrity during normal use but readily degradable in a biologically rich environment.

Polymers are the backbone of our life. Both material and the biological matter are standing on the strong bonds that these amazing macromolecules hold together. Various polymers are used as food, clothing, shelter and fuel. They are the building blocks of all living systems i.e. plants, animals and microorganisms. The biological world is made up of a number of polymers such as proteins, polysaccharides, polynucleotides, polyamides and polyphenols. Some of the earliest polymers known to man were silk, wool, cotton, cellulose, gums and starch. All these polymers have contributed towards human civilization. The advent of plastics or man made polymers started a new era in the polymer world (Ojumu et al., 2004).

The changes from the stone age to bronze age to iron age took several thousands of years. But plastic age developed rapidly after the steel age. These highly versatile polymers have gradually replaced the other polymers in a myriad number of applications. They have influenced our economy, standard of living and health conditions, transportation, pharmaceuticals, building and construction and power sector. Their influence on the economic sector is unparalleled. They make or break economies and have transformed the quality of our society. Infact they have permeated every facet of human life (Shivaram and Singh, 2003).

3.1.1 CLASSIFICATION OF POLYMERS

a. Natural polymers or Biopolymers

Natural polymers are referred to those polymers that are formed in nature during the growth cycles of living organisms. They are also called as biopolymers. Based on the origin biopolymers may be further classified into plant, animal and microbial-based (Fig 3.1.1).
Apart from this, polymers are generally classified into seven classes (Table 3.1.1). They are nucleic acids, proteins (polyaminoacids), polysaccharides, polyhydroxyalkanoates, polyphosphates, polyisoprenoids and polyphenols. An eighth type, polythioester has been recently included (Eversloh et al., 2001).

![Classification of polymers](image)

**Figure 3.1.1 Classification of polymers**

### b. Synthetic or Man-made plastics

These polymers are made from hydrocarbons derived from petroleum. Depending on its ultimate form and use these synthetic polymers are classified as follows (Gowariker et al., 1996):

**i. Plastics:**

Polymers, which can be molded or formed into any state of our choice.

**ii. Elastomers:**

Elastomers are popularly called as rubbers.

**iii. Fibres or liquid resins:**

Some of the well-known synthetic fibres are nylon, polyesters, polypropylene and acrylics.
Figure 3.1.2: Classification of biopolymers based on their origin.

TABLE 3.1.1: Classes of biopolymers and their occurrence.

<table>
<thead>
<tr>
<th>Class</th>
<th>Synthesis in</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prokaryotes</td>
<td>Eukaryotes</td>
</tr>
<tr>
<td>Nucleic acids</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Proteins; Polyaminoacids</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Polysaccharides</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Polyhydroxyalkanoates</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Polyphosphate</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Polyisoprenoids</td>
<td>No</td>
<td>Plants, some fungi</td>
</tr>
<tr>
<td>Lignin</td>
<td>No</td>
<td>Only plants</td>
</tr>
</tbody>
</table>

Source: Modified from Eversloh et al., 2001

Synthetic / manmade polymers are further divided into two types,

a. Water-soluble
b. Water-insoluble

Water-soluble polymers are specialty polymers with functional groups that affect water solubility such as carboxyl, hydroxyl and amido group. Water-insoluble polymers are
referred to as commodity plastics. They include polymers such as polyethylene, polypropylene, polystyrene, poly vinyl chloride (PVC), poly ethyleneterephthalate (PET) and Nylon 6,6. Some synthetic polymers are biodegradable but most commodity plastics are non-biodegradable (Kawai, 1995).

3.1.2 Growth of Plastics and its implications on the Society

Plastics are made from oil, natural gas, coal and salt. The polymer / plastic growth worldwide has been steady around 6 per cent per annum which is much higher than the GDP (gross domestic product) growth rate of 3.3 per cent. The annual world production of polymer materials was around 150 million tons in 1996 with the average per capita consumption of plastics in developed countries ranging from 80-100 kg per year. The demand for polymers was poised to increase from 2.6 million metric tons per annum (MMTPA) to 4.2 MMTPA by the year 2002 and is expected to cross 7.7 MMTPA by the year 2007.

The plastic consumption in China was estimated to be 16 million tons in 2000, fifth in the world after USA, Japan, Germany and South Korea. In USA, the annual per capita consumption of plastics is 90 kg and in the European union it is 60 kg and the world average is 15 kg (Market assessment).

In India, the consumption of plastics has been trebling every decade. The present consumption has grown up to 4 million kilotons and is likely to go up to 12 million kilotons by 2010. Indian per capita consumption was only 1.9 kg, the lowest in the world but was projected to reach 3.2 kg by 2002 (Market assessment).

However demand for plastics reached 4.3 MT in the year 2001-2002 and is expected to reach 8 million tons by 2006-07 (Biodegradable plastics). Major end uses of plastics in India are for agricultural and water management 25%, construction 25%, electronics 17%, packaging 15%, transportation 15%, and others 3%. There are 8 major polymer producers in India. Indian petrochemical corporation Ltd. (IPCL), Haldia petrochemicals Ltd. (HPL), Reliance industries Ltd. (RIL), Gas authority of India Ltd. GAIL, Natural Organic Chemicals Industry Ltd. NOCIL), Polyolefins Ltd., (PIL), Southern petrochemicals and Industrial Corporation (SPIC) and ICI/ Oswal industries are the major ones. Among the various polymers produced, LDPE is 1, 80,000 MTPA, linear LDPE is 9,70,000 MTPA, HDPE is 3,60,000 MTPA and PP and PS is 9,00,000 MTPA (Market assessment). The present market in India is 25,000 crore (Biodegradable plastics).
The fear of depletion of wood resources has established plastics as the material of choice in many applications. In food sector, various characteristics of plastics such as low density, ready sealability, resistant to break, appearance, impermeability to oxygen and water vapour, low temperature and flexibility have contributed for its large usage (Levy, 1993). Nearly one fifth of (about 3.5 - 4 million tons) the total plastic consumption worldwide is used as packaging, of which half is foamed plastics such as expanded polystyrene (Ren, 2003). Plastic shopping bags are the most ubiquitous consumer item on earth. Their light weight, low cost and water vapour resistance make them very convenient for carrying groceries, clothing or any other routine purchase that it is hard to imagine life without them. The first plastic bag was introduced in USA in 1957 for sandwiches, fruits and vegetables. Plastic trash bags appeared around the world around 1960 and they took off by 1970 as HDPE (Halweil, 2004).

3.1.3 “White Pollution”- Hazards of Plastics

Unfortunately, these highly durable, versatile and extremely useful materials cause adverse effects on the environment after its use. Plastics are not biodegradable. They accumulate in the environment and harm the ecosystem.

The United States of America discards approximately 25 million tons of plastic household waste every year and most of it is landfilled where it remains undegraded for years. In China, the annual plastic waste generation is 4 million tons with only 10% recovered or recycled and about 20-30% is incinerated or landfilled. (Hankermeyer and Tjeerdema, 1999). In Europe and Japan there are only a few sites left that can be landfilled (Risch, 1991).

Plastics lead to adverse affects on environment during and after use. These polymers which are synthesized chemically by polymerization contribute towards air pollution and waste management problems (Porier et al., 1995). Plastics threaten animal life. Some well-known cases that are reported are the death of cows due to choking after consumption of plastics. Millions of marine animals are also killed every year due to toxicity caused by plastic debris or entanglement with them. The plastic packages are indiscriminately thrown away from houses, trains and shops. Some drain into drainage and block them. If plastics are incinerated they produce poisonous gases like carbon monoxide, chlorine etc. The burning of PVC results in production of hydrogen chloride, a corrosive pollutant. Americans throw away 100 billion plastic grocery bags each year.
These are becoming more and more common in poorer nations as well. Bags produced in Asia account for one quarter of those used in wealthy nations (Halweil, 2004). Accumulated plastic film residues in the soil have caused significant decrease in agricultural yield. Plastic wastes floating on rivers and lakes are increasingly threatening fishery, navigation, and operation of hydropower plants, irrigation and other public works. Moreover, as over 99% of plastics are fossil fuel origin, their rapid increase will put further pressure on the already limited non-renewable resources on earth (Ren, 2003).

Because plastics account for 20% of volume of solid municipal waste, there is increasing motivation for society to find alternate disposal methods for them. Overall there is no concerted effort done for plastic management.

### 3.1.4 Plastic Waste Management

Domestic organic wastes including plastics are usually disposed off in landfills. However, burying of waste is no longer considered an ecologically acceptable method. Scott (2000) has suggested an alternative option for waste management. The method indicates the need for mechanical recycling, energy recovery and biological recycling of wastes.

The United Nations Environmental program (UNEP) defines waste management as taking all practical steps to ensure that wastes are managed in a manner which will protect human health and the environment against the adverse effects. According to Ren (2003), the major principles considered in such waste management are:

![Waste Management Strategy](image)

**Figure 3.1.3 Waste Management Strategy**
3.1.5 ‘Reduce, Reuse, Recycle and Recovery of energy’

In Europe as well as worldwide, legal actions support environmentally sound waste management systems. European parliament and council of directive 94/62/EC of 20 December 1994 on packaging and packaging waste, states clearly that the prevention of waste should be ‘a first priority’, while reuse, recycling and other forms of recovery are ‘additional fundamental principles’ for the reduction of final disposal of such wastes. This packaging directive also highlights the key role played by public participation for the success of waste management (Ren, 2003). The concept of taxing plastic bags as means of curbing plastic waste is starting to become popular worldwide. The introduction of consumption tax of Euro 0.15 per bag on plastic bags by Irish government in 2002, led to a reduction of 90% in the first month of operation (Klemz, 2002). However the enthusiasm on tax in UK has waned (Bennet, 2003). Taiwan is the largest Asian country to drive restrictions and even ban on plastic disposables with environmental protection and administration banning disposable tableware, including polystyrene foam containers and plastic bags (Moore, 2003).

In China, the law of solid waste pollution prevention and control defines the responsibility of producers, sellers and users in recovery and recycling. It says that producers and users should choose easily recyclable, disposable and environmentally safe materials as packaging (Ren, 2003). There are reports on the ban of plastic bags in Bangladesh as well as in Bhutan (Moore, 2003). Government of India (the Ministry of environment and forests) has issued a notification dated 2nd September 1999 under the title ‘Recycled plastics manufacture and usage’, prohibiting the use of recycled plastic carry bags for carrying, storage and packing of ready to eat food items. Some cities in India have seen limited success at banning plastic bags. According to Stevens, ”In ignoring nature’s way of building strong materials we have for many applications over engineered our plastics for stability with little consideration of their recyclability or ultimate fate and ended up transforming irreplaceable resources to mountain of wastes” (Stevens, Green plastics).

In conclusion, the lack of biodegradability, growing water and land, surface litter problem, air and pollution of incinerated plastics has raised worldwide concern on use of plastics. Amidst the proplastic arguments, there still is a strong need for the look out for alternatives for synthetic plastics and to replace them steadily. All this has promoted worldwide research to develop new biodegradable alternatives to plastics.
3.1.6 Biodegradable Alternatives

Biodegradable polymers or bioplastics are important and interesting areas that are being looked out as alternatives for synthetic plastics. These are a new generation of materials able to significantly reduce the environmental impact in terms of energy consumption and green house effect. ISO 472 -1988, a standard authority defines a biodegradable plastic as, ‘a plastic designed to undergo a significant change in its chemical structure under specific environmental conditions resulting in a loss of some properties that may vary as measured by standard test methods appropriate to the plastics and application in a period of time that determines its classification’. The change in chemical structure results from the action of naturally occurring microorganisms.

Biodegradation is a process by which bacteria, fungi, yeasts and their enzymes consume a substance as food source so that its original form disappears (Chandra and Rustgi, 1998).

Biodegradable polymers are classified into two broad groups. They may be of natural origin or synthetic or man made. Further biopolymers are classified based on their origin and method of production as follows:

3.1.7 Plant Based Polymers

i. Starch

Starch is an inexpensive agricultural resource and is industrially produced in large quantities (7 million tons per year in Europe). Nearly 50% of starch is used for non-food applications and about 30% of the starch production are industrially used for making films. Starch, a polysaccharide has a mixture of linear and branched polysaccharide with the latter predominating. It is a repeating unit of mixture of two polymers, amylose and amylopectin. Amylose is a predominantly linear (to highly branched) polymer, comprised of (1- 4) α -D-linkages with number average molecular weight in the range of several thousands. Amylopectin is highly branched with intermittent (1–6) linkages. Its molecular weight is of the order of several millions and can be as high as 50 millions. Starch is a renewable degradable carbohydrate polymer that can be purified from various sources by environmentally sound processes. It is found in high amounts in plants like corn (maize) potato and wheat. Three generations of starch as bioplastics have come through history:
1. Starch as only a filling material along with synthetic plastics. Here the biodegradable part of plastic was only 5-20%.

2. Hydrophilic synthetic polymer base filled with 50-80% starch.

3. Starch as plastics with 100% starch.

Starch itself has a severe limitation. It is water-soluble, hence will swell and deform upon exposure to moisture. Therefore blending with hydrophobic synthetic polymers chemically modifies them. Starch only plastics are highly suitable in cushioning electrical goods where the packages are kept dry. Thermoplastic starch (TPS®) has been developed by BIOTEC®, Germany. TPS® derivatives, starch esters, bioplastic granules (BIOPLAST) and films (BIOFLEX) are being produced (Lorcks, 1998).

**ii. Cellulose derivatives**

Cellulose is a structural polysaccharide and is the constituent of higher plants. It is the most abundant natural polymer on earth and is a linear polymer of anhydroglucose. It is highly crystalline, fibrous and insoluble in water in its native form (Petersen *et al.*, 1999). It can either be used in its original form or modified into cellulose acetate, cellulose butyrate or cellulose nitrate.

Cellophane a regenerated form of cellulose was the first transparent, flexible packaging film that was developed. Since the introduction of synthetic thermoplastic packaging films in 1950’s cellophane sales have dropped by 90% inspite of having good tensile strength, elongation, excellent printability and good machinability (Petersen *et al.*, 1999). Moreover for film production cellulose is dissolved in an aggressive toxic mixture of sodium hydroxide and carbon disulfide (Xanthation) and then recast into sulphuric acid to produce a cellophane film. These films are sensitive to moisture, not heat sealable, manufactured at the cost of trees and are highly inflammable. In order to improve its moisture barrier properties blends such as cellulose acetate films, cellulose ether and ethyl cellulose films are manufactured. These have better moisture, oil, gas and grease resistant properties. But modification of cellulose films is costly and difficult. Also gas and moisture barrier properties of cellulose acetate are not optimal with respect to food packaging (Petersen *et al.*, 1999).

Apart from these, corn zein, wheat gluten, soy protein etc., have been exploited as packaging materials. The barrier, vitamin adhesion and anti microbial carrier properties of
zein film coatings have been used on a variety of foods. Zein is also used on pharmaceuticals and for production of water-soluble pouches for dried foods. Wheat gluten and soy protein is used in sausage casings.

3.1.8 Animal Based

i. Chitin and Chitosan

Chitin is the main structural component of shells of crustaceans, molluscs, and insects. It also forms parts of jaws and body spines of certain worms, and is found in cell walls of fungi and in some algae. It is almost as common as cellulose and this ubiquity has made it useful in some potential uses. Chitosan is a derivative of chitin that is deacetylated. Chitin is a poly-β-(1-4) linked N-acetyl-D-glucosamine widely found in nature. It is produced commercially by deacetylating chitin obtained from shellfish waste. Chitosan films are clear, tough, flexible and have good oxygen barrier properties. They can be obtained by casting from aqueous solution. Chitosan-based coatings can protect foods from fungal decay and modify the atmospheres of fresh fruits.

ii. Collagen

Collagen is a fibrous, structural protein in animal tissue that can be converted into edible and biodegradable films. These are not very strong and tough, but have reasonably good mechanical properties. They do not have good moisture barrier properties. These are commercially successful as edible films. Gelatin, casein and whey proteins are also used as biodegradable packaging materials.

3.1.9 Microbial Based

i. Microbial extracellular Polysaccharides

Polysaccharides are condensation polymers of monosaccharides resulting in the formation of glycosidic linkages by elimination of water. Microorganisms produce several polysaccharides. The first microbial polysaccharide to be commercialized was dextran. Dextran is a α-1, 6-glucopyranoside polymer.

Bacteria such as *Alcaligenes faecalis* var. *Myxogenes*, *Azotobacter vinelandii* and *Beijerinkia indica* produce curdlan, bioalgin, and PS-7 respectively.

The only microbial polysaccharide that has reached the maximum commercialization is xanthan gum; an exopolysaccharide produced from bacterium *Xanthomonas campestris*. 
Xanthan has a cellulose type main chain and trisaccharide graft chains containing glucuronic acid.

Pullulan is a microbially produced polysaccharide; this water-soluble extract is a neutral glucan and is synthesized by a fungus *Aureobasidium pullulans*. Pullulan is composed of 1,6-linked maltotriose units. It is a α-D-glucan in which maltotriose or maltotetrose units (linked) are coupled through α 1,6 bonds to form a polymer whose molecular weight is dependent on the cultural conditions. Although resistant to amylases these can be degraded by pullulanase from *Enterobacter aerogenes* strains. The main use is because of its capacity in film formation. Films formed by 5-10% solutions have low permeability to oxygen especially when compared to other commercially available materials such as cellophane or polypropylene. Plasticizers are necessary for further processing.

All the above are being used as biodegradable alternatives to synthetic plastics in various applications but subject to their limitation of water solubility.

**ii. Polylactic acid (PLA)**

Polylactic acid is derived from lactic acid for which carbohydrates in sugar beets, potatoes, wheat, maize and milk are the source. Polylactic acid is a substance familiar to the human body, as we ourselves produce it by every muscle contraction. The body can break it down. PLA can be processed through the injection moulding, foil blowing and deep drawing. PLA may be applied as a coating. PLA is water-resistant but cannot withstand high temperatures (>55°C). In comparison to starch biopolymer the degradation process is very slow. However, within a composting facility it can be broken down in 3 to 4 weeks. PLA is commercially available as LACEA (polylactic acid) (Hiraishi and Khan, 2003).

**iii. Polyhydroxyalkanoates (PHA)**

PHAs are a group of microbial polyesters formed intracellularly in a number of bacteria. These polymers have properties similar to synthetic plastics and have gained much attention and importance as future biodegradable plastics (Lee, 1996). Many of these are also commercially available as BIOPOL (PHBV) and BIOGREEN (PHB) (Hiraishi and Khan, 2003).
iv. Other Polymers

Apart from these polyesters, the only other polyester existing in living organism are polymalic acid which is water soluble polyester occurring in lower eukaryotes. Cutin and suberin are water insoluble polyesters occurring in plants (Kolattukudy, 2001).

Other important biopolymers known are alginates, carrageenan, pectin, lipid films and protein films. Among these microbial polymers are gaining much importance as bioplastics.

3.1.10 Challenges for Bioplastics

Use of these biodegradable alternatives brings additional challenges, which needs to be addressed with at most importance. Biodegradability is not a favourable property in many of the applications. Development of a biodegradable plastic is challenging because it has to perform better than synthetic plastics in all aspects and also be biodegradable. The issues of energy consumption air and water pollution and production of waste during the entire lifecycle of the product is questioned. Apart from this the economical aspect of the process from raw material to end products and the land utilized for the process is seriously addressed (Scott, 2000). Hence to achieve the benefits of biodegradable plastics there is a need to address the potential areas where it can be implemented. The applications of biodegradable polymers have focussed on three major areas (Chandra and Rustgi, 1998):

Figure 3.1.4 Applications of bioplastics

Biodegradable plastics have been developed as surgical implants and as drug delivery agents for controlled and long-term release of drugs to targeted sites. These biomaterials are also being used as absorbable surgical sutures, for use in the eye, bone fixation devices, vascular grafts, and artificial skin. Biopolymers are also used in controlled release of pesticides, nutrients, agricultural mulches and planting containers. Most of the biopolymers are developed into packaging materials especially as food packaging materials (Chandra and Rustgi, 1998). Biodegradable plastic packaging is slowly being adopted by food service
companies for use as films for sandwich wraps or for packaging fresh products such as salads, pasta or bakery goods (Pierce, 2004).

Bioplastics are being considered not necessarily for biodegradable aspects but also because of their performance. Environmental friendliness is considered as an additional benefit and hence there is a lot of investment in the area. An interaction between research and implementation is necessary to make a significant impact on the food-packaging sector. Cargill Dow LLC, which manufactures polylactic acid (PLA) is producing more than 300 million pounds and economically competing with the costs of polyethylene terephthalate (PET). Wilkinson Manufacturing and Wild oaks Markets Inc are manufacturing thermoformed food containers and plastic containers (Pierce, 2004). Companies that are currently involved in the production of microbial polymers are listed in Table 3.1.2.

A fully biodegradable polymer is defined as a polymer that is completely converted by living organisms, usually microorganisms, to carbon dioxide, water and humic material. Biodegradable materials under development include polylactides, polyglycolic acids, polyhydroxyalkanoates (PHAs), aliphatic polyesters, polysaccharides and their co-polymers and/or blends (Steinbuchel, 1991). Amongst these, PHAs are of particular interest because they possess thermoplastic characteristics and resemble synthetic polymers to a larger extent.

Table 3.1.2 List of companies involved in producing biodegradable packaging

<table>
<thead>
<tr>
<th>Material</th>
<th>Trade Name of the product</th>
<th>Raw materials</th>
<th>Manufacturing company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomer</td>
<td>Polyhydroxybutyrates (PHBs)</td>
<td>Biomer Germany</td>
<td></td>
</tr>
<tr>
<td>Biogreen</td>
<td>Polyhydroxybutyrate (PHB)</td>
<td>Mitsubishi Gas Chemical Japan</td>
<td></td>
</tr>
<tr>
<td>Biopol</td>
<td>PHB/ (PHV) Polyhydroxyvalerate</td>
<td>Metabolix, Cambridge Massachusetts USA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Curdlan</td>
<td>Takeda Chemical Industries</td>
<td></td>
</tr>
<tr>
<td>Nodax</td>
<td>Aliphatic polyesters principally PHA</td>
<td>Proctor &amp; Gamble USA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pullulan</td>
<td>Hyashibara co USA</td>
<td></td>
</tr>
</tbody>
</table>

(Compiled from: Materials list, Friendly packaging)
It is well understood that synthetic plastics are highly resistant to microbial
degradation in landfills. This has become a problem for municipalities worldwide because
municipal landfills lose capacity because of the accumulation of synthetic plastics. PHAs are an
intracellular storage material synthesized by a variety of bacteria, and are materials of interest because
they have some properties similar to synthetic plastics. Plastics produced from PHAs have been
reported to be truly, fully biodegradable (Page, 1992). In addition, the degradation product of PHA is a
common intermediate compound in all higher organisms. Therefore, it is plausible that it is
biocompatible to animal tissues and PHA may be used in surgical applications without any toxicity.

Different genera of prokaryotic microorganisms (Eubacteria and Archea) synthesize
polyhydroxyalkanoates (PHAs) as water insoluble intracellular particles (Zinn et al., 2001 and
Anderson and Dawes, 1990). Production of polyester particles is induced when carbon source is
in excess quantity or growth conditions have been imbalanced by declining nitrogen source or
other factors (Reddy et al., 2003 and Rehm, 2003). Once the limiting nutrients are available,
these compounds are degraded by intracellular enzymes and carbon and energy supply (Tanio
et al., 1982 and Shirakura et al., 1986). Polyhydroxyalkanoates (PHA) polymers are
biodegradable when exposed to environmental conditions and thereby have many applications in
agriculture, food industry, medicine and pharmacy (Zhao et al., 2003 and Parlane et al., 2009).
The culture conditions have main effects to induce PHA production by bacteria. Moreover,
different genera or different subpopulations of single genus do not have the same physiological
response when exposed to the same culture conditions (Muller and Babel, 2003).

Polyhydroxyalkanoates (PHAs) are a class of bacterial storage compounds that have
received considerable attention in recent years because of their potential use as biodegradable
thermoplastics (Byrom, 1987). Within this family, a large amount of research has been
conducted on the homopolymer poly-(3- hydroxybutyrate) (PHB) and the copolymer poly-(3-
hydroxybutyrate- co-3-hydroxyvalerate) (PHB-co-V) (Steinbuchel and Schlegel, 1991). The
former polymer was discovered in 1926 and has been the prototypical PHA, while the latter
was more recently discovered, in 1983 (Findlay and White, 1983), and has attracted interest
because of its enhanced flexibility over PHB. This polymer is primarily a product of carbon
assimilation (from glucose or starch) and is employed by microorganisms as a form of energy
storage material accumulated intracellularly to be metabolized when other common energy
sources are not available.

105
PHAs are linear polyesters produced in nature by bacterial fermentation of sugar or lipids. More than 100 different monomers can be combined within this family to give materials with extremely different properties. They can be either thermoplastic or elastomeric materials, with melting-points ranging from 40 to 180°C.

The most common type of PHAs is PHB (poly-beta-hydroxybutyrate). PHB has properties similar to those of (polypropylene) PP, however it is stiffer and more brittle. A PHB copolymer called PHBV (polyhydroxybutyrate-valerate) is less stiff and tougher, and it is used as packaging material.

It is well known that production and storage of PHAs are integral and essential parts of the excess biological phosphorus removal (EBPR) mechanisms. It is less well known by the wastewater treatment community that PHA production by bacteria also is used for the manufacture of biodegradable plastics. Plastics production is typically accomplished using pure cultures with the goal of maximizing the amount of stored PHAs at the end of a nutrient limited or oxygen limited phase.

3.1.11 Potential benefits of the biopolymer to society and the environment

i. Environmental impacts

- Recycling plastic as an alternative to environmental pollution and landfill, usually requires more energy compared to creating new plastic. A biopolymer with the ability to biodegrade, such as PHB, removes the need to consider the less energy efficient recycling method.
- Burning waste plastic to harness energy is an option towards the solution to the landfill issue, but this releases toxic gases and increases carbon dioxide concentration in the atmosphere. Conversely, biological polymers form part of a natural cycle whereby carbon dioxide and water are used during photosynthesis and released during natural decomposition.
- The ability to synthesise PHB from a wide variety of carbon rich sources means that a secondary use or market can be found for some waste products. Using substrates such as industrial food waste and molasses from sugar processing reduces the need for the treatment and disposal of such wastes.
• The complete changeover to PHB from normal petroleum derived plastics would reduce landfill volumes by approximately 20%, given this is the percentage composition of plastics in our rubbish. This would reduce overall volumes of pollution.

ii. Societal impacts

• PHB and other biopolymers have revolutionised the medical industry. PHB is biocompatible with human blood and tissues, and readily reabsorbs into the body objects such as implants and threading. The biopolymer can also be used as a material for slow releasing drugs. Improvements in this field are inevitable.
• Petrol derived plastics can be carcinogenic. Examples include those containing benzene and vinyl chloride. PHB is a safer material for use in containers and drink bottles where this is an inconclusive concern.
• Reducing the volume of landfill by 20% has the social benefit of increasing overall domestic, commercial and industrial land use. It also reduces the public ‘eyesore' the landfill creates.
• Production of PHB using food substrates can have negative societal impacts. A higher demand for substrates which form the staple diet of developing countries may reduce the ability of these countries to purchase this food. Such a consequence would worsen the food shortages of these developing countries.

3.1.12 Disposal

It biodegrades in microbially active environments in 5-6 weeks. The action of some enzymes produced by microbes solubilizes PHB which is then absorbed through the cell wall and metabolised. PHB is normally broken down to carbon dioxide and water when degraded in aerobic conditions. In absence of oxygen the degradation is faster, and methane is also produced. PHB is not degraded in biologically inactive systems such as sanitary landfills.
Figure 3.1.5  Comparison of biotic and abiotic mechanism of degradation of polymeric material (Gu, 2003)

The focus of this work is to successfully screen the bacteria that would produce PHAs from the biofilm obtained from Rotating Biological Contactors (RBCs) used to treat wash water from the distillery unit. The bacterial strains ultimately selected, *Enterobacter aerogenes, E. cloacae* and *Escherichia coli* were used to produce PHAs (as explained later).
Review of Literature
3.2 REVIEW OF LITERATURE

Environmental microbiology is the study of microorganisms which exist in natural or/and artificial environment. It is a fascinating field of science and the origin of this field rests in the observations of Antony van Leeuwenhoek. During the last few decades we have begun learning how to harness microbial biosynthetic and degradative activities. This harnessing, including the intentional manipulation of microbial activities, constitutes the basis of microbial biotechnology, whereby we direct the activity of microorganisms within both natural and artificial environments for varieties of purposes. As an example, we utilize microorganisms as tools to degrade both natural and anthropogenic materials in wastewaters digesters, composters, landfills, natural terrestrial environments and natural or artificial aquatic ecosystem.

There is a well known saying that “Everything touched by King Midas turned to gold”. By a sort of inversion process, pretty well everything modern men touch, including themselves, turns to a waste product sooner or later. Wastes are usually discarded into water, with or without processing. Presently, water is becoming a rare commodity, and the available water sources are inadequate to meet the essential basic needs of man, which is mainly due to increased industrialization of developing countries. Improper disposal methods and inadequate control of toxic effluents from different industries have led to the widespread contamination of surface as well as ground water and have made the water resources polluted for usage (Odum, 1969).

3.2.1 General Properties of Polymers

In Greek ‘Poly’ means ‘many’ and ‘mer’ means part. Polymers are a class of giant molecules (macromolecules) that are composed of smaller units. Simple units are referred to as monomers and more complicated building blocks are referred to as ‘repeating units’. The process by which the monomers are assembled into polymers, either chemically or biologically is referred to as polymerization. When only one type of monomer is present, the polymer is referred to as a homopolymer. A copolymer is formed when two or more different monomers are linked together. Polymers can be linear or branched. They may be further classified as crystalline or amorphous. The following properties influence the physical property of a polymer:
1. Monomer composition
2. Type of linkage.
3. Size or molecular weight.

The most important characteristic of a polymer molecule is their chain length, since that determines whether they have any usefulness at all. In general, characteristics such as melting temperature and glass transition are strongly dependent on the chain length up to degrees of polymerization of approximately 100. For example the viscosity of molten polymer varies as degree of polymerization, owing to entanglements between neighbouring chains in the melt. Copolymerization is used to modify properties such as crystallinity, solvent resistance, permeability and toughness, which generally vary with copolymer composition (Odum, 1969). Some of the important properties and characteristics of polymers that should be understood are:

3.2.2 Melting point

Polymers exist only as solid or liquid and never as gas as they decompose before reaching their boiling point. The temperature at which there is a change in the polymer state is called the melting point.

3.2.3 Glass transition temperature

The glass transition temperature is an important parameter of a polymeric material. It is used as a measure for evaluating the flexibility of a polymer molecule and the type of response that would exhibit to mechanical stress. The temperature below which a polymer is hard and above which, it is soft, is called glass transition temperature.

3.2.4 Crystallinity

Crystallinity is expressed in terms of that fraction of the sample, which is crystalline. The density of the crystalline component is higher than the amorphous component. Other properties such as density, modulus, hardness, permeability and heat capacity will be affected by crystallinity.

3.2.5 Tensile strength

Tensile strength is a measure to withstand forces that tend to pull it apart to determine the extent to which the material stretches before breaking.
3.2.6 Biodegradability

Biodegradability is the ability of the polymer to be utilized as a nutrient source by microorganisms and converted into carbon dioxide, biomass, and water.

3.2.7 Polyhydroxyalkanoates (PHA)

The first PHA to be discovered was the short chain length PHA, poly-(3-hydroxybutyrate), P(3HB), in *B. megaterium* by a French scientist Francois Lemoigne in 1926 (Lemoigne, 1926) and is one of the most well studied PHA. Mcl-PHA was only discovered in 1983 when *P. oleovorans* was grown in octane (Dekoning, 1995). Since the first discovery of PHA, more than 90 different genera of Archae and Eubacteria has been reported to accumulate PHAs (Zinn *et al.*, 2001). The molecular structure of the PHA produced is directly dependent on the organism used, culture conditions for the organism’s growth and the carbon feed.

Polyhydroxyalkanoates (PHAs) represent a complex class of storage polyesters that are synthesized and deposited as insoluble cytoplasmic inclusions by a wide range of Gram-positive and Gram-negative bacteria, and by some Archaea (Renner *et al.*, 1996). It was reported that these PHAs are accumulated as discrete granules and are believed to play a role as sink for carbon and reducing equivalents in microbes (Madison and Huisman, 1999). PHAs are accumulated by bacteria under unbalanced growth conditions, especially when the carbon substrate is in excess of other nutrients such as nitrogen, sulfur, phosphorus or oxygen (Madison and Huisman, 1999; Kim and Lenz, 2001; Reddy *et al.*, 2003). They are polyesters of various hydroxyalkanoate monomers. Among the completely biodegradable plastics, PHAs have been drawing much attention because of their similar material properties to conventional plastics and complete biodegradability (Lee and Chang, 1995).

Since the discovery of PHB in 1926 as storage inclusions in *Bacillus megaterium* (Lemoigne, 1926), over 300 different bacteria, including Gram-negative and Gram-positive species, have been reported to accumulate various PHAs (Steinbüchel, 1991; Steinbüchel and Pieper, 1992; Steinbüchel and Valentin, 1995, Braunegg *et al.*, 1998; Madison and Huisman, 1999, Zinn *et al.*, 2001 and Berlanga *et al.*, 2006). Other than PHB, more than 140 different PHA constituents are known (Steinbüchel, 1991).

Polyhydroxyalkanoates (PHAs) are the polymers of hydroxyalkanoates that accumulate as carbon/energy or reducing-power storage material in various microorganisms (Satoh, 1998). PHAs are stored in the bacterial cytoplasm as inclusion bodies (Lee, 1995) and
they are synthesized and accumulated intracellularly as distinct granules, usually under unfavorable growth conditions, such as feast and famine regime, limitation of nitrogen, phosphorus, sulphur, magnesium or oxygen in the presence of excess carbon source (Poirier, 1995).

Basically, PHAs can be broadly subdivided into three groups based on the number of carbon atoms present in its monomer units (Steinbuchel, 2001):

(a) Short-chain-length PHAs consisting of 3-5 carbon atoms (PHA SCL).
(b) Medium-chain-length PHAs consisting of 6-14 carbon atoms (PHA MCL).
(c) Long-chain-length PHAs consisting of more than 14 carbon atoms (PHA LCL).

### 3.2.8 Polyhydroxyalkanoates in Nature

PHAs are accumulated in the cells as discrete granules, the size and number per cell vary depending on the different species. The granules appear as highly refractive inclusions under electron microscopic observation. In *Alcaligenes eutrophus*, 8 to 13 granules were observed per cell, with diameter ranging from 0.2 to 0.5μm (Byrom, 1994). As PHAs are insoluble in water, the polymers are accumulated in intracellular granules inside the cells and the polymerization of these soluble intermediates into insoluble molecules prevents the leakage of valuable compound out of bacterial cell (Preiss, 1984). Phospholipids and proteins form a layer over the surface of a PHA granule and in the interface of a granule, the most dominant compound seen is Phasin, a class of proteins known to influence the number and size of PHA granules.

Bacteria have developed mechanisms that allow them to survive nutrient starvation and to tolerate exposure to multiple stress agents prevalent in natural environments. Accumulating PHAs is a natural way for bacteria to store carbon and energy, when nutrient supplies are imbalanced. The stored PHA can be degraded by intracellular depolymerases and metabolized as carbon and energy source as soon as the supply of the limiting nutrient is restored (Byrom, 1994).

The marine environment provides a virtually untapped resource for novel bacteria and possibly polymers. Occurrence of polyhydroxyalkanoates (PHA) accumulating microbes have been reported from various environments including mangroves (Doan et al., 2012), marine sediments (Findlay and White, 1983, Odham et al., 1986, Wang and Bakken, 1998, Rawte and Mavinkurve, 2001, Choi et al., 2004 and Arun et al., 2009) antartic areas (Ayub et al., 2004), soil (Chan et al., 2006; Sabat et al., 1998), estuarine detritus (Herron et al., 1978), sewage
sludges (Reddy et al., 2009), ponds (Yellore and Desai, 1998), palm-oil mill effluent pond (Alias and Tan, 2005) and integrated-farming pond (Redzwan et al., 1997).

Although several bacteria are reportedly known to synthesize and accumulate PHA, a few groups like the methanogenic bacteria and lactic acid bacteria are unable to synthesise these polymers. In most bacteria, PHAs are deposited as prokaryotic inclusion in the cell and these inclusions are only one among many other inclusions. In some bacteria like *E.coli*, the PHA were found as complexes of PHB-Ca2+-polyphosphate molecules and this complex aids in transport Ca2+ ions out of the cell (Madison and Huisman, 1999).

The PHAs are ubiquitous in nature as they are also found in several eukaryotic cells including plant and animal tissues (Reusch, 1989). In human plasma, they can be found associated with lipoproteins and serum albumin (Madison and Huisman, 1999). The lipid molecules and albumin are thought to be acting as transporters of PHB, the smallest known PHA, through the blood, with albumin being the major carrier. Madison and Huisman (1999) point out the possible roles of PHB in large eukaryotic organisms, as it is highly insoluble in aqueous solutions.

Lee and Choi (1999), in their studies on biological phosphate removal process, considered PHA production from waste product as a coupled process for reducing the amount of organic waste. In biological phosphorus removal process, bacteria accumulating polyphosphate, uptakes carbon substrates and accumulate these as PHA under anaerobic condition. Under aerobic condition this accumulated PHA is utilized for energy generation.

### 3.2.9 Chemistry of the PHAs

PHAs are a family of optically active biological polyesters, containing (R)-3-hydroxyalkanoic acids (HA) monomer units. The 3-HA are all in the R configuration due to the stereospecificity of the polymerizing enzyme, PHA synthase.

Of all the biodegradable plastics being studied, those that have generated the most interest are the poly(3-hydroxyalkanoates) or PHAs which are made by bacteria. Like all plastics, PHAs are polymers, long molecules made up of many small subunits (monomers) which have been joined together. These water-insoluble storage polymers are biodegradable, exhibit thermoplastic properties and can be produced from renewable carbon sources. The composition of the polymer synthesized is governed by two main factors, i.e. the bacterial strain being used and the carbon source utilized to grow the bacteria. (Valappil, 2008)
In the case of PHAs, the monomers are 3-hydroxyalkanoates. An alkanoate is simply a fatty acid which is a linear molecule containing just carbon and hydrogen (an alkane) with a carboxyl group at one end (making an alkanoate). Furthermore, these monomers have a hydroxyl group (OH) at the 3rd carbon (the beta position), making these beta or 3-hydroxyalkanoates. The hydroxyl group of one monomer is attached to the carboxyl group of another by an ester bond; these plastics are thus polyesters. As is shown in Figure 3.2.1, the polyester linkage creates a molecule which has 3- carbon segments separated by oxygen atoms. The remainder of the monomer becomes a side chain off the main backbone of the polymer. The pendant group (R in Figure 3.2.1) varies from methyl (C1) to tridecyl (C13) (Poirier et al., 1995). The carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighboring monomer. This polymerization reaction is catalyzed by the host’s PHA synthase. Most of the PHAs encountered in nature are poly(beta-hydroxybutyrate) (PHB), in which the monomer unit is hydroxybutyric acid and the side chain is a methyl group. Other monomer units occur in nature, and many others can be produced in the laboratory by feeding unusual carbon sources to bacteria. Most PHAs, even what we call PHB, are actually copolymers, and contain some amount of another type of monomer unit.

Short-chain length PHAs (scl-PHAs) consists of 3–5 carbon atoms, whereas 6–14 carbon atoms are present in Medium-chain length PHAs (mcl-PHAs). The difference lies in the substrate used and specificity of the enzyme PHA synthase to bring hydroxyalkanoates to a certain range. There is a third group of PHAs named long-chain-length PHAs (lcl-PHAs) comprised of 15 and above carbon atoms, which are given less consideration compared to the rest (Steinbuchel and Valentine 1995).

PHAs vary in their mechanical properties depending on the composition of the monomeric units. The scl-PHAs are generally considered as thermoplastics, whereas mcl-PHAs are elastomers. PHB, the main candidate among scl-PHAs displays a similar degree of crystallinity and melting point ($T_m$) as polypropylene (PP), but is stiffer and more brittle than PP. Its copolymerization with hydroxy valerate (HV) monomers reduces its stiffness and increases its toughness, giving a product with desirable properties for commercial applications. The mcl PHAs are semicrystalline elastomers with a low $T_m$, low tensile strength and high extension to break and can be used as biodegradable rubber. Hence, it is
important to study the mechanical and physical properties of such commercially important polymers before their use in the industry.

\[
\begin{align*}
R = \text{CH}_3, & \quad \text{Poly(3 hydroxybutyrate)} \\
R = \text{CH}_2-\text{CH}_3, & \quad \text{Poly(3-hydroxyvalerate)}
\end{align*}
\]

3.2.10 Physical Properties of PHAs

The composition of the PHA has a direct effect on the physical properties of the plastic, in. PHB, with its short methyl side chain, is a very crystalline and very brittle polymer. Industrially, it is difficult to use because the temperature at which it melts is very close to the temperature at which it begins to decompose. Its high degree of crystallinity causes it to crack easily. As a result, the PHA used commercially is PHBV, a copolymer of hydroxybutyrate and hydroxyvalerate (5 carbons long). PHBV is a random copolymer, meaning that the monomer units do not occur in the chain in any particular order. PHBV can still crystallize, but it produces a much more supple plastic and melts at a lower temperature, making processing easier. PHB and PHBV have properties similar to polypropylene, and
bottles made from these polyesters feel just like "normal" plastic. The flexibility increases with sidechain length throughout the PHA family, largely because of a loss of crystallinity. Polymers composed mostly of hydroxyoctanoate, an 8-carbon monomer, are elastic. Longer side chain polymers are so soft that they are gummy or glue-like. This remains one of the potential values of PHAs, that by feeding bacteria an appropriate substance, a PHA with specific desirable properties can be produced.

The properties of PHB (homopolymer of Poly-3-hydroxybutyrate), PHBV (PHB + 20% hydroxyvalerate monomers) and PHB4B (PHB + 16% 4-hydroxybutyrate monomers) and PHBHx (PHB + 10% mcl monomers) are compared with PP and is represented in the Table below (Tsuge, 2002).

PHAs show a high degree of polymerization, as high as 30,000. They are generally lipophilic substances and are insoluble in water. They are soluble in chlorinated hydrocarbons such as chloroform, methylene chloride, 1,2-dichloroethane or 1,1,2-trichloroethane and in propylene carbonate. In certain solvents, their solubility may vary according to the chemical composition eg. PHB is insoluble in petroleum ether where as PHO is soluble in this organic solvent (Timm et al., 1990). They are optically active and piezoelectric in nature. The molecular weight of the polymers is in the range of $2 \times 10^5$ to $3 \times 10^6$ daltons, based on the type of microorganism and growth conditions (Byrom, 1992).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PHB</th>
<th>PHBV</th>
<th>PHB4B</th>
<th>PHBHx</th>
<th>PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melting temperature (°C)</td>
<td>177</td>
<td>145</td>
<td>150</td>
<td>127</td>
<td>176</td>
</tr>
<tr>
<td>Glass transition – temperature (°C)</td>
<td>2</td>
<td>-1</td>
<td>-7</td>
<td>-1</td>
<td>-10</td>
</tr>
<tr>
<td>Crystallinity (%)</td>
<td>60</td>
<td>56</td>
<td>45</td>
<td>34</td>
<td>50-70</td>
</tr>
<tr>
<td>Tensile strength (MPa)</td>
<td>43</td>
<td>20</td>
<td>26</td>
<td>21</td>
<td>38</td>
</tr>
<tr>
<td>Extension to break (%)</td>
<td>5</td>
<td>50</td>
<td>444</td>
<td>400</td>
<td>400</td>
</tr>
</tbody>
</table>

3.2.11 The Biology of PHA

In nature, prokaryotic microorganisms respond to sudden increases in essential nutrients in their usually hostile environment by storing important nutrients for survival during prolonged period of starvation (Sudesh et al., 2000). PHAs are one such storage compound. PHAs are usually produced when carbon sources are in excess. The carbon
sources are assimilated, converted into hydroxyalkanoate (HA) compounds and finally polymerized into high molecular weight PHAs and stored as water insoluble granules in the cell cytoplasm. PHAs are an excellent storage compound because their presence in the cytoplasm, even in large quantities does not disturb the osmotic pressure of the cell. PHA granules can be observed as refractile granules under phase contrast light microscope. When thin sections of cells containing PHAs are viewed under transmission electron microscope, the granules appear as electron transparent, discrete, spherical particles with clear boundaries. The number and sizes of granules per cell differ depending on the PHA-producer microorganisms and their growth stage. In Wautersia eutropha (formerly known as Alcaligenes eutrophus), 8-13 granules per cell with sizes ranging from 0.2-0.5 m were detected (Byrom, 1992). PHA granules could be stained with Sudan Black (Schlegel, 1970) and more specifically by Nile Blue A, exhibiting a strong orange fluorescence. Nile Blue A is a more specific dye than Sudan Black B as it does not stain glycogen and polyphosphate. Both stains however can stain lipid bodies.

3.2.12 Biosynthesis of PHA

Polyhydroxyalkanoates are polyesters of hydroxyalkanoates (HAs) having the general structural formula shown in Figure. Numerous bacteria can synthesize and accumulate PHAs as carbon and energy storage materials or as a sink for redundant reducing power under the condition of limiting nutrients in the presence of excess carbon (Steinbüchel, 1991). Three metabolic phases of the biosynthesis of PHA in bacteria can be distinguished. First, a carbon source suitable for biosynthesis of PHA must enter the cell from the environment (Sangkharak and Prasertsan, 2012). This is achieved either by a specific transport system located in the cytoplasmic membrane or by diffusion of the compound into the cell. Second, anabolic or catabolic reactions, convert the compound into a hydroxyacyl coenzyme A thioester which is a substrate of the PHA synthase. Third, PHA synthase, which is the key enzyme of PHA biosynthesis, uses these thioesters as substrates and catalyzes the formation of the ester bond with the concomitant release of coenzyme A (Figure 3.2.2).

At present it cannot generally be excluded that the PHA synthases also use other thioesters of HA as substrates. Phase II is of most importance, since during this phase the carbon source is converted into a suitable substrate for the PHA synthase. Many bacteria are able to convert acetyl-CoA in two steps via acetoacetyl-CoA to D (-)-3- hydroxybutyryl-CoA
giving rise to poly(3HB). Regarding the application of precursor substrates, the most simple type of reaction is the conversion of a HA, which is provided as a carbon source to the cells, by a thikinase or a coenzyme A transferase into the corresponding HA-coenzyme A thioester, such as, for example, the conversion of 4HB into 4-hydroxybutyryl-coenzyme A. If the carbon source is not a precursor substrate, and if the carbon source is first converted into a central intermediate of metabolism, a complex sequence of reactions may be required to obtain PHA consisting of HA other than 3HB (Steinbuchel, 1996).

In most bacteria PHAs are synthesized and intracellularly accumulated under unfavorable growth conditions such as limitation of nitrogen, phosphorus, magnesium, or oxygen in the presence of excess carbon (Anderson and Dawes, 1990). It is, therefore, important to develop cultivation strategies that can simulate these conditions for the efficient production of PHA. Some bacteria such as *Alcaligenes latus* and a mutant strain of *Azotobacter vinelandii* are known to accumulate PHA during growth in the absence of nutrient limitation. Selection of a microorganism for the industrial production of PHA should be based on several factors including the cell’s ability to utilize an inexpensive carbon source, growth rate, polymer synthesis rate, and the maximum extent of polymer accumulation. The yield of PHA on carbon source is important not to waste substrate to non-PHA material. An equation that predicts the overall yield of PHA on several carbon sources has been derived and can be used for the preliminary calculation of PHA yields (Yamane, 1996). Recovery of PHA should also be considered because it significantly affects the overall economics.
3.2.13 PHA biosynthesis in Eucaryotic Cells

PHAs being ubiquitous are also reported in several plant and animal tissues. The synthesis of PHB in *Saccharomyces cerevisiae* has been demonstrated by expressing the PHB synthase gene from *Cupriavidus necator* (Leaf et al., 1996). PHB production in *Saccharomyces cerevisiae* cells required only the PHB polymerase gene from *C. necator* introduced into the cells. However, in case of recombinant *E.coli*, the introduction of the complete PHB pathway was necessary for PHA formation to occur. The PHA production was also found to be very low, ie, only 0.5% of the CDW and is very low compared to bacterial production of PHAs (Poirier et al., 1995) introduced a modified *phaC1* gene from *Pseudomonas aeruginosa* into *S. cerevisiae* (Zhang et al., 2000) engineered the synthesis of PHA polymers composed of monomers ranging from 4 to 14 carbon atoms in *S. cerevisiae*.
PHA production in an insect cell was reported in cabbage looper cells by the introduction of \textit{phbC} gene from \textit{R. eutropha} using a Baculovirus system (Williams \textit{et al.}, 1996). PHA production in an insect, \textit{Spodoptera frugiperda} cells using a baculovirus has also been reported (Williams and People, 1996). Though PHA production in insect cells has been reported, the percentage of PHA accumulation is found to be very low compared to that of the microbial system (Madison and Huisman, 1999).

Synthesis of PHA in plants was first demonstrated in 1995 by the accumulation of PHB in the cytoplasm of cells of \textit{Arabidopsis thaliana} (Poirier \textit{et al.}, 1995). In order to produce PHA in large quantities from cheap resources, several researchers have been investigating the possibility of producing PHB in transgenic plants. Synthesis of PHAs in crops is also an excellent way of increasing the value of the crops (Poirier, 1995; Somerville and Bonetta, 2001). Since β-ketothiolase, the first enzyme of PHA synthesis is present in the cytoplasm of higher plants, only the reductase and the PHA synthase are required to synthesize PHA in plant cells (Poirier \textit{et al.}, 1995). Even though transgenic \textit{Arabidopsis thaliana} plants harboring the \textit{C. necator} PHA biosynthesis genes was constructed, these plants accumulated low level of PHB granules in the nucleus, vacuole and cytoplasm. Poirier \textit{et al.}, (1995) has reported that the accumulation of PHB in the nucleus of plant cell could affect its growth. The plastid was suggested to be the ideal location for the PHB accumulation because it is the location of high flux of carbon through acetyl-CoA (Nawrath \textit{et al.}, 1994). Recently, the genetically engineered genes of \textit{Cupriavidus necator} were successfully targeted to the plant plastids and the enzymes were active in the plastids (Nawrath \textit{et al.}, 1994).

PHB synthesis has been demonstrated in the cells of cotton fibres. In this case, the PHA produced was used as an intracellular agent that modifies the heat exchange properties of the fibre. The PHA biosynthesis genes like \textit{phaA}, \textit{phaB} and \textit{phaC} genes from \textit{C. necator} were expressed in transgenic cotton under the control of a fibre specific promoter (John and Keller, 1996). In \textit{Nicotiana tabacum}, PHB was synthesised through the co-expression of the \textit{phaB} gene from \textit{C. necator} and the PHA synthase from \textit{Aeromonas caviae} (Nakashita \textit{et al.}, 1999). PHB has been produced in Rape seed (\textit{Brassica napus}) by expressing the PHB genes of \textit{C. necator} (Houmiel \textit{et al.}, 1999; Valentin \textit{et al.}, 1999).
3.2.14 Microbial synthesis of PHA

In microbial communities, the factors inducing PHA synthesis are species specific; for some PHA producers it is deficiency of biogenic elements like nitrogen, phosphates in the medium whereas for others this is oxygen deficiency (Braunegg et al., 1998).

Approximately 140 different hydroxyalkanoic acids are known to be incorporated into PHAs (Steinbüchel, 1996), with microbial species from over 90 genera being reported to accumulate these polyesters (Zinn et al., 2001).

*Cupriavidus necator*, (previously known as *Hydrogenomonas eutropha*), *Alcaligenes eutrophus*, *Ralstonia eutropha* and *Wautersia eutropha* (Vandamme and Coenye 2004; Vaneechoutte et al., 2004), has been the most extensively studied and commonly used bacterium for PHA production. In the 1980s, a glucose-utilizing mutant of *C. necator* was employed by Imperial Chemical Industries (UK) for the industrial production of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)], which was sold under the trade name of BiopolTM (Luzier, 1992).

**Table 3.2.2 Some natural bacterial genera that are PHA accumulators**

<table>
<thead>
<tr>
<th>Zobellella</th>
<th>Delftia</th>
<th>Comamonas</th>
<th>Sinorhizobium</th>
<th>Klebsiella</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azospirillum</td>
<td>Rhizobium</td>
<td>Rhodobacter</td>
<td>Rhodopseudomonas</td>
<td>Listeria</td>
</tr>
<tr>
<td><em>Enterobacter</em></td>
<td>Thermus</td>
<td>Paracoccus</td>
<td>Azoarcus</td>
<td>Amaricoccus</td>
</tr>
<tr>
<td>Paracoccus</td>
<td>Thauera</td>
<td>Pseudomonas</td>
<td>Vibrio</td>
<td>Azotobacter</td>
</tr>
<tr>
<td>Alcaligenes</td>
<td>Cupriavidus</td>
<td>Staphylococcus</td>
<td>Chromobacterium</td>
<td>Streptococcus</td>
</tr>
<tr>
<td>Pseudoaltermonas</td>
<td>Burkholderia</td>
<td>Bacillus</td>
<td>Halomonas</td>
<td>Methylobacterium</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>Aeromonas</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 3.2.3 Some bacterial strains producing PHAs

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Carbon sources</th>
<th>PHA content (% dry cell weight)</th>
<th>PHAs</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alcaligenes eutrophus</em></td>
<td>Butyric +pentanoic acid</td>
<td>43 to 55</td>
<td>P(3HB-3HV)</td>
<td>Yoshiharu <em>et al.</em>, 1988</td>
</tr>
<tr>
<td><em>A. eutrophus</em></td>
<td>Pentanoic acid</td>
<td>46</td>
<td>P(3HB-3HV)</td>
<td>Yoshiharu <em>et al.</em>, 1988</td>
</tr>
<tr>
<td><em>A. eutrophus</em></td>
<td>Butyric acid</td>
<td>48</td>
<td>P(3HB)</td>
<td>Yoshiharu <em>et al.</em>, 1988</td>
</tr>
<tr>
<td><em>A. eutrophus</em></td>
<td>Glucose</td>
<td>54</td>
<td>P(3HB)</td>
<td>Yoshiharu <em>et al.</em>, 1988</td>
</tr>
<tr>
<td><em>A. eutrophus</em></td>
<td>Olive oil</td>
<td>47</td>
<td>P(3HB)</td>
<td>Minoru <em>et al.</em>, 1992</td>
</tr>
<tr>
<td><em>A. eutrophus</em></td>
<td>Corn oil</td>
<td>39</td>
<td>P(3HB)</td>
<td>Minoru <em>et al.</em>, 1992</td>
</tr>
<tr>
<td><em>A. eutrophus</em></td>
<td>Palm oil</td>
<td>40</td>
<td>P(3HB)</td>
<td>Minoru <em>et al.</em>, 1992</td>
</tr>
<tr>
<td><em>Rhizobium trifolii</em></td>
<td>Mannitol</td>
<td>40 to 50</td>
<td>-</td>
<td>Vincent <em>et al.</em>, 1962</td>
</tr>
<tr>
<td><em>A. latus</em></td>
<td>Sucrose</td>
<td>50 to 80</td>
<td>P(3HB)</td>
<td>Yamane <em>et al.</em>, 1996 ; Wang and Lee, 1997</td>
</tr>
<tr>
<td><em>Azotobacter vinelandii</em></td>
<td>Glucose + fish peptone</td>
<td>79.8</td>
<td>P(3HB)</td>
<td>Page and comish, 1993</td>
</tr>
<tr>
<td><em>Azotobacter vinelandii</em></td>
<td>Valeric acid</td>
<td>78</td>
<td>P(3HB-3HV)</td>
<td>Page <em>et al.</em>, 1992</td>
</tr>
<tr>
<td><em>Azotobacter vinelandii</em></td>
<td>Propionic acid</td>
<td>75</td>
<td>P(3HB)</td>
<td>Page <em>et al.</em>, 1992</td>
</tr>
<tr>
<td><em>Azotobacter vinelandii</em></td>
<td>Butyric acid</td>
<td>78</td>
<td>P(HB)</td>
<td>Page <em>et al.</em>, 1992</td>
</tr>
<tr>
<td><em>Chromobacterium violaceum</em></td>
<td>Valeric acid</td>
<td>62</td>
<td>P(3HV)</td>
<td>Steinbuchel and Schmack, 1995</td>
</tr>
<tr>
<td><em>Methylobacterium organophilum</em></td>
<td>Methanol</td>
<td>52</td>
<td>P(3HB)</td>
<td>Kim <em>et al.</em>, 1996</td>
</tr>
<tr>
<td><em>Protomonas extorquens</em></td>
<td>Methanol</td>
<td>64</td>
<td>P(3HB)</td>
<td>Suzuki <em>et al.</em>, 1986</td>
</tr>
<tr>
<td><em>Pseudomonas oleovorans</em></td>
<td>n-Octane</td>
<td>33</td>
<td>P(3HHx-3HO)</td>
<td>Preusting <em>et al.</em>, 1993</td>
</tr>
<tr>
<td><em>Erwinia sp.</em></td>
<td>Palm oil</td>
<td>46</td>
<td>P(3HB)</td>
<td>Majid <em>et al.</em>, 1999</td>
</tr>
<tr>
<td><em>Erwinia sp.</em></td>
<td>Palm oil + propionic acid</td>
<td>40</td>
<td>P(3HB-3HV)</td>
<td>Majid <em>et al.</em>, 1999</td>
</tr>
<tr>
<td><em>Erwinia sp.</em></td>
<td>Palm oil + valeric acid</td>
<td>34</td>
<td>P(3HB-3HV)</td>
<td>Majid <em>et al.</em>, 1999</td>
</tr>
</tbody>
</table>

P(3HB): poly(3-hydroxybutyrate);  
P(3HV): poly(3-hydroxyvalerate);  
P(3HB-3HV): copoly(3-hydroxybutyrate-3-hydroxyvalerate);  
P(3HHx-3HO): copoly(3-hydroxyhexanoate-3-hydroxyoctanoate)

PHA is accumulated by numerous bacteria but can also be found in eukaryotic organisms such as yeasts, plants and animals (Reusch, 1989). Whereas there is still an
ongoing discussion about a putative function of PHA in higher organisms. In bacteria PHA functions as a carbon and energy storage material and/or as a sink for redundant reducing power (Steinbuchel, 1991). The synthesis is most effective under conditions of limiting nutrients in the presence of an excess of carbon source. Depending on the bacterial host, one of four different biosynthetic pathways have been discovered to produce PHAs. Introductions to each of these different biosynthesis routes are found in the literature (Steinbuchel and Pieper, 1992).

Table 3.2.4 Microorganisms Capable of Glycogen and Glycogen-Like Reserve Accumulations

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Microorganism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeromanas formicans</td>
<td>Aeromanas hydrophila</td>
</tr>
<tr>
<td>Aerobacter aerogenes</td>
<td>Agrobacterium tumefaciens</td>
</tr>
<tr>
<td>Aphanocapsa 6308</td>
<td>Arthrobacter crystallopoietes</td>
</tr>
<tr>
<td>Arthrobacter viscosus</td>
<td>Bacillus cereus</td>
</tr>
<tr>
<td>Bacillus megaterium</td>
<td>Bacillus stearothermophilus</td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td>Chromatium vinosum</td>
</tr>
<tr>
<td>Chlorobium limicola</td>
<td>Citrobacter freund</td>
</tr>
<tr>
<td>Clostridium botulinum</td>
<td>Clostridium pasteurianum</td>
</tr>
<tr>
<td><strong>Enterobacter aerogenes</strong></td>
<td><strong>Enterobacter cloacae</strong></td>
</tr>
<tr>
<td>Escherichia aurescens</td>
<td>Klebsiella pneumoniae</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Micrococcus luteus</td>
</tr>
<tr>
<td>Mycobacterium amegmatis</td>
<td>Mycobacterium phlei</td>
</tr>
<tr>
<td>Mycobacterium smegmatis</td>
<td>Mycobacterium tuberculosis</td>
</tr>
<tr>
<td>Pasteurella pseudotuberculosis</td>
<td>Pseudomonas Pseudoflava</td>
</tr>
<tr>
<td>Rhodocyclus purpureus</td>
<td>Rhodomicrobium vannelli</td>
</tr>
<tr>
<td>Rhodopseudomonas acidiphila</td>
<td>Rhodopseudomonas capsulatus</td>
</tr>
<tr>
<td>Rhodopseudomonas globiformis</td>
<td>Rhodopseudomonas gelatinosa</td>
</tr>
<tr>
<td>Rhodopseudomonas palustris</td>
<td>Rhodopseudomonas sphaeroides</td>
</tr>
<tr>
<td>Rhodopseudomonas viridis</td>
<td>Rhodospirillum fulvum</td>
</tr>
<tr>
<td>Rhodospirillum molasichianum</td>
<td>Rhodospirillum rubrum</td>
</tr>
<tr>
<td>Rhodospirillum tenue</td>
<td>Rhodospirillum photometricum</td>
</tr>
<tr>
<td>Salmonella enteritidis</td>
<td>Salmonella montevideo</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>Sarcina lutea</td>
</tr>
<tr>
<td>Serratia liquefaciens</td>
<td>Serratia marcescens</td>
</tr>
<tr>
<td>Shigella dysenteriae</td>
<td>Streptococcus mitis</td>
</tr>
<tr>
<td>Streptococcus mutans</td>
<td>Streptococcus salivarius</td>
</tr>
<tr>
<td>Streptococcus sanguis</td>
<td>Synechococcus 6031</td>
</tr>
</tbody>
</table>
3.2.15 Significance of Microbial Synthesis of PHAs

The synthesis of PHAs can be achieved by either chemical or biological synthesis. The majority of biosynthesis of PHAs is performed using microorganisms. In contrast PHA synthesis by chemical method is not wide spread due to a mixing of R and S stereoisomers within the polymer chain. This has significant implications for the biodegradability of polymers, as PHB depolymerases fails to catalyse the hydrolysis of S-PHB linkages (Kemnitzer et al., 1993; Hocking and Marchessault, 1994), while the bacterial PHAs, possessing 100% R stereoisomers, were completely biodegradable in nature (Hocking and Marchessault, 1994).

In microbes, only (R)-isomers are accepted as substrates for the polymerizing enzyme, as PHA synthase enzyme is highly specific towards R stereoisomeric substrates. In microbes, all the PHAs that have been characterized so far, are invariably of R- configuration.

High levels (10–40% (w/w) of dry weight) of polymer inside the plant have a negative effect on the growth and development of the plant. Therefore plant cells have the ability to produce only low yields (<10% of dry weight) of PHA. At present, this problem has not been overcome (Bohmert et al., 2002). In contrast, PHAs are accumulated within bacteria to levels as high as 90% (w/w) of the dry cell mass (Madison and Huisman, 1999). In addition to the slower growth of the transformed plant cells than the native cells, there occurred instability of the phbB and phbC genes in transformed plant cells (Hahn et al., 1997). The PHA synthesis in eukaryotic cells like insect cells and yeasts reported low yield. The microbial production of PHAs thus becomes more significant than any other mode of PHA production.

Isolation of diverse PHAs producing bacteria via different enrichment techniques can help to identify novel and more efficient PHA producers. This can lead to better PHA yields in a short period of time, thus cutting down production costs. The screening of large number of organisms that accumulate PHA with a combination of monomers, yielding the desirable quality in a sufficiently large amount is necessary as the chemical composition of PHAs depends mainly on the bacterial strain.

3.2.16 Recovery of PHA

Following the fermentation, cells containing PHAs are separated by conventional procedures such as centrifugation, filtration, or vortexing. After the biomass harvesting, cells are disrupted to recover the polymers. A number of different methods have been developed for the recovery of PHA. The first method that has most often been used involves extraction
of P (3HB) from biomass with solvent. The solvents employed include chloroform, methylene chloride, propylene carbonate, and dichloroethane. Due to the high viscosity of even dilute PHA solutions, about 20 portion of solvent are required to extract 1 portion of polymer (Byrom, 1992). The large amount of solvent required makes this method economically unattractive, even after the recycling of the solvent (Holmes and Lim, 1990).

Several other methods that have been developed involve the use of sodium hypochlorite for the differential digestion of non-PHA cellular materials (Berger et al., 1989). Although this method is effective in the digestion of non-PHA cellular materials, it causes severe degradation of P (3HB) resulting in a 50% reduction in the molecular weight. The use of sodium hypochlorite together with chloroform significantly reduced degradation of PHA (Hahn et al., 1994). It was suggested that chloroform immediately dissolves the isolated P (3HB) by hypochlorite, and thus protects polymer from degradation. Normally, polymer purity of greater than 95% is obtained by hypochlorite treatment.

Excluding the recovery process, the economics of PHA production are largely determined by the substrate cost and PHA yield. The efficiency of substrate conversion is important, and can be predicted from the physiology and biochemistry involved in the PHA synthesis. Among the various nutrients in the fermentation medium, the carbon source contributes most significantly to the overall substrate cost in PHA production. A number of carbon sources, including carbohydrates, oils, alcohols, acids and hydrocarbons, can be used by various bacteria (Yamane, 1993). The theoretical yield (the yield based on the reaction stoichiometry) of P(3HB) has been estimated for several of these carbon substrates (Yamane, 1993). Regeneration of nicotinamide nucleotides, which are used as cofactor for PHA synthesis, has been taken into account in this analysis. It was also suggested that the overall yield, which is the yield in actual fermentation, would be roughly proportional to the theoretical yield and PHA content. Because of their low price, crude carbon substrates such as the cane and beet molasses, cheese whey, plant oils and hydrolysates of starch (corn and tapioca), cellulose and hemicellulose can be excellent substrates to several bacteria utilizing them.

3.2.17 Structure of PHA Granules

The structure of PHA granules has not been fully determined but the major constituent of granules is PHA, often PHB, with small amounts of protein and lipid (Griebel et al., 1968).
In vivo the hydrophobic polyester core is largely amorphous, (De Koning and Lemstra, 1992) with water as a component that prevents crystallization by acting as a plasticizer (Horowitz and Sanders, 1994). After isolation, PHA is often crystalline.

Initial studies, including electron microscopy in the 1960s, (Ellar et al., 1968) have shown the polyester core to be surrounded by a 4 nm boundary layer, which most likely comprises a phospholipid monolayer (Mayer et al., 1996) with embedded and attached proteins (Steinbuchel et al., 1995 and Mayer and Hoppert, 1997). While most data seem to be consistent with a monolayer, alternative membrane models, for example, comprising inner and outer protein layers sandwiching phospholipids, have been suggested (Stuart et al., 1998). More recent electron microscopy data indicated that the thickness of the surface layer surrounding the PHA granules to be 14 nm, which the authors took as an indication of the size of the associated proteins (Jendrossek et al., 2007). However, it cannot entirely be ruled out that the boundary layer primarily consists of proteins and that attachment of membrane material is only an isolation artifact.

Table 3.2.5 Chemical composition of purified PHA granules

<table>
<thead>
<tr>
<th>Granule composition</th>
<th>Dry weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA</td>
<td>97.7</td>
</tr>
<tr>
<td>Protein</td>
<td>1.87</td>
</tr>
<tr>
<td>Lipid</td>
<td></td>
</tr>
<tr>
<td>a. Acetone extractable</td>
<td>0.21</td>
</tr>
<tr>
<td>b. Alcohol-ether and ether extractable</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Source: Griebel et al., 1968
Figure 3.2.3 Different representations of PHA granules.

(A) TEM image of bacterial cell filled with PHA granules. Bar, 500 nm. (Journal of Bacteriology, 2005, reproduced with permission from American Society for Microbiology.)

(B) TEM image of single PHA granule isolated from bacterial cell zooming in on the paracrystalline-like layer of particles covering the granule. Bar, 50 nm. (Applied and Environmental Microbiology, 2007, reproduced with permission from American Society for Microbiology.)

(C) Schematic depiction of a PHA granule with granule associated proteins. (Reproduced with permission, from Rehm, 2003)

In addition to Electron Microscopy (EM), a variety of techniques have been used to investigate PHA, including wide-angle X-ray scattering (Kawai, 1995) nuclear magnetic resonance spectroscopy, and confocal microscopy. Using wide-angle X-ray scattering, Kawaguchi and Doi confirmed that PHA in native granules is amorphous, even after isolation, and that certain treatments seemed to initiate crystallization, presumably by removing a lipid component. Recently, contrast-variation small-angle neutron scattering was used to probe granule organization, and results were consistent with the phospholipid
monolayer model (Russell, 2006). Atomic force microscopy (AFM) allows imaging at nanoscale while being rapid and less damaging to preparations than EM. Recently, analysis of PHA granules by AFM has shown an additional network layer with globular areas, most likely also incorporating structural phasin proteins (Dennis et al., 2008). AFM was also used to show porin-like structures in the surrounding membrane, which were suggested to provide a portal to the amorphous polymer core and be the site of PHA metabolism and depolymerisation (Dennis et al., 2003).

Investigation of PHA granules is strongly influenced by the preparation technique because denaturation and crystallization of PHA often occurs through physical stress such as excessive sonication, (Dennis et al., 2008) freeze-thaw cycles, or exposure to solvents, detergents, or alkalis (Griebel and Merrick, 1971). To avoid denaturation during the purification process, PHA granules can be purified using mechanic (e.g., French Press) or enzymatic cell lysis followed by density gradient centrifugation. These techniques should allow accurate analysis and consistent end-use of PHA granules.

3.2.18 Quantitative Analysis using Gas Chromatography

Gas chromatography (GC) has been used to a great extent by other laboratories in order to confirm PHA production from bacterial cells. The principle behind GC is that each compound will leave the column at a different rate. Therefore, a different amount of time is taken for each compound to leave the column. This concept is called retention time (Oehmen et al., 2005). It is using this logic that different PHBs and copolymers can be measured (Figure 3.2.4).

![Figure 3.2.4 Separation of poly-β-hydroxybutyrate (3HB) and poly-β-hydroxyvalerate (3HV) using gas chromatography techniques (Oehmen et al., 2005).]
3.2.19 Other types of PHA

There are many types of PHA. The material properties and hence the application of the PHAs vary depending on the monomers composition. Polyhydroxybutyrate, P (3HB), is the most well known and well characterized PHA.

3.2.20 Other Properties of PHAs

All PHAs possess some properties which recommend them for some applications and make them interesting to industry such as:

<table>
<thead>
<tr>
<th>Table 3.2.6 Properties of PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>They are thermoplastics and/or elastomeric compounds which can be processed with apparatus already used by the plastic manufacturing industry to mimic some of the most important petrochemical polymers.</td>
</tr>
<tr>
<td>Exhibit a rather high degree of polymerization ranging from 105 to almost 107 Da</td>
</tr>
<tr>
<td>Non-toxic</td>
</tr>
<tr>
<td>At least poly(3HB) and poly(3HB-co-3HV) possess piezoelectric properties</td>
</tr>
<tr>
<td>All PHAs are biodegradable</td>
</tr>
</tbody>
</table>

Extensive reviews of bacterial PHAs have been performed by Lafferty et al., (1988); Anderson et al., (1990); Doi and Abe, (1990), Lee, (1996); Sasikala and Ramana, (1996) and Braunegg et al., (1998). PHAs are an intracellular storage material synthesized by a wide variety of bacteria. They are composed mainly of poly-beta-hydroxybutyric acid (PHB) and poly-beta-hydroxyvaleric acid (PHV), although other forms are possible. Lafferty et al., (1988) stated that the accumulation of PHA can be stimulated under unbalanced growth conditions, i.e., when nutrients such as N, P or sulfate become limiting, when oxygen concentration is low, or when the carbon: nitrogen (C: N) ratio of the feed substrate is high. According to Doi, (1990), when growth conditions are unbalanced, acetyl-CoA cannot enter
the tricarboxylic acid (TCA) cycle to obtain energy for cells due to high concentrations of NADH. The high concentrations of NADH inhibit the enzyme citrate synthase leading to an increase in the level of acetyl-CoA. Acetyl-CoA is then used for PHA biosynthesis by a sequence of enzymatic reactions. PHA can serve as a carbon or energy source for microorganisms during starvation periods. In EBPR systems, PHA is formed and stored during the anaerobic period by phosphorus accumulating organisms (PAOs) when no electron acceptors are available for growth mechanisms. This permits them to sequester most of the substrate and out-compete organisms that do not have phosphorus and PHA storage capabilities.

3.2.21 The enzymology of PHA production and organisation of the PHA biosynthetic genes

PHA production is catalysed by the enzyme PHA synthase which uses, hydroxyacyl-CoA, (HACoA) molecules as substrate. The reaction catalysed by this enzyme is shown in Figure 2.5. The PHA synthase enzyme is encoded by the phaC gene (Rehm, 2003).

![Figure 3.2.5 Reaction catalysed by the enzyme PHA synthase (Rehm, 2003).](image)

Currently the sequences of 88 PHA synthases have been carried out including two from the halobacterial species *Haloarcula marismortui* and *Haloferax mediterranei* (Grage *et al.*, 2009). Based on the amino acid sequences, the substrate ranges of the enzymes, molecular mass and subunit compositions, PHA synthases have been grouped into four classes i.e. Class I, II, III and IV (Rehm, 2003; Grage *et al.*, 2009).

The Class I PHA synthase (prototype: *Cupriavidus necator*) consist of only one type of subunit (PhaC). Its molecular weight ranges between 61 and 73 kDa and catalyzes polymerization of short chain length hydroxyacidCoAs, HACoAs. In this class, the genes encoding for the PHA synthase (*phaC*), β-ketothiolase (*phaA*) and NADPH dependent acetoacetyl-CoA reductase (*phaB*) constitute the *phaCAB* operon (Peoples and Sinskey, 1989). The β-ketothiolase enzyme catalyses the condensation of two acetyl-CoA molecules to
form the acetoacetyl-CoA. This acetoacetyl-CoA molecule formed is then converted to (R)-3-hydroxybutyryl-CoA by the NADPH dependent acetoacetyl-CoA reductase (Peoples and Sinskey, 1989).

The Class II PHA synthase (prototype: *Pseudomonas aeruginosa*) is encoded by two different genes, *phaC1* and *phaC2*, again 61 to 73 kDa in size. Each catalyse the polymerisation of medium chain length hydroxyacetyl CoA substrates, to form mcl-PHAs (Rehm, 2003). The two *phaC* genes are separated by the *phaZ* gene, encoding the PHA depolymerase. Further downstream of the synthase operon, the *phaD* gene is collinearly located along with the genes *phaI* and *phaF*, which are transcribed in the opposite direction. Both *phaF* and *phaI* code for granule associated structural proteins phasins (Ren *et al.*, 2005). The *phaF* gene is also involved in the transcriptional regulation of *phaC1* gene expression (Prieto *et al.*, 1999). PhaF encoded by *phaF* is a granule associated protein which in the absence of substrates for PHA production remains free in the cytoplasm as no PHA granule is formed. Thus it binds to the promoter region upstream of the *phaC1* gene thereby inhibiting the transcription of the *phaC1* gene. However, in the presence of PHA producing substrates, the phaF is bound to the PHA granule and therefore the *phaC1* gene gets expressed (Prieto *et al.*, 1999; Kessler and Palleroni, 2000). The *phaD* gene product plays a role in the regulation of the size and number of PHA granules formed (Klinke *et al.*, 2000).

The Class III PHA synthase (prototype: *Allochromatium vinosum, Thiocystis violacea, Thiocapsa pfennigii*) is a heteromer containing two subunits of 40 kDa, each encoded by the genes *phaC* and *phaE*. The subunits do not show any homology to each other. Both are required for the functional activity of the PHA synthase which catalyses the polymerization of short chain length HACoAs (Liebergesell *et al.*, 1992). *phaP* codes for the granule associated structural protein called phasins. These proteins have been observed to have an influence on the granule size, number and also on the molecular weights of the synthesized PHA (Klinke *et al.*, 2000). *phaA* and *phaB* encodes for the β-ketothiolase and an NADPH dependent acetoacetyl-CoA reductase, respectively, as described above.

The Class IV PHA synthase (prototype: *Bacillus megaterium*) is a heterodimer containing two subunits of 40 kDa and 20 kDa each, encoded by the *phaC* and *phaR* genes respectively. This enzyme catalyses the polymerisation of scl-HACoA substrates to form Short chain length (scl)-PHAs. In this class, the *phaC* and *phaR* genes are separated by the
phaB gene. Here the phaRBC operon is transcribed as a tricistronic operon. The phaP and phaQ genes located upstream are transcribed in an opposite direction. Here too, the PhaP proteins are associated with the PHA granules and are called phasins. In Cupriavidus necator, PhaP have been found to regulate P(3HB) production by regulating the surface/volume ratio of P(3HB) granules and by interacting directly with the PHA synthase (York et al., 2001 a, b). PhaQ in B. megaterium, is found to be a transcriptional regulator that negatively controls expression of the phaQ and phaP genes (Lee et al., 2000).

3.2.22 Uses and applications of PHAs

Commercial production of PHA is so far only viable by fermentative biotechnological processes. Several PHAs, such as, in particular, the homopolyester poly (3HB), the copolyester poly(3HB-co-3HV) and PHAs consisting of 3-hydroxyoctanoate, 3-hydroxydecanoate and a few other medium-chain-length 3-hydroxyalkanoates, poly(3HAMCL), have been manufactured into various materials, and applications in various areas have been revealed. Whereas the use of poly(3HB) and poly(3HB-co-3HV) as biodegradable bioplastics was established some time ago, other applications such as the manufacturing of latex paints, and specifically medical applications including retard materials and use as scaffolding material for tissue engineering due to their biocompatibility, are currently under development (Steinbuchel, 1991).

Isolated and purified PHAs can be used as such or in combination with other materials such as starch, cellulose fibers, glass fibers or synthetic plastics to obtain compounded materials. The purified material can be processed through transterification, melted in the presence of synthetic polyesters, by modification of the side chains or by crosslinking through energized irradiation and chemical reagents. Another usage of the PHAs can be done as a source to obtain enatiomeric pure hydroalkanoic acids upon chemical or enzymatic cleavage for the synthesis of various chemicals. There have also been applications for bacterial cell mass containing a high fraction of PHAs as binders for other fibrous materials (Steinbuchel, 1991).

PHAs have been used in the manufacture of bottles, containers and fibers for biodegradable materials (Holmes, 1985). They were tested as water-resistant moisture barriers for food packaging, replacing polyethylene or aluminum (Hanggi, 1995; Amass et al., 1998). Putative applications of PHAs are the use as osteosynthetic materials, bone plates, surgical
sutures and other surgical materials (Zhang et al., 1995, Saad et al., 1999). PHAs could also be used for the controlled release of drugs and other chemicals in medicine, pharmacy, agriculture and food industry (Gursel and Hasirci, 1995; Pouton and Akhtar, 1996). Another promising development is the use of mcl-PHA as chiral monomer source (Witholt and Kessler, 1999).

Other applications for such polyesters can be found in agriculture (e.g. as cold frame sheeting, seed capsules, or formulations for the controlled release of pesticides or herbicides). A process for elimination of nitrates from drinking water using PHB has also been developed (Steinbuchel, 1991).

Recently attempts have been made to extend the field of application of P(3-HB) and P(3-HB/3-HV) by preparing degradable block copolymers with polystyrene and polyethers, or “blends” with polyethylene, polystyrene, polyvinyl chloride, or polyethylene oxide act as a gas barrier in a similar way to polyvinyl chloride or polyethylene terephthalate (Holmes, 1985). Because of these properties, P (3- HB) could well play an important role as a packaging material. Nowadays this is the only widespread application of this material, mostly used to produce shampoo bottles that degrade themselves within compost in a short span of time (Steinbuchel, 1991).

However, industrial applications of P (3HB) have been hampered knowing to its low thermal stability and excessive brittleness upon storage (Lee, 1996). The copolymer of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV), P(3HB-co-3HV), is more flexible and tougher than the P(3HB). It can be used to make various products, including films, coated paper, board, compost bags, disposable food service ware and moulded products such as bottles and razors and also be used for biomedical applications (Lee, 1996).

Besides that, there have recently discovered 4-hydroxybutyrate, P(4HB). The P(4HB), has been found to be useful in the biomedical applications (Martin and Williams, 2003). It was used for tissue engineered heart valve scaffold and viable ovine blood vessels (Chen and Wu, 2005). Also, a high molecular weight copolymer of 3HB and 4HB [P(3HB-co-4HB)] containing 0–100 mol% of 4HB, can be produced by Comamonas acidovorans with a controlled degradation rate (Saito and Doi,1994), making them ideal candidates for biomedical applications such as tissue engineering (Martin and Williams, 2003). Other applications of PHA are shown in Table 3.2.7.
### Table 3.2.7 Other possible application of PHA

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Packaging films, bags and containers</td>
</tr>
<tr>
<td>2</td>
<td>Biodegradable carrier for long term dosage of drugs, medicines</td>
</tr>
<tr>
<td>3</td>
<td>Insecticides, herbicides, or fertilizers</td>
</tr>
<tr>
<td>4</td>
<td>Disposable items such as razors, utensils, diapers, or feminine</td>
</tr>
<tr>
<td>5</td>
<td>Hygiene products</td>
</tr>
<tr>
<td>6</td>
<td>Surgical pins, sutures, staples, and swabs</td>
</tr>
<tr>
<td>7</td>
<td>Wound dressing</td>
</tr>
</tbody>
</table>

#### 3.2.23 Biodegradability of PHA

The prime distinguishing feature of PHAs from synthetic plastics is its biodegradability. The extent of biodegradation is dependent on a number of factors such as microbial activity of the environment, the exposed surface area, moisture, temperature, pH and molecular weight of the PHA (Boopathy, 2000). PHA biodegradation is also influenced by its polymer composition and crystallinity (Lee, 1996) and the nature of its monomer units. Copolymers containing PHB monomer units have been found to degrade more rapidly than either PHB or 3HB-co-3HV copolymers.

Microorganisms secrete enzymes like PHA hydrolases and PHA depolymerases that break down the PHAs into its monomeric hydroxyacids, which are utilized as a carbon source for growth. The enzyme activity depends on the PHA composition and the environmental conditions (Choi et al., 2004; Jendrossek and Handrick, 2002). PHA degrading enzymes have been reported in various microorganisms and they are ubiquitous in nature. Biodegradation of PHAs in nature, distribution of PHA degrading microbes and enzymes involved in PHA degradation have been very well reviewed by several researchers (Tokiwa and Calabia, 2004; Jendrossek, 1998; Nishida and Tokiwa, 1993). Biodegradation of PHA under aerobic conditions results in the release of carbon dioxide and water, whereas in anaerobic conditions the degradation products are carbon dioxide and methane (Santhanam and Sasidharan, 2010).

Madison and Huismman, (1999) reported that the degradation rate of a piece of PHB varies from a few months (in anaerobic sewage) to years (in seawater). Studies have shown...
that 85% of PHAs were degraded in seven weeks (Johnstone, 1990; Flechter, 1993). UV light is found to accelerate the degradation of PHAs (Shang et al., 2003). They are compostable over a wide range of temperatures, at a maximum of around 60°C, with moisture content of 55%. PHA is also reported to degrade in aquatic environments (Lake Lugano, Switzerland) within 254 days at temperatures not exceeding 6°C (Johnstone, 1990). Within mammals, the polymer is hydrolysed only slowly (Pouton and Akhtar, 1996). Even more important than biodegradability of PHAs is the fact that their production is biological and based on renewable resources (Braunegg et al., 1995).

![Figure 3.2.6 Biodegradability of bioplastics](image)

3.2.24 Biodegradation in the environment

In nature, the microbial population present in a given environment and temperature also contribute to the biodegradability of the polymer. Microorganisms from the families Pseudonocardiaeae, Micromonosporaceae, Thermomonosporaceae, Streptosporangiaceae and Streptomycesaceae predominantly degrade P(3HB) in the environment. These microbes secrete extracellular enzymes that solubilise the polymer and these soluble products are then absorbed through their cell walls and utilised. Some PHA producing bacteria are able to degrade the polymer intracellularly. During intracellular degradation, the polymer is ultimately broken down to acetyl-CoA which under the aerobic conditions enters the citric acid cycle and is oxidised to CO₂ (Lee and Chang, 1995). The enzyme involved in the degradation of the PHAs is the PHA depolymerase encoded by phaZ (Koller et al., 2008).
Figure 3.2.7 Shampoo bottles made of BIOPOL, Progress of degradation of P(3HB-3HV) bottles when incubated during the summer (average temperature, 20°C) in aerobic sewage sludge with treatment for 0, 2, 4, 6, 8, and 10 weeks (from left to right).


3.2.25 National Status of PHA Research

In 2002, Rawte and his co workers reported the incidence of marine and mangrove bacteria accumulating polyhydroxyalkanoates on the mid-west coast of India. Higher incidence of PHA accumulating microbes was reported from samples from marine environments, including sediments from mangroves and from the Bombay high oil fields. The presence of PHA accumulation in Chromobacterium violaceum, Vibrio neries and Listeria sp. were reported and various physico-chemical factors affecting PHA accumulation by marine bacteria were studied (Rawte and Mavinkurve, 2004). Microbial production of PHB by marine microbes isolated from various marine environments along south east and south west coastal regions of India were reported (Arun et al., 2009). In their studies, the most potential PHA producers were identified as Vibrio sp. and Bacillus sp. It was interesting that the hunt for novel PHA producers are mainly focused from the marine environments. PILA synthase gene (phaC1 gene) of indigenous Pseudomonas sp. LDC-5 was successfully cloned in Escherichia coli by Sujatha and Shenbagarathai, (2006). This recombinant E. coli was found to accumulate medium chain length PHA and can be used for the large-scale production of this polymer.

Khardenavis et al., (2007) studied the biotechnological conversion of agro industrial wastes into PHB. Ramadas et al., (2009) reported PIUB production in B. sphaericus NCIM 5149 and optimized the bioprocess variables enhancing PHA production by central composite design. Nair et al., (2009) reported the accumulation of PHB in a phenol degrading
*Alcaligenes sp.* d2 under phenol stressed condition. Grafting of medium chain length polyhydroxyalkanoates into carboxymethyl chitosan (CMCH) using ceric ammonium nitrate as an initiator was done and its biodegradation studies were carried out by Nair *et al.*, (2009). These CMCH grafted mcl PHA is proposed to have applications in the field of medicine such as tissue engineering and drug-delivery systems.

In Kerala, research works related to the studies of PHAs including the isolation, characterization and bioprocess development of novel PHA producing microbes were extensively carried out in different research institutions Universities including Cochin University of Science and Technology, Cochin; Mahatma Gandhi University, Kottayam; NIIST, Trivandrum; Rajiv Gandhi Center for Biotechnology, Trivandrum; University of Kerala, Trivandrum etc. In India, majority of the research centers are fruitfully engaged in research works on biopolymers including PHAs. However, none of the research works have been extended into the large scale industrial production of PHAs. To date, there are no companies reported to be engaged in industrial production of any PHAs in India.
Materials and Methods
3.3 MATERIALS AND METHODS

The difficulty of screening and selecting a specific bacterium is that any bacterium chosen or screened will have positives and negatives associated with it. A variety of factors will be considered in the selection of the bacterium. For example, Gram-positive bacteria may be favored because of their ability to produce large amounts of peptidoglycan which can aid as a co polymer. However, Gram-negative bacteria have thinner cell walls and would be easier to get the PHAs extracted from, as well as produce higher PHA yields. In either case, the Gram-positive and/or the Gram-negative bacteria chosen will need to utilize specific sugars, be able to grow in non optimal pH, and able to produce high yield PHAs. In this chapter we will be discussing the methods used to screen the isolated cultures form the Rotating biological contactor, for the production of PHA.

In this work small amount of biofilm was scrapped from the Rotating biological contactor (RBC) treating washwater of Trichy distilleries and Chemical Limited, Sangillyandapuram, Tiruchirapalli, Tamil Nadu in a sterile glass containers, tapered tightly, processed for for various biochemical analysis and stored at −20°C for further analyses. All chemicals and reagents used in the study were procured from Merck chemicals, India and the composition for all the medium used for growing the strains were given in Appendix I separately.

3.3.1 Isolation of bacterial strains from the Rotating biological contactor (RBC):

A single colony on Luria Bertani medium (LB medium) was picked with a sterile toothpick into 50-mL demineralized water in a 1.5-mL Eppendorf tube.

Minimal Salts Medium (MSM) was prepared and 50ml of MSM broth was taken in each Erlenmeyer flasks and autoclaved at 121°C for 20 min. 100μl of fresh bacterial inoculum were inoculated in each flask and incubated for a week at 37°C to detect the accumulation of PHAs.

3.3.2 PHA production and extraction in shake flask cultures:

Two methods, namely the pretreatment and plating method and the direct plating method were applied for bacterial isolation. In the pretreatment and plating method, 1 g or 1 ml of the sample was added to 10 ml of nutrient broth (NB) under different pHs, NaCl concentrations and temperatures. Samples were incubated at 30°C and 37°C at 200 rpm for 24
h. Then 100 μl/mg of these pretreated samples were added to nutrient agar (NA) under the aforementioned conditions for 24 h.

In direct plating, 1 g or 1 ml of each sample was added to 10 ml sterile distilled water. One hundred microliters of the sample was spread directly on Nutrient agar (NA) plates under different experimental conditions (as mentioned above). Incubation was done at 37°C for 24 - 48 hrs. Single colonies of isolates were picked and streaked on fresh PHA detection agar plates. Sugars tested were glucose, xylose, arabinose, galactose, and mannose. The cells were grown over a 48 hour period at 30°C. PHAs were monitored using the Nile Blue A technique (0.5 μg/ml) (Spiekermann et al., 1999, Porwal et al., 2008, Chaudhry et al., 2011). After overnight incubation, plates were observed under UV light. Colonies producing fluorescence were purified and streaked on fresh NA plate, for further study.

The positive control was grown on Nitrate-limited minimal medium NRLM with fructose as both cultures were known to grow on this sugar; the negative control was NRLM (in Appendix I).

A 10 mL innoculum from a pre-culture with an optical density (OD) of 0.5 at 600 nm of strain 1.1 was added to 90 mL minimal medium with each different carbon source at 2 % in place of glucose in separate flasks with the remaining medium composition as described above. The flasks were incubated at 37°C for more than 3 days at 200 rpm. Samples were collected at 2 h intervals from 0 to 78 h. Cells were collected by centrifuging samples taken at regular intervals and drying at 55°C to constant weight. PHA was extracted from cultures using the sodium hypochlorite method of Ramsay et al., (1994). PHA content was determined by dividing the weight of PHA obtained after extraction by the cell dry weight (CDW) and expressed in terms of percentage content of CDW.

### 3.3.3 Nile blue A staining Staining techniques

Nile blue A staining Staining techniques found by Ostle and Holt, (1982), with some modification, in order to see which strains from ATCC produce the PHAs. The microscope used was a Zeiss Axioplan 2 with Acridine Orange filter set and differential interference contrast (DIC); the wavelength was 360-460nm, and UV intensity was 63-95%. This method allowed for quick identification and also allow for elimination of some bacterial species. The method also allowed for other potential species to move forward to the second objective of carbon source utilization and maximization of utilization of the sugars present in the biomass.
3.3.4 Extraction of PHA by osmotic cell lysis methodology:

PHA extraction was done by solvent extraction methods (Arnold et al., 1999). The broth containing bacterial cells were subjected to centrifugation at 10000 rpm for 15 min (Sorval, centrifuge). The pellet was suspended in distilled water at 5°C for 24 h. This condition ensures the lysis of cells. This lysed suspension was centrifuged at 5000 rpm for 30 min. The pellet was washed 5–10 times with the same volume of distilled water. Finally, a pure white substance was obtained. This indicates that, there were almost no cells or cell membranes left since they have freed from pink colour. The final pellet was dried in an oven at 80°C until a constant weight was attained. The white dust resulting from this treatment was dissolved in chloroform. Most of the material dissolved readily and the undissolved remains were removed by filtration. Finally, the chloroform was evaporated at room temperature and a thin film of polyhydroxyalkanoates was obtained.

Selection of the most suitable strain, only *Enterobacter aerogenes*, *E. cloacae* and *Escherichia coli* were chosen for further experimental work as these strains were able to utilize all the carbon sources tested when supplied one by one in the minimal medium; maximum cell density and PHA accumulation was achieved with various easily available cheap substrates as carbon source.

3.3.5 Extraction of PHA

The polymer was extracted from this dried bacterial biomass using various methods as described below.

a. **Extraction using dispersion of chloroform (CHCl₃) and sodium hypochlorite (NaOCl)**

In this method of extraction, two different hypochlorite concentrations, hypochlorite to chloroform ratios and incubation times were used. In the first method the dried bacterial biomass was incubated (orbital shaker from Stuart Scientific Orbital Shaker, S150) in a dispersion containing 30% NaOCl and CHCl₃ in a 1:1 ratio at 30°C for two and a half hours and 150 rev min⁻¹.

In the second improvised extraction method the dried bacterial biomass was incubated in a dispersion containing 80 % NaOCl and CHCl₃ in a 1:4 ratio at 30°C for two and a half hours and 150 rev min⁻¹. It was then centrifuged at 4000 g for 18 minutes following which three layers were formed. The topmost layer was that of hypochlorite, middle layer contained
the cell debris and the bottom layer was the CHCl$_3$ containing the dissolved polymer. Polymer was then precipitated by introducing this CHCl$_3$ layer into 10 volumes of ice cold methanol with continuous stirring.

b. Chloroform extraction

The dried biomass was incubated in CHCl$_3$ for 24 hrs at 30°C with vigorous shaking. The CHCl$_3$ solution was then concentrated using a rotary vacuum evaporator (Perkin Elmer USA) followed by polymer precipitation in 10 volumes of chilled methanol with continuous stirring.

3.3.6 Quantification procedures

a. Optical density (OD) and Dry cell weight (DCW)

As per the method reported by Grothe et al., (1999) and Patwardhan and Srivastava, (2004), optical density (OD) of the suitably diluted cell suspension was measured at 600 nm (Grothe et al., 1999) against a media blank in a spectrophotometer (Shimadzu UV-1700, Suzhou Instruments Manufacturing CO., Ltd., Suzhou, China). Dry cell weight was determined gravimetrically. Culture samples were centrifuged (4000 rpm, 15 min, 4°C) and the supernatant was refrigerated for further analysis.

The cell pellet was re-suspended in deionized water, recovered after re-centrifugation (4000 rpm, 15 min, 4°C), dried to constant weight (90°C, 24 h), cooled in a desiccator, and then weighed to determine Dry cell weight (DCW).

The following formula was used for the detection of percentage of PHA:

$$% \text{ PHA} = (\frac{\text{Amount of PHA}}{\text{Amount of biomass}}) \times 100$$

For PHA analysis, cells were harvested by centrifugation, washed twice in water, and lyophilized. The dried material was then weighed (approximately 15 to 30 mg) and transferred to a glass tube. The material was extracted four or five times with warm (65°C) methanol to remove lipids, free fatty acids, and acyl- CoA, including 3-hydroxyacyl–CoA, while PHA, which is insoluble in methanol, remains associated with the cells. After centrifugation and removal of the residual methanol, the material was suspended in 0.5 ml of chloroform to which 0.5 ml of methanol containing 3% sulfuric acid was added. The mixture was heated at 95°C for 4 h and cooled down on ice. One milliliter of 0.1% NaCl was added to each tube, and the mixture was vortexed vigorously and centrifuged at 5,000 rpm for 5 min.
The chloroform phase was harvested and dried over anhydrous MgCl₂. Identification of monomers present in plant PHA was facilitated by the use of commercial 3-hydroxy acid standards and purified bacterial PHAs.

3.3.7 PHA identification using Gas Chromatography–Mass Spectrometry (GCMS)

Samples for GC analysis were prepared as described by Riis and Mai, (1988). Forty mg each of dry cell mass obtained from natural carbon media resulting in optimized PHA production, was put in 50 ml sealable glass centrifuge tubes. Two ml 1,2-Dichloroethane (DCE), 2 ml propanol mixed solution (1 volume hydrochloric acid and 4 volume propanol) and 200 μl of internal standard solution prepared by adding 2.0 g benzoic acid in 50 ml propanol were added to each sample in a 50ml sealable glass centrifuge tube. The tubes were incubated in a convection oven for 2 h at 100°C and shaken once every 30 min. After cooling the tubes to room temperature, 4 ml deionized (DI) water was added and the tubes were shaken for 30 sec before allowing to settle by gravity. The bottom organic phase was directly injected into the GCMS.

Standards were prepared by dissolving 200 mg PHB extracted from dry cell mass of the optimized run using synthetic media, in a 10 ml volumetric flask by keeping in a convection oven for 2 h at 100°C. After cooling to room temperature, the solution was made up to 10 ml by adding DI water. Two hundred μl, 400 μl, 600 μl and 800 μl of this mixture were taken and treated in the same way with DCE, propanol and benzoic acid as mentioned above.

GC analysis for confirming the presence of PHA in the broth was carried out with the help of an Agilent 5975 GC-MS in electron ionization (EI) mode equipped with a HP-5MS 30m x 250um x 0.25um column with helium at a flow of 1 ml/min was used. Initial temperature of 50°C was held for 3 min before ramping to 325°C at 15°C/min and holding for 5 min. A 3 min solvent delay was used.

3.3.8 Confocal imaging of inclusion bodies of Enterobacter sp.

Thin smear of Enterobacter aerogenes and E. cloacae resulting in enhanced PHB production in various carbon sources of our study were placed on a slide and dried. The slide was then immersed in 1% Nile blue A aqueous solution in a shallow container and stained for 40 min by placing in a shaking incubator (55°C, 50 rpm). The slide was dried again before 5 μl of water was put on the slide and a cover slip placed over it. A confocal microscope (LSM
CARL ZEISS MICROIMAGING LLC., NY, U.S.A) with Zeiss Axio Observer Z1 inverted microscope and Zeiss Plan Apochromat 63x objectives (NA 1.4 oil immersion) was used to obtain images of inclusion bodies of *Enterobacter sp.* at the Cellular and Molecular Imaging Facility (CMIF) in the department of Plant Biology at NCSU. The inclusion bodies were excited at 488 nm (argon laser) and fluorescence emission was determined from 492 nm to 625 nm.

### 3.3.9 PHA inclusions detected by electron microscopy.

Wild-type and recombinant cells grown for 3 days in media containing oleic acid were analyzed by transmission electron microscopy (TEM). While both types of cells showed the presence of numerous oil bodies (given in R & D), only recombinant cells producing PHA showed the presence of small electron-lucent inclusions within membrane-bound organelles.

The apparent size of these inclusions is in the range of 0.1 to 0.2 µm in diameter. Both the size and general appearance of these inclusions, as seen by TEM, are very similar to PHA granules found in bacteria as well as in transgenic plants indicating that, similar to PHA synthesized in these other hosts, PHA produced in yeast accumulates in the form of inclusions.

### 3.3.10 Characterization and identification of bacteria

#### a. DNA Extraction

Microbial cells were harvested by centrifugation, and cell pellets were suspended in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) containing 2 mg of lysozyme per ml in a total volume of 5 ml. Samples were incubated at 30°C for 30 min, which was followed by addition of 1.2 ml of 0.5 M EDTA and protease (final concentration, 0.2 mg/ml) and an additional 30 min of incubation. After addition Cell Lysis buffer of 0.7 ml of 10% (wt/vol) sodium dodecyl sulfate (SDS), 1.8 ml of 5 M NaCl, and 1.5 ml of 10% CTAB-NaCl solution (10% [wt/vol] cetyl trimethyl ammonium bromide in 0.7 M NaCl), the mixtures were incubated at 65°C for 20 min. Samples were extracted with chloroform-isooamyl alcohol (24:1, by volume), and the supernatants were transferred to clean tubes. DNA was precipitated by addition of 0.6 volume of isopropanol and pelleted by centrifugation (48,000 x g, 15 min). DNA pellets were rinsed with 70% (wt/vol) ethanol, dried, dissolved in TE buffer, and quantified by absorption spectrophotometry at 260 nm (Jensen *et al.*, 1991).
During the extraction of the PHA from the cultivated bacterial strains, there are some precaution procedures that must be taken seriously. Because the extraction used solution that are dangerous which are some volatile and corrosive, the use of lab coat, latex glove and mouth mask must be used. Some solution such as hot chloroform, ethanol, and concentrated sulfuric acid must be handling with care.

The addition of sulphuric acid in the last step of extraction of the PHA is very concentrated and it cannot be read by UV-Spectrophotometer at 235nm. So, the concentrated sulfuric acid that contains PHA must be diluted first. Silica cuvette was used because the normal cuvette can react with the sulfuric acid and can interfere with the reading of UV-Spectrophotometer.

a. **Primer details and PCR conditions.**

Primers (100 ng each forward and reverse primer) designed to anneal to conserved regions in cyanobacteria were used to amplify bacterial methyl transferase gene in Enterobacter species. Each 50 µl PCR mix contained ~50-100 ng DNA template, 0.1 picomole each forward and reverse primer, 0.25 mM dNTPs (Invitrogen), 2.5 mM MgCl₂ and 2.5 U Platinum Taq DNA polymerase (Invitrogen) in the manufacturer’s buffer. The reaction was run at 95°C for 5 min initial denaturation followed by 95°C for 1 min denaturation, 1 min annealing at 59°C, 1 min extension at 72°C and a final extension at 72°C for 5 mins for 30 cycles. A reaction mixture without DNA template was used as negative control. PCR products were routinely visualized by running 10 µl of PCR mixture on 1% agarose gels (Bio-Rad, Richmond, CA) in 0.59 Tris–borate–EDTA (TBE) buffer stained with ethidium bromide (0.0001%).

At the end of one cycle, the targeted sequences on both strands have been copied. When the three step cycle is repeated, the two strands from the first cycle are copied to produce four fragments. These are amplified in the third cycle to yield eight double stranded products. Thus each cycle increases the number of target DNA molecules exponentially. After approximately 30 cycles of PCR, the DNA region flanked by the primers will have been amplified approximately a billion-fold (Jensen et al., 1991). For identification of related sequences, a database alignment using BLAST algorithm was performed.
Table 3.3.1 Steps involved in PCR Reaction

<table>
<thead>
<tr>
<th>First step</th>
<th>The target DNA containing the sequence to be amplified is heat denatured to make it single stranded.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Second step</td>
<td>The temperature is lowered so that the primers can hydrogen bond or anneals to the DNA on the both sides of the target sequence. Because the primers are very small and are present in excess, the targeted DNA strands anneal to the primer rather than to each other.</td>
</tr>
<tr>
<td>Final Step</td>
<td>DNA polymerase extends the primers and synthesizes copies of the target DNA sequence using dNTPs. Only polymerase able to function at high temperatures employed in the PCR technique can be used.</td>
</tr>
</tbody>
</table>

3.3.11 Multiple sequence alignment and phylogenetic tree construction

All the nucleotide sequences were converted into PASTA format and multiple sequence alignment for the assembled nucleotide sequences was done by using the Clustal X program (Thompson et al., 1997) in BIOEDIT software (Hall, 1999). Aligned sequences were imported into an MEGA5: Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0 (Tamura et al., 2007) software for further analysis. The ends of the alignment were trimmed to obtain equal lengths for all sequences and the aligned sequences were converted into MEGA format for carrying out phylogenetic analysis. The phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) using nucleotide based TN84 evolutionary model for estimating genetic distances based on synonymous and nonsynonymous nucleotide substitutions. Statistical support for branching was estimated using 1000 bootstrap steps.

3.3.12 Production of PHA – Overall view

The production of PHAs involves four major steps (Figure 3.3.1), these are:

1. Culturing of the organisms in suitable growth and PHA production medium.
2. Harvesting of the cultures at desired specific time points and then lyophilisation.
3. Extraction of PHAs from the dried bacterial biomass.
4. Characterisation of PHAs.
Figure 3.3.1 Flowchart for the production of PHAs.
Results and Discussions
3.4 RESULTS AND DISCUSSIONS

It is less well known that PHA production by the bacterial community also is used for the manufacture of biodegradable plastics. Therefore, the goal of this work was to improve PHAs production by isolation and selection for prominent PHA-producing bacteria from natural sources like biofilm of RBC treating wash water from a distillery unit under various selective conditions. The superior isolates were selected and identified for their potential to produce new PHAs containing new monomeric constituents using inexpensive carbon sources. Also, this study is different from other studies in another aspect. Strains of Enterobacter aerogenes and E. cloacae isolated from biofilm of RBC treating washwater from the Trichy distilleries and Chemical Ltd, Sangilliyanapuram, Trichy, Tamilnadu were firstly presented among PHA synthesizing bacteria utilizing easily available cheap carbon sources.

3.4.1 Screening of potential PHA producer

Totally 5 bacterial strains were isolated from the biofilm of Rotating Biological Contactors (RBCs), of which only Enterobacter aerogenes, E. cloacae and Escherichia coli showed well flourished growth. While analyzing for the other properties that these organisms posses apart from this biodegradation, it was found out that they have an interesting character of synthesizing PHA under stress conditions. So we carried out further study based on this property which plays an important role for finding out a solution to plastic pollution. To get an overview of the occurrence and diversity of polyhydroxyalkanoate-producing bacteria, they are grown in PHA detection agar (PDA Agar). Glucose and sucrose were used as carbon sources for screening and optimization experiments. PHA producers were detected macroscopically by observing the turbidity on PDA. Producers were further screened by Sudan black B staining (Anderson & Dawes, 1990) and Nile blue A staining (Wang & Bakken, 1998). Viable colonies were directly observed under UV light and fluorescence to detect the accumulation of PHAs (Spiekermann et al., 1999) for taking photographs. Enterobacter aerogenes and E. cloacae showed characteristic granules, indicating the possible presence of PHA(s) in the cells. This could possibly be due to the ability of the strain to metabolize glucose efficiently and result in to the production of high biomass with the accumulation of PHA. On the other hand, although E. coli does not naturally produce PHA, this bacterium is considered to be appropriate host for generating higher yields of the
biopolymer because of its fast growth and the ease with which it can be lysed. Recombinant
*E. coli* cultured under optimal conditions has been shown to accumulate PHB up to 85% of
the cell dry weight. The morphology and biochemical characteristics of the isolate then are
observed. The PHAs producing strains can be identified by sequencing (partial sequences) of
their 16S rDNA as described by Edwards *et al.*, (1989).

**3.4.2 Screening of PHA granules using Nile Blue A staining method and TEM**

Our strain, *Enterobacter aerogenes* and *E. cloacae* tested positive for presence of
lipophilic PHA granules (positive Nile blue A staining). PHA granules were observed as
Pink/orange florescence under UV light by PHA producer isolate *Enterobacter aerogenes &
E.cloacae* by using Nile blue A staining method (Plate 4.1, 4.2 & 4.3). The PHA granules in
*Enterobacter aerogenes* and *E. cloacae* were photographed with a TEM (Plate 3.4.8).

![Plate 3.4.1 Colony morphology of PHA producing bacteria on PDA medium.](image)

A – *Enterobacter aerogenes*; B – *E.cloacae*; C - Control
Plate 3.4.2 (A) Pink/orange florescence under UV light by PHA producer isolate Enterobacter aerogenes & E. cloacae with Nile blue A staining by viable colony method.

Plate 3.4.3 (B) No florescence under UV light by Non-PHB producer with Nile blue A staining by viable colony method.
Plate 3.4.4 *Enterobacter* strains accumulating PHAs stained with Nile blue A (arrow) after culturing on modified complex and minimal medium at 30°C for 48hrs observed under fluorescence microscope.

Plate 3.4.5 Microscope image of the strain *Enterobacter* sps. with polymer inclusions when grown on dodecanoate
Plate 3.4.6 PHA production by *E. sp.* grown in a Nitrate-limited Minimal medium with glucose (NRLM-G) at pH 6 and 30°C. Cells were observed during stationary phase with Nile Blue A: (A) using differential interference contrast microscopy (400X cells appear green) and (B) epifluorescence microscopy 1000X.

Plate 3.4.7 Cells with PHA inclusions inside
Plate 3.4.8 Observation of PHA granules in *Enterobacter species* under phase-contrast and transmission electron Microscope (TEM).

Plate 3.4.9 PHA granules in cell stained with Nile blue A and observed under simultaneous fluorescent (1a) and visible light (1b) (*Enterobacter strain*)
3.4.3 *Enterobacter sp*– DNA isolation and Gel picture:

Lane-1: 1kb ladder

Lane-2, 3: Approx 2.1 Kbp PCR product

The amplification yields the partial gene of methyl transferase, which indicated the presence of the gene in the organism of study.

3.4.4 Observation of PHA granules

PHA granules have been recognized by their affinity for the dye Sudan black B and Nile Blue A, which is a presumptive test for the presence of PHA (Smibert and Krieg, 1981). Therefore, the colonies grown on biofilm of RBC treating wash water in distillery (with 1.5% agar) were stained with Nile Blue A using thin smears (Weibuli, 1953). The stained preparations were examined under the microscope to determine cellular PHA accumulation in this study. After the detection of PHA production capability of our strain by Nile Blue A staining method, it was confirmed using transmission electron microscopy (TEM). The sample preparation for TEM was done as described by Chien *et al.*, (2007). Bacterial cells in
broth at stationary phase were centrifuged (at 8,000 g for 13 min), washed and fixed with in potassium phosphate buffer (pH 7.0) and then prefixed with 4% paraformaldehyde and 2.5% glutaraldehyde (Michael Knoll et al., 2009). The Strain cells were then fixed with 1% osmium tetroxide and 1.5% potassium ferricyanide in phosphate buffer (pH 7.0), stained with 1% uranyl acetate and examined under the TEM (Jeol 100 SX).

3.4.5 Determination of biomass

The biomass was harvested by centrifuging 100 ml of culture (10,000 rpm, 15 min, 4°C) and the cell pellet was washed in distilled water and dried to constant weight (105°C, 24 h) (Ram Kumar Pandian et al., 2009). Biomass of PHA accumulated in Enterobacter aerogenes and E.cloacae was discussed in Results and Discussions of Chapter 5.

3.4.6 PHA extraction and quantification

The method used for PHA extraction and quantification was adapted from the gravimetric method employed by Kim et al., (1994). Once the final pellet of PHA (shown in Plate 3.4.11 & 3.4.12) was obtained and weighed, the yield of PHA could be estimated.

Quantification of polyhydroxyalkanoates was performed by sodium hypochlorite method (Berger et al., 1989).

Plate 3.4.11 Three different phases appeared after centrifuge
Plate 3.4.12 PHA pellet obtained from our carbon source

3.4.7 Identification analysis of PHA samples using confocal microscope and Gas Chromatography–Mass Spectrometry (GC-MS)

Inclusion bodies in cells from fermentation broth of all carbon sources in our study with partial nutrient addition was visually examined using confocal microscopy. PHA inclusion bodies can be distinguished in all the cells which appear as green fluorescence (Plate 3.4.13). The images in this study were similar to the ones taken by Ostle and Holt, (1982), for PHA granules in *Azotobacter chroococcum* stained with Nile blue A. The size (~2 μm in diameter and 2.5 μm in length) and shape of *A. latus* is consistent with that observed by Palleroni and Palleroni, (1978) and Holt *et al.*, (1994). Visual estimation of the percentage of cells with PHA inclusion bodies using Skimmed milk and latex from *Calotropis* and *Opuntia*, and banana powder as carbon sources are (86.59% of Dry Cell Weight (DCW), 83.25% of DCW, 82.84% of DCW and 81.52% of DCW respectively. PHA obtained by all the media with partial nutrient addition was confirmed by GC analysis.
Plate 3.4.13 PHA inclusion bodies in *Enterobacter species* obtained from one of the carbon Media of our study, stained with Nile blue A (top left), digital image correlation (top right) and overlay of both (bottom left) observed with a confocal microscope. Scale bar, 10.0 μm.

PHA inclusion bodies in *Enterobacter sp.* obtained from skimmed milk powder, stained with Nile Blue A (top left), digital image correlation (top right) and overlay of both (bottom left) observed with a confocal microscope. Scale bar, 10.0 μm.

Anthony Paladino, (2009) successfully extracted PHAs and peptidoglycan from the cell of *Bacillus megaterium* a Gram-positive PHA-producing bacterium.

Nur Ceyhan and Guven Ozdemir, (2011) isolated and identified *Enterobacter aerogenes* (designated *E. aerogenes* 12Bi) by using biochemical and phylogenetic characterization from domestic wastewater which produces PHA.

PHA has been industrially produced by pure cultures including *A. latus, A. vinelandii, P. oleovorans*, recombinant *A. eutrophus* and recombinant *E. coli* (Grothe *et al.*, 1999).

Nighat *et al.*, (2011) isolated and purified twelve different genera from the samples collected from Molasses, oil/ghee and sewerage contaminated soils. They reported biochemically some of the PHA producers showed affiliation to *Citrobacter, Listeria, Streptococcus,*
Enterobacter and Escherichia species. In soil samples contaminated with molasses (sugar industry waste), 95% and in samples contaminated with oil/ghee industry waste all bacterial isolates showed PHA accumulation ability.

The following Table shows PHAs contents accumulated by various microorganisms. It was found that Alcaligenes spp. contains a maximum of 96% PHAs in its cell.

**Table 3.4.1 Variations of PHAs contents in various microbial strains**

<table>
<thead>
<tr>
<th>Microbial strain</th>
<th>Weight/ dry cell mass (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ralstonia eutropha</em> (reclassified Alcaligenes eutrophus)</td>
<td>96</td>
</tr>
<tr>
<td>Azospirillum</td>
<td>75</td>
</tr>
<tr>
<td>Azotobacter</td>
<td>73</td>
</tr>
<tr>
<td>Baggiatoa</td>
<td>57</td>
</tr>
<tr>
<td>Leptothrix</td>
<td>67</td>
</tr>
<tr>
<td>Methylcystis</td>
<td>70</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>67</td>
</tr>
<tr>
<td>Rhizobium</td>
<td>57</td>
</tr>
<tr>
<td>Rhodobacter</td>
<td>80</td>
</tr>
</tbody>
</table>

(Available from www: [http://members.rediff.com/jogsn/bp6.htm](http://members.rediff.com/jogsn/bp6.htm))

Kuno Jung, (1999) synthesized Poly (3-hydroxyalkanoates) (PHAs) from organic solvents by *Pseudomonas oleovorans*.

Supporting data for PHA production in telluric environments were provided by Wang and bakken (1998), who screened 63 soil bacteria for PHA production.

Attia et al., (2010) isolated and screened 30 PHA producing bacterial isolates sofor from contaminated urban and hilly areas which were Gram negative and belonged to *Pseudomonas, Citrobacter, Klebsiella, Escherichia and Enterobacter* genera.

Chenyu et al., (2012) in their work utilized *Azotobacter vinelandii* for polyhydroxyalkanoates Production From Low-cost Sustainable Raw Materials like sugar beet molasses.

Wu et al., (2001) isolated *Bacillus sp.* JMa5 from molasses contaminated soil for PHB production using sugar cane molasses.

Narancic et al., (2012) reported that only one isolate, *Pseudomonas sp.* TN301 among seven bacterial strains from the river sediment exposed to petrochemical industry effluents could accumulate mcl-PHA from naphthalene to 23% of cell dry weight.

In recombinant *E. coli*, the potential of PHA synthesis from whey has also been exploited using various common PHA producing bacteria, such as *Ralstonia eutropha* DSM545 (Marangoni et al., 2002), *Pseudomonas hydrogenovora* (Koller et al., 2008), *Thermus thermophilus* HB8 (Pantazaki et al., 2009) and wild strains, such as *Methyllobacterium sp.* ZP24 (Yellore and Desai, 1998 and Nath et al., 2008) and *Hydrogenophaga pseudoflava* DSM1034 (Koller et al., 2011).

Antony et al., (2011) reported the accumulation of 94% of PHA/dry weight of the organism *Enterobacter cloacae* SU – I grown in 8gm/l lactose – containing medium.

Paramjeet Khandpur et al., (2012) extracted Polyhydroxyalkanoates (PHA) from *Pseudomonas aeruginosa* and *Bacillus subtilis*.

**Vijaya Abinaya et al., (2012)** isolated Polyhydroxyalkanoates (PHA) accumulating bacteria were from rubber plant growing areas of Kerala. They identified *Bacillus cereus* yielded PHA of 0.436 g/l, amounting to 13.77% (w/w) of dry cell weight.

Majid, (2008) in her work screened eleven strains of bacterial isolated from activated sludge in PHA-producing reactor for their ability to produce PHA in a minimal medium supplemented with glucose as carbon source and grow at 30°C.

Usman Arshad et al., (2007) demonstrated that the 9 strains that were selected from contaminated soil namely, *Pseudomonas, Citrobacter, Enterobacter, Klebsiella, Escherichia* and *Bacillus* genera were all PHA producers at varying levels.

Ilona Gasser et al., (2009) in their work confirmed the hypothesis that the rhizosphere is an interesting hidden reservoir for PHA producers.

Annarita Poli et al., (2011) reported the composition, biosynthesis, and production of PHAs by different archaeal species.
Yu-Hong Wei et al., (2011) in their work determined the Optimal carbon/nitrogen ratio for PHB production in *Cupriavidus taiwanensis* 184, 185, 186, 187, 204, 208, 209 and *Pseudomonas oleovorans* ATCC 29347.

Sreya Kumbhakar et al., (2012) carried out Screening of root nodule bacteria for the production of PHA and the study of parameters influencing the PHA accumulation.
Conclusion
3.5 CONCLUSION

The goal of this work was to improve the production of polyhydroxyalkanoate (PHA) by isolation and selection of prominent PHA producing bacteria from natural sources like biofilm of rotating biological contactor (RBC) treating wash water from Trichy Distilleries and chemicals limited, Sangaliyandapuram, Trichy under various selective conditions. Apart from reducing the organic load in washwater released from the distillery unit, through literature review it was evident that both the strains *Enterobacter aerogenes* and *E. cloacae* present in the biofilm of RBC had another interesting property of synthesizing PHA under nutrient limitation. Thus in the present work, *Enterobacter aerogenes* and *E. cloacae* were screened extensively for PHA production The superior isolates were selected and identified for their potential to produce PHAs by growing them in PHA detection Agar (PDA). PHA granules have been recognized by their affinity for the dye Nile Blue A which confirms the presence of PHA in the above mentioned two strains. After the detection of PHA production capability of these two strains by Nile Blue A staining method, it was confirmed using transmission electron microscopy (TEM).