ISOLATION, SCREENING AND SELECTION OF EFFICIENT POLY-β-HYDROXYBUTYRATE (PHB) SYNTHESIZING BACTERIA

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I. 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 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.

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 (Steinbüchel, 1991). Amongst these, PHAs are of particular interest because they possess thermoplastic characteristics and resemble synthetic polymers to a larger extent.

Plastics produced from PHAs have been reported to be truly biodegradable in both aerobic and anaerobic environments (Page, 1995), unlike many of the "so-called" biodegradable plastics made synthetically. PHAs are composed mainly of poly-beta-hydroxybutyric acid (PHB) and poly-beta hydroxyvaleric acid (PHV), although other forms are possible. More than 80 different forms of PHAs have been detected in bacteria (Lee, 1996). Only two forms of PHAs, *i.e.*, PHB homopolymer and 3HB-3HV copolymer are commercially produced by Zeneca, United Kingdom.

Lafferty *et a*l. (1988) stated that the accumulation of PHA by microorganisms can be stimulated under unbalanced growth conditions, *i.e.*, when nutrients such as nitrogen, phosphorus or sulfate become limiting, when oxygen concentration is low, or when the C:N ratio of the feed substrate is higher. PHB is accumulated by numerous microorganisms and is the best characterized PHA (Madison and Huisman, 1999). PHB was first discovered in bacteria (Lemoigne, 1926). It is an unique intracellular polymer accumulated under stress conditions but with excess carbon source. During strvation, PHA serve as carbon and energy source and is rapidly oxidized thereby retarding the degradation of cellular components, combating the adverse conditions as in rhizosphere (Okon and Itzigsohn, 1992). A number of bacteria such as *Azotobacter*, *Bacillus,* Archaebacteria, Methylobacteria, *Pseudomonas* have been found to synthesize PHA to varying levels. *Ralstonia eutropha* (formerly *Alcaligenes eitrophus*) has been the subject of much published research work because it can accumulate PHAs upto 80 per cent dry weight (Lee, 1996). Though accumulation of PHA is not a prerequisite, they are reported to assist the sporulation process in *Bacillus* spp. (Cool and Canon, 1999). In nitrogen fixing, PHA accumulating bacteria, *Azospirillum* (Okon and Itzigsohn, 1992) and *Azotobacter* sp. (Page, 1992) fixed nitrogen even in the absence of exogenous carbon. PHA, as a reduced compound, acted as a sink for excess electrons, especially in nitrogen fixing bacteria by providing protection to the oxygen-labile nitrogenase (Page*,* 1992; Okon and Itzigsohn, 1992).

The production of biodegradable plastics on a large scale is limited because of the relative expense of the substrate and low polymer production. According to Yamane (1993), the higher production costs, especially raw material costs, make it difficult for PHA plastics to compete with conventional petroleum-based plastics in the commercial market place. Hence, alternative strategies for PHA production are being investigated. PHA production costs could be reduced by several means by using cheaper substrates such as starch, whey (Kim *et al.,* 1994) or enhancement of product yield eg., by using recombinant *E. coli* (Lee, 1996). There have been some investigations on the possibility of producing PHB in transgenic plants (Lee, 1996; Nawrath *et al.,* 1994)

PHAs are natural thermoplastic polyesters, which can be used for manufacture of disposable items such as razors, utensils or different personal hygiene products (Lee, 1996). They can be used in the manufacture of latex paints (Steinbüchel and Hein, 2001). PHAs also

promise to be a new source of small molecules, some of which have potential applications as biodegradable solvents. These include β-hydroxy acids, 2-alkenoic acids, β-hydroxyalkanols, β-acyllactones, β-amino acids, and β-hydroxyacid esters (Williams and Peoples, 1996). PHAs, being biodegradable and biocompatible, have applications in medical therapeutics. PHAs can be used to fabricate three-dimensional, porous, biodegradable heart valve scaffold (Sodian *et al.,* 2000), bone fracture fixation (Galego *et al.,* 2000), manufacture of surgical pins, sutures, staples, swabs, fixation rods and cardiovascular stents (Scholz, 2000). PHAs can be used as carriers for long term slow release of drugs, insecticides, herbicides and fertilizers and in wound dressing (Lee, 1996).

The success in the biodegradable plastic strategy largely depends on the isolation of potent PHA producing bacteria and optimizing culture parameters for maximum PHA biosynthesis.

Keeping these points in view, the following objectives were addressed in the present study.

1. Isolation of PHB producing bacteria from different environmental samples.
2. Screening for high PHB producers from the isolated bacteria.
3. Optimization of cultural parameters for maximum PHB yields.
4. Use of cheaper substrates for production of PHB.
5. Scale up studies.

II. REVIEW OF LITERATURE

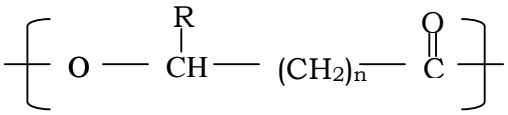
Poly-□-hydroxybutyrate (PHB) is a natural, biodegradable polymer accumulated in the form of intracellular granules by a large variety of bacteria. The polymer can be made in to films, fibers, sheets and even moulded in to the shape of a bag and bottle, besides special applications in medical industry as absorbable medical implants. The main factor preventing large scale production and commercialization of PHB is its high cost of production. Use of less expensive substrates, improved cultivation strategies and easier down stream processing methods are required for reducing the cost. The current status of the research on this subject is highlighted herein.

2.1 POLYHYDROXYALKANOATES (PHA)

PHAs are among the most investigated biodegradable polymers in recent years. They are superior to other biodegradable polymers because of the large number of different monomer constituents that can be incorporated. At present, about 150 different hydroxyalkanoate units have been identified. The most common is poly-□– hydroxybutyrate (PHB).

The PHA are non-toxic, biocompatible and biodegradable thermoplastics that can be produced from renewable resources. They have a high degree of polymerization; are highly crystalline, optically active, isotactic, piezoelectric and insoluble in water. These features make them highly competitive with polypropylene, the petrochemically derived plastics.

PHAs are polyesters of hydroxy alkanoates with the general structural formula as shown in Fig. 1.



n varies from 600 to 35000

R = hydrogen Poly (3-hydroxypropionate)

R = methyl Poly (3-hydroxybutyrate)

R = ethyl Poly (3-hydroxyvalerate)

R = profile Poly (3-hydroxyhexanoate)

R = pentyl Poly (3-hydroxyoctanoate)

R = nonyl Poly (3-hydroxydodecanpate)

Fig. 1 Structure of PHA (Lee, 1995)

2.1.1 Poly □-hydroxybutyrate (PHB)

The first and the best known member of PHA, the homopolymer poly(3-hydroxybutyrate) (PHB) was discovered by Maurice Lemoigne in 1925 (Jackson and Srienc, 1994). Approximately 150 different hydroxyalkanoic acids are at present known, as constituents of bacterial storage polyesters (Steinbüchel and Valentin, 1995). PHAs, synthesized by many Gram positive and Gram negative bacteria as storage compounds, are deposited as insoluble inclusions in the cytoplasm (Steinbüchel, 1991). These water insoluble PHAs exhibit rather high molecular weights, thermoplastic and/or elastomeric features and some other interesting physical and material properties. Plastics produced from PHAs have been reported to be truly, fully biodegradable (Page, 1995). Microbes belonging to more than 90 genera including aerobes, anaerobes, photosynthetic bacteria, archaebacteria and lower eukaryotes are able to accumulate and catabolise these polyesters.

PHB is an aliphatic homopolymer of poly-□-hydroxibutyric acid with a melting point of 179 ℃ and is highly crystalline (80%). It can be degraded at the temperature above its melting point. PHB has some properties similar to polypropylene with three unique features: thermoplastic processability, 100% resistance to water, and 100% biodegradability (Hrabak, 1992).

Byrom (1990) summarized two major advantages of the PHB-PHV copolymer over the PHB homopolymer. Firstly, the copolymer has a lower melting point. However, HV content in the copolymer does not significantly decrease the temperature at which molecular weight degradation occurs. This important feature allows the copolymer to be processed with a larger range of temperature conditions. Secondly, it has a lower flexural modulus or level of crystallinity, which makes it tougher and more flexible. However, a disadvantage of having a low crystallization is that it takes a longer cycle time in the processing step, e.g., the injection-moulding process (Hrabak, 1992).

PHAs are a family of optically active biological polyesters, containing *(*R)-3HA monomer units (Anderson and Dawes 1990). The 3-hydroxyalkanoic acids are all in the *R* configuration due to the stereo specificity of the polymerizing enzyme, PHA synthase. At the C-3 or β- position, an alkyl group, which can vary from methyl to tridecyl is positioned. This alkyl chain can be saturated, aromatic, unsaturated, halogenated or with branched monomers (Garcia *et al.,* 1999; Arkin *et al.* 2000).

2.1.2 Classification of PHA

PHAs are classified as scl – PHAs, mcl – PHAs and lcl – PHAs based on the length of the hydroxyalkanoic acid monomers (Steinbüchel and Pieper, 1992). Scl – PHAs are composed of C3 to C5, 3-hydroxy/4-hydroxy fatty acids. Poly(3-hydroxybutyrate) (PHB), the first of the PHAs studied extensively, falls in this group of PHAs. mcl – PHAs are composed of C6 to C16, 3-hydroxy fatty acids. This group includes polymers such as poly(3-hydroxyhexanoate) P(3HHx), poly(3-hydroxyheptanoate) P(3HHp), poly(3-hydroxyoctanoate) P(3HO), poly(3-hydroxydodecanoate) (3HDD) etc., The term lcl – PHAs is reserved for the PHAs composed of more than C163-hydroxy fatty acids. These classes are also referred to as scl-, mcl- and lcl-PHAs.

2.1.3 Importance of PHB to microorganisms

PHB granules act as energy reserve materials when nutrients such as nitrogen and phosphorous sources are available in limiting concentrations in the presence of excess carbon source.

Senior and Dawes (1971) proposed that PHAs could serve as a sink for reducing power and, therefore, be regarded as a redox regulator within the cell.

Encystment of *Azotobacter* cells in lab conditions was linked to PHB accumulation (Stevenson and Socolofsky, 1973). However, later it was shown that in natural habitats, accumulation of large amounts of PHB was not an absolute requirement for encystment (Thompson and Skerman, 1979).

Anderson and Dawes (1990) observed that the presence of PHB in a cell retarded the degradation of cellular components such as RNA and proteins during nutrient starvation frequently, but not universally. PHB enhanced survival of some but not all the bacteria and also served as a carbon and energy source for spore formation in *Bacillus* sp.

Steinbuchel (1991) reported that PHAs were stored as intracellular cytoplasmic inclusions. And, these inclusions, as a reduced polymer, provided the advantage that they become unavailable as a carbon source for the competing organisms, and that they were osmotically inert and did not, therefore, affect the osmotic pressure of the cells.

Steinbuchel (1991) reported that the PHA biosynthesis in *Ralstonia eutropha* and *Rhodospirillum rubrum* included pyridine nucleotide dependent reduction of acetoacetyl-CoA. Thus, PHB is a sink for reducing equivalents and can be considered to be a fermentation product.

By contrast, in *Pseudomonas oleovorans* and *P. aeruginos*a, oxygen deficiency exerted a negative effect on the accumulation of PHA. Because in these organisms either reducing equivalents were formed or energy in the form of ATP was consumed.

In *Rhizobium* and *Bradyrhizobium japonicu*m, during the bacteroid stage, the nitrogen fixation apparatus competed with PHB formation for reducing equivalents (Povolo *et al.,* 1994).

2.1.4 Biosynthesis of PHA

PHA production starts in response to stress imposed on cells, usually by nitrogen or phosphorus limitation, although in the presence of abundant carbon source. Under these

conditions (PHA accumulation phase), the cells do not grow or divide but instead divert their metabolites towards the biosynthesis of hydoxyalkyl-CoA (HA-coA). HA-CoA is an activated monomeric precursor that is polymerized by the enzymatic action of PHA synthase to form a PHA polyester. Being insoluble in water, PHA begins to form amorphous and nearly spherical granules that gradually fill the cells and force them to expand. The final number of PHA granules in a typical PHA producing cell of *Ralstonia eutropha* was ten (Anderson and Dawes, 1990; Ballard *et al.,* 1987) and the diameter was about 500 nm when growth ceased.

Most of the organisms synthesize PHA using *R. eutropha* PHA biosynthetic pathway. The biosynthesis pathways of *R. eutroph*a, *Zoogloea ramiger*a and *Azotobacter beijerinckii* are well established (Doi, 1990). Firstly, a substrate is condensed to acetyl-coenzyme A (acetyl-CoA). Two moles of acetyl-CoA are then used to synthesize one mole of PHB.

Rhodospirillum rubrum PHA biosynthetic pathway is similar to the *R. eutropha* pathway but two enoyl-CoA hydratases are involved in the second step of catalyzing the conversion of L-3-hydroxybutyryl-CoA to D-3-hydroxybutyryl-CoA via crotonyl-CoA (Anderson and Dawes, 1990; Doi, 1990; Lee, 1996).

Pseudomonas oleovorans PHA biosynthetic pathway is found in *P. oleovorans* and most *Pseudomonas* from the rRNA homology group I (Lee, 1996). They produce medium-chain-length (MCL) PHAs from MCL-alkanes, alcohols, or alkanoates. According to Doi (1990), production of short-chain-length (SCL) PHAs, i.e., PHB homopolymer and PHB-PHV copolymer could also be produced by these organisms but the production were less than 1.5%. This PHA biosynthesis involves the cyclic-□-oxidation and thiolytic cleavage of fatty acids, *i.e*., 3- hydroxyacyl-CoA, and intermediates of the □-oxidation pathways are used for PHA biosynthesis.

Most pseudomonads from the rRNA homology group I except *P. oleovorans* also produce MCL-PHAs using *P.aeuroginosa* PHA biosynthetic pathway. The pathway used in these organisms is called the *P. aeruginosa* PHA biosynthetic pathway. Steinbuchel (1996) reported that MCL-PHAs produced by this pathway were from unrelated substrates, e.g., gluconate or actetate. PHA was synthesized from acetyl-CoA via fatty acid synthetic pathways.

Wang and Lee (1997) have classified bacteria that are used for the production of PHAs into two groups, based on the culture conditions required for PHA synthesis. The first group that includes *Ralstonia eutropha*, Methylotrophs, and *Pseudomonas*, require the limitation of an essential nutrient element in the presence of an excess carbon source for efficient synthesis of PHAs. The second group which includes *Alcaligenes latu*s, *Azotobacter vinelandii* and recombinant *Escherichia coli*, does not require nutrient limitation for PHA synthesis and can accumulate PHA during growth. However, even in *Alcaligenes latus* PHB synthesis is enhanced by nitrogen limitation suggesting that this growth during PHA accumulation is not an efficient process.

2.15 Genetic basis for PHA formation

Umeda *et al.* (1998) reported that in *Acinetobacter sp., Alcaligenes latus, Pseudomonas acidophila,* and *Ralstonia eutropha,* the phbCAB genes are in tandem on the chromosome although not necessarily in the same order. In *Paracoccus denitrificans, Rhizobium meliloti* and *Zooglea ramigera*, the *phb*AB and *phb*C loci are unlinked. The PHA polymerase has two subunits in *Chromatium vinosum, Thiocystis violacea*, and *Synechocystis* encoded by *phb*E and *phb*C genes (Hein *et al.,* 1998).

The PHB biosynthetic genes *phbA* (for 3- ketothiolase), *phbB* (NADPH-dependent acetoacetyl-CoA reductase), *phbC* (PHB synthase) from acetyl-CoA are clustered and are presumably in one operon *phb*CAB. The loci encoding the genes for PHA formation have been characterized from 18 different species (Madison and Huisman,1999). The diversity of P(3HB) biosynthetic pathways implies how far the *pha* loci have diverged. The *phb* (genes encoding enzymes for scl-PHA) and *pha* (genes encoding enzymes for mcl-PHA) are not necessarily clustered and the gene organization varies from species to species.

2.1.6 Enzymology of PHB production

The biosynthetic pathway of PHB consist of three reactions catalyzed by three different enzymes (Fig. 2). The first reaction consists of the condensation of two acetylcoenzyme A (acetyl-CoA) molecules in to acetoacetyl-CoA by β-ketoacylCoA thiolase

(encoded by *phb*A). The second reaction is the reduction of acetoacetyl CoA to (R) -3-hydroxybutyryl-CoA by an NADPH dependent acetoacetyl–CoA dehydrogenase (encoded by *phb*B). Lastly, the (R)-3-hydroxybutyryl-CoA monomers are polymerized in to PHB by P (3HB) polymerase or synthase, encoded by *phb*C (Huisman *et al.,* 1989).

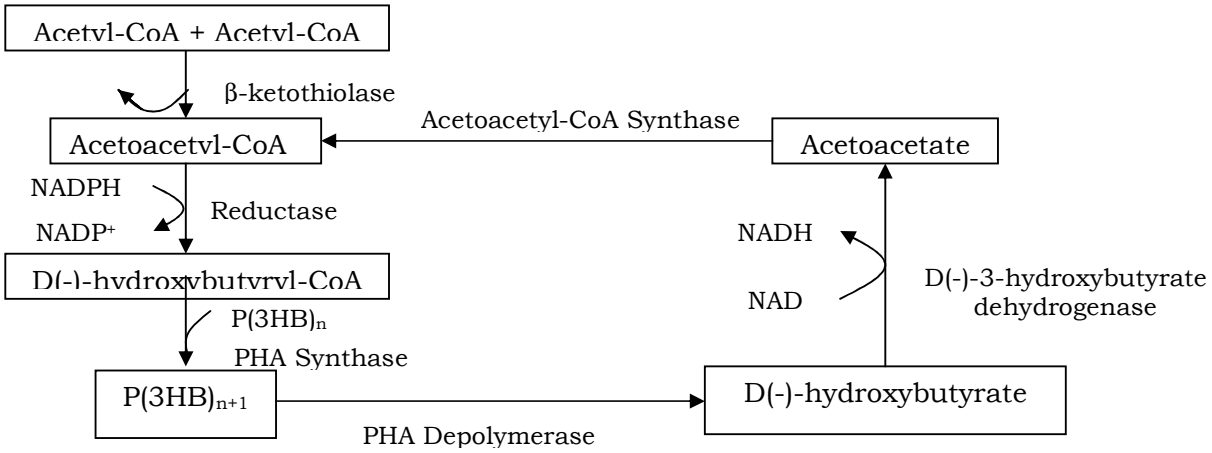


Fig. 2 : Metabolic pathway involved in the synthesis and breakdown of PHB in *R. eutropha* (Lee *et al.,* 1994)

2.2 ISOLATION OF PHB PRODUCING MICROORGANISMS

2.2.1 PHB production by bacteria

Page (1992) observed that *Azotobacter vinelandii* was not fit for commercial production because it produced PHA with low yield and formed capsules. Strain UWD of this organism, however, was of interest because it was a capsule-negative mutant and produced PHA content of approximately 70-80%.

Byrom (1992) has discussed on the industrial production of PHA in ICI, UK. *Ralstonia spp.* was the organism of choice for ICI because it produced an easily extracted PHA with high molecular weight. PHA production from *Azotobacter* and methylotrophs were also investigated. However, PHA with low yield and molecular weight was produced from methylotrophs and was difficult to extract also. *Azotobacter* was not an organism of interest because it used carbon substrate for polysaccharide synthesis rather than for PHA production. *R. eutropha* produced 70-80% polymer under phosphate limiting conditions.

A good candidate for PHA production would be a culture that can store high PHA concentrations while growing on an inexpensive growth substrate. Lee (1996) also suggested that the growth rate of a microorganism and its polymer synthesis rate were factors that should be considered when selecting a potential candidate of PHA production.

Yamane *et al.* (1996) studied the production of PHA by *A. latus* using sucrose as the feed substrate. Higher cell concentration (142 g/L) was obtained in a short culture time (18 h) and PHB content at the end of the culture time was 50%. They concluded that the inoculum size reduced the culture time. They compared the culture time required for the production of PHB by *R. eutropha* fed with glucose when the same technique (pH-stat fed-batch) was used. The culture time required for *R. eutropha* was longer (30 hours) to obtain high cell concentration (122 g/L). However, a higher PHB production of 65 per cent was obtained. The average molecular weight of PHB obtained in this study was 3.16×10^5.

Renner *et al.* (1996) studied the production of copolymer by 13 bacteria from the rRNA superfamily III. They reported that different bacteria were able to produce PHAs with different PHV/PHB compositions when grown on the same substrate.

Rawte and Mavinkurve (1998) isolated PHB producing bacteria from mangroves along the Mandovi estuarine ecosystem. Preliminary screening of the isolates grown on tributyrin agar for accumulation of PHBs was done microscopically using the fluorescent dye

Nile Blue A. Out of 65 PHB producing isolates, 60 were able to fix atmospheric nitrogen and grew luxuriantly on nitrogen free media.

Lokesh *et al.* (2005) carried out the isolation of PHA producing bacteria and characterized the potent PHA producer as *Sphingomonas* spp. as confirmed by 16s rDNA sequencing. In their study, the strain was grown on different sugars and organic acids and its ability to accumulate PHA was analyzed. The strain could accumulate PHA when grown on disaccharides, aldohexose, sugaralcohols and some organic acids, but failed to assimilate ketoses, pentoses and starch. Among the sugars tested, PHA yield was found to be high with sucrose or mannose, contributing to 55-60 per cent of cell biomass.

Pal and Paul (2000) isolated aerobic free living nitrogen fixing bacteria from natural environments. Systematic screening of these isolates has indicated that nearly 70 per cent of isolates of the genus *Azotobacter* were capable of accumulating PHB. The PHB contents of majority of the strains ranged from 25-47 per cent of cell dry weight, while only 7 isolates accumulated PHB accounting to more than 50 per cent of their cell dry weight. One of the promising strains of *Azotobacter chroococcum* has been shown to accumulate the polymer accounting nearly to 70 per cent of cell dry weight, when grown under optimized conditions.

Tajima *et al.* (2003) isolated a gram-positive bacterium (designated as strain no INT005) from a field soil that accumulated polyhydroxyalkanoate (PHA) from a grass field soil. The PHA productivities of strain INT005 were higher than those of *Bacillus megaterium* and *Ralstonia eutropha* at 37-45 ^{0}C and the PHA exhibited moderate thermostability.

Mukhopadhyay and Paul (2003) reported the potentialities of phototropic purple non sulphur bacteria for PHB synthesis. They isolated 30 organisms from water and sludge samples collected from different water bodies of West Bengal by enriching under phototropic microaerophilic conditions. Systematic screening of these isolates for PHB production led to the identification of five strains with PHB content ranging from 10-15 per cent of cell dry weight, when grown in acetate containing medium under a light intensity of 10000 lux. Carbon sources like acetate and butyrate were most suitable for PHB accumulation. However, the presence of nitrogen source in the growth medium was found to be inhibitory for PHB accumulation although the growth was enhanced. Phosphate and sulphate limiting conditions enhanced the polymer accumulation by the isolates. They also evaluated the effects of physical factors like pH, light intensity on polymer accumulation.

Ayub *et al.* (2004) isolated *Pseudomonas* sp. 14-3, a strain from Antarctic environments that accumulated large quantities of polyhydroxybutyrate (PHB) when grown on octanoate. This isolate was characterized on the basis of phenotypic features and partial sequencing of its 16s ribosomal RNA gene.

Sujata *et al.* (2005) isolated PHB producing bacteria from different lcoations such as garden soil, tannery effluents, sewage sludge and field soil. They obtained higher PHB positive strains from sewage sludge and tannery effluents compared to other sources.

Yilmaz *et al,* (2005) isolated 29 strains of *Bacillus* from the grassland soils of Ankara, Turkey and were identified as *B. brevis*, *B. sphaericus*, *B. cereus*, *B. megaterium*, *B. circulans*, *B. subtilis*, *B. licheniformis* and *B. coagulans*. Poly-*β*-hydroxybutyrate (PHB) production by these strains was determined by the spectrophotometric method, and they found that PHB production ranged from 1.06–41.67% (w/v) depending on the dry cell dry weight. The highest PHB production and productivity percentage was found in *B. brevis* M6 (41.67% w/v).

2.2.2 PHB producion by cyanobacteria

Sudesh *et al.* (2001) observed that *Synechocystis* pcc6803 accumulated PHA. The PHA content was about 5% of cell dry weight. They showed that the biosynthesis could be improved by introducing multicopies of heterologus *pha* synthase gene. Nile blue A staining and freeze fracture electron microscopy revealed the presence of many PHA inclusions in the cell cytplasam.The relatively low weight of PHA in the cyanobacterium when compared to other bacteria was probabaly due to the small size and mass. They also reported that PHA synthesizing ability of the cyanobacterium might, in fact, be quite similar to that was shown by most bacteria in nature.

2.2.3 PHB production by yeasts

Safak *et al.* (2002) isolated 15 strains of yeasts from Kombucha tea and identified them as *Saccharomyces cerevisiae, Candida krusei, Kloeckera apiculata* and *Kluyveromyces africans.* They were evaluated for PHB production. PHB accumulation in these strains were found to be between 0.50 and 16.67 per cent. They examined the effect of different carbon and nitrogen sources on the accumualtion of PHB in *Rhodotorula glutinus* Var. glutinus 60 and *S. diastaticus* 27. Different N sources tested did not have any influence on PHB production in *R. glutinus* Var. glutinus 60. On the other hand, when the strain was grown in mannitol medium as the carbon source, the PHB production was observed to be higher than the control and the PHB production was increased to 21.95 per cent.

2.2.4 PHA production by recombinant bacteria

Metabolic engineering is being intensely explored to introduce new metabolic pathways to broaden the utilizable substrate range, to enhance PHA synthesis and to produce novel PHA. Recombinant *E.coli* strains harbouring the *Alcaligenes eutrophus* PHA biosynthesis genes in a stable high-copy-number plasmid have been developed and used for high productivity (Zhang *et al*., 1994). Since *E. coli* can utilize various carbon sources, including glucose, sucrose, lactose and xylose, a further cost reduction in PHA is possible by using cheaper substrates such as molasses, whey and hemicellulose hydrolysate (Lee *et al*., 1994).

Natural PHA- producing bacteria have a long generation time and relatively low optimum growth temperatue. These are often hard to lyse and also contain pathways for PHA degradation. Bacteria such as *E. coli* are incapable of synthesizing or degrading PHA; however, *E. coli* grows fast, even at higher temperature and is easy to lyse. Faster growth will enable it to accumulate a large amount of polymer. The easy lysis of the cells save the cost of the purification of PHA granules (Steinbuchel and Schegel,1991; Wang and Bakken,1998; Madison and Huisman,1999). Hence, *E. coli* has been used to transfer PHA genes.

PHB production has been studied mostly in recombinant *E. coli* cells harbouring PHA synthesizing genes from *R. eutropha* (Lee *et al.,* 1994; Zhang *et al.,* 1994; Kidwell *et al.,* 1995). Introduction of *Pha*C_{RS} gene from *Rhodobacter sphaeroids* into *E. coli* does not support PHB synthesis (Ji Hoe and Lee, 1997). PHB accumulation also failed in recombinat *E. coli* cells harbouring *Pha*C_V from *Chromobacter violaceum* and *Pha*B_{Re} and PhaRe genes from *R. eutropha* (Kolibuchuk *et al.,* 1999). While the PHB synthesizing genes from *R. eutropha* have been transferred to *E. coli,* The *Pha*C genes from other organisms like *R. sphaeroides* and *C. violaceum* apparently did not function in the *E. coli* genetic background.

2.3 SCREENING METHODS FOR PHB ACCUMULATION IN BACTERIA

2.3.1 Staining methods for PHB detection

While isolating PHB-accumulating bacteria from nature, it is necessary to screen rapidly a wide collection of bacteria in a short time. Stains specific to PHB are made use of in the detection of the granules. Viable colony staining technique has been suggested as a method for rapid screening of PHB accumulating bacteria.

Hartman (1940) was the first to suggest the use of Sudan black B, as a bacterial fat stain. Subsequently, Burdon *et al*, (1942a) confirmed the greater value of this dye and modified the procedure for demonstrating intracellular fatty material in bacteria by preparing microscopic slides of bacteria stained with alcoholic Sudan black B solution and counterstained with safranin.

Lewis (1941) gave the procedure for demonstrating intracellular fatty material in bacteria by preparing, from suspensions of the organisms in alcoholic Sudan black B solution, dried films counterstained with safranin. Previously, it was thought that dried, fixed films were entirely unsuitable for fat stains.

Ostle and Holt (1982) reported that Poly-□-hydroxybutyrate granules exhibited a strong orange fluorescence when stained with Nile blue A. Heat-fixed cells were treated with 1% Nile blue A for 10 min and the excitation was observed at an excitation wavelength of 460 nm. Glycogen and polyphosphate did not stain. Nile blue A stain appeared to be a more specific stain for poly-β- hydroxybutyrate than Sudan black B.

Kranz *et al.* (1997) described the colony screening and selection systems to analyze the production of PHAs in *R. capsulatus*. Screening with Nile red dissolved in acetone distinguished between PHA producers and non producers.

Spiekermann *et al.* (1999) recommended the use of a sensitive viable colony staining method using Nile red for direct screening of bacteria that accumulate PHA. The direct inclusion of 0.5□g /ml of dye in the medium did not affect the growth of the cells but allowed the detection of the presence of PHAs in the viable colonies at any time during growth. The PHA producers exhibited strong fluorescence when observed under UV light.

Juan *et al.* (1998) employed viable colony screening method for the rapid detection and isolation of PHB producing exopolysaccharide deficient mutants from wild type of *Rhizobium meliloti*. For this, 0.02% alcoholic solution of Sudan black B was applied to stain bacterial colonies which were grown on LB agar plates and the plates were kept undisturbed for 30 min. The dye was then decanted and plates were gently rinsed by adding 100% ethanol. Colonies unable to incorporate the sudan black B appeared white, while PHB producers appeared bluish black.

2.3.2 PCR techniques for detection of PHB producers

Sheu *et al.* (2000) employed Colony PCR and semi-nested PCR techniques for screening polyhydroxyalkanoate (PHA) producers, that were isolated from different environments. Three degenerate primers were designed based on multiple sequence alignment results and were used as PCR primers to detect PHA synthase genes. The sensitivity limit of the colony PCR was 1x 10^5 viable cells for *Ralstonia eutropha*. The results have suggested the application of this PCR protocol for rapid detection of PHA producers from the environment .

Sujatha *et al.* (2005) used the colony PCR to detect the PHA synthase genes from bacterial strains isolated from various ecosystems. For this, they isolated the genomic DNA as described by Sambrook *et al*., (1989) and used as a template. Colony PCR was performed following the procedure of Sheu *et al.,* (2000). In each PCR analysis, 30ng of genomic DNA of Nile red and Nile blue A positive strains and three degenerate primers *phac*F1, *phac*F2 and *pha*-CR4 were used to detect PHA synthase genes. PCR amplified DNA fragments were observed by agarose gel electrophoresis in 1 per cent agarose gel. The amplified DNA fragments were visualized by UV illumination.

2.4 SUBSTRATES AND GROWTH CONDITIONS FOR PHB PRODUCTION

Suzuki *et al.* (1986) reported that *Pseudomonas* sp. grown in methanol as sole carbon and energy source had produced 66% of PHB on dry weight basis. In order to obtain the high PHB content, a proper medium composition was utilized. In this study, concentrations of phosphate and ammonium were maintained at low levels. Nitrogen deficiency was found to be the most effective way to stimulate the accumulation of PHB. The limitation of dissolved oxygen concentration was found to decrease the rate of biomass growth and PHB production. This finding was contradictory to the results reported by others.

Sillman and Casida (1986) studied the cyst formation versus PHB accumulation in *Azotobacter vinelandii* ATCC12837. When inoculated in Burks agar medium containing 0.2 mg of glucoe/ml, it accumulated PHB prior to cyst formation. Addition of low levels of NH_4cl, urea or adenine caused an increment in cyst formation but PHB formation was reduced. Hypoxanthine and inosine addition also stimulated cyst formation, although PHB formation was not reduced. It was concluded that cyst formation by *Azotobacter* did not require accumulation of more than minimal amounts of PHB.

Doi *et al.* (1990) opined that the structure and composition of PHA, as well as its physical and thermal properties could be controlled by composition and concentration of feeding substrates. They conducted experiments on PHA production by *R. eutropha* using various types of substrate. The copolymer of 3-hydroxybutyrate and 4-hydroxybutyrate (3HB-4HB) was obtained from 4-hydroxybutyric acid, butyrolactone, 1,4-butanediol, 1,6-hexanediol, 1,8- octanediol, 1,10-decanediol, or 1,12-dodecanediol.

Poul *et al.* (1990) studied the effect of different carbon sources at a fixed C:N ratio of 15:1. The PHA accumulation was the most significant in the late logarithmic to stationary phase of growth, with the highest quantities of PHB synthesized on malate, as compared with

acetate, pyruvate, lactate and fructose. Higher levels were found to accumulate when lower PO_2 conditions were maintained during growth.

Byrom (1990) stated that a problem was experienced using the wild-type of *R. eutropha* on propionate as carbon source *i.e.*, only about one third of propionate was incorporated into the HV unit of the copolymer. The mutant strain, PS-1, was found to utilize propionate more effectively. A propionate fraction of 80% or greater was incorporated into the HV unit of the copolymer by the mutant strain.

Alcaligenes latus could store PHA up to 80% under normal growth condition. Therefore, one-step PHA production process could be used with this organism (Hrabak, 1992). According to Doi (1990), two stage fed batch culture is the most widely used technique to maximize high concentrations of both cell and PHB. In first stage of growth phase, optimum nutritional conditions were used to develop a high biomass concentration. Then, a selected nutrient was limited to stimulate PHA production in the second stage or accumulation phase.

Steinbuchel and Pieper (1992) studied the production of PHB-PHV copolymer by *R. eutropha* strain R_3 under nitrogen limitation. PHA contents were 47%, 35.7%, 29.5%, 21.5% and 43.2% when fructose, gluconate, acetate, succinate and lactate were used as carbon sources, respectively. When magnesium or sulphur was a limiting condition and fructose was used as the sole substrate, *R. eutropha* strain R_3 could accumulate PHA of 45% or 47% with the PHV fraction of 7% or 6%, respectively.

Daniel *et a*l. (1992) reported that *Pseudomonas 135* when grown in an ammonium-limited fed batch culture using methanol as the sole carbon and energy source, it could produce maximum PHB (55%). When the cells were cultivated on Mg^{2+} and PO_4^{3-} limited media, the production of PHB were 42.5% and 34.5%, respectively.

Kim *et al.* (1994) reported that a fed-batch culture of *R. eutropha* NCIMB 11599 with a glucose concentration control and nitrogen limitation gave PHB production of 121 g/l. When nitrogen limitation was used to induce PHB accumulation, NaOH solution, was added to the medium for pH control. However, due to the significant cell lysis caused by the toxicity of NaOH solution, high-cell-density fermentation was impossible. In addition, it was very important to maintain phosphate and magnesium ion levels above 0.35 g/l and 10 mg/l respectively (Asenjo *et al.* 1995). Ryu *et al.* (1997), therefore adopted phosphate limitation strategy to induce PHB accumulation where the pH was controlled by addition of NH_4OH. Under these conditions, 232 g/l of PHB were obtained.

Shimizu *et a*l (1994) investigated the PHA production from *R. eutropha* H16 (ATCC 17699) fed with butyric and valeric acids. Optimum conditions for PHB production using butyric acid by this organism were at the concentration of 3 g/l butyric acid and a pH of 8.0. PHV or other PHAs was not reported in this study. A PHB content of 75 % was obtained under these conditions, while lower PHB contents were achieved when pH was kept at 8.0. When the butyric acid concentrations were kept at 0.03, 0.3 and 10 g/l, PHB contents of 44%, 55%, and 63%, respectively were obtained.

Bourque *et al.* (1995) investigated on the production of PHB by *Methylobacterium extorquens* ATCC 55366 using methanol as the sole carbon and energy source in a fed-batch fermentation system. The production of PHB between 40% and 46% on a dry-weight basis was produced by *M. extorquen*s. In addition, biomass production and the growth rate of *M. extorquens* were affected by the mineral composition supplied. The absence of (NH4) SO_4 or $MnSO_4$ and the absence of a combination of $CaCl_2$, $FeSO_4$, $MnSO_4$ and $ZnSO_4$ had a negative impact on biomass production and the specific growth rate of this bacterial strain.

Parshad *et al.* (2001) tested 37 isolates and mutants of *Azotobacter chroococcum* for PHB production using Sudan black B staining method. With 2 per cent glucose and 15 mMol/l ammonium acetate, PHB production was found to be maximum at 36 and 48 hours of growth under submerged cultivation and under stationary cultivation respectively. They also observed that PHB production was higher on sucrose and commercial sugar as compared to glucose and mannitol. Among inorganic nitrogen sources, they found ammonium acetate (15 mMol/l) to be the best for PHB production.

Ghatnekar *et al.* (2002) studied PHB production from *Methylobacterium* SPV-49. They evaluated different carbon sources. Maximum accumulation of PHB was observed with glucose as the carbon source. Methanol and sugars such as sucrose and lactose also induced PHB accumulation. The effect of C:N ratio on polymer accumulation was studied.

Different strategies for the extraction and recovery of the polymer from the cells were compared. A non solvent based method using a high pressure homogenizer in the presence of SDS was found to be most satisfactory. Maximum recovery of 98 per cent was achieved by homogenizing the cells at 400 kg cm^{-2} in 5 per cent SDS solution.

Saha and Paul (2003) reported that poly (3-hydorxybutyric acid) and copolymer of poly (3-hydroxybutyrate-Co-3-hydroxyvalerate) [P (3HB-Co-3Hv)] were accumulated by numerous bacteria as energy reserve material under conditions of unbalanced growth in the presence of excess carbon source. They also reported that *A. chroococcum* MAL-201 (MTCC 3853) was found to accumulate P(3 HB) accounting 68.4% of cell dry weight from glucose (2% w/w) in modified Norris nitrogen free medium. It produced co-polymer P(3HB-Co-3HV) when propionate and valaretae were used as sole source of carbon at low concentration (0.01-0.1% w/w). The PHA content of cells was less than 10 per cent of cell dry weight but the HV content of the polymer showed wide range of variability ranging from 5.94 to 3.65 mole per cent. Supplementation of glucose with propionate and valerate as precursors at mid log phase of growth, increased the total PHA content upto 68.9 per cent of cell dry weight with 19.31 mol per cent HV.

Mahishi *et al.* (2003) studied the optimization of PHB production by recombinant *E. coli* (ATCC : PTA-1579) harbouring the PHA synthesizing genes *Pha*C_{Sa} and *Pha*B_{Sa} from *Streptomyces aureofaciens* NRRL 2209. Effects of different carbon and nitrogen sources on PHB accumulation by recombinant *E. coli* were studied. Among the carbon sources used, glycerol, glucose, palm oil and ethanol supported PHB accumulation. No PHB accumulation was observed in recombinant cells when sucrose or molasses was used as the carbon source. Yeast extract and peptone, a combination of yeast extract and peptone and corn steep liquor were used as nitrogen sources. The maximum accumulation (60% cell dry weight) was measured after 48 hr of cell growth at 37^0C in a medium with glycerol as the sole carbon source and yeast extract and peptone as the nitrogen sources.

Khanna and Srivastav (2004) optimized the cultural parameters for the growth of *R. eutropha* NRRL814690 in the presence of nutrients, to decrease the production cost of PHB. With fructose and ammonium sulphate as C and N sources respectively, *R. eutropha* exhibited a maximum biomass of 3.25g/L with PHB concentration of 1.4g/L in 48 h. To determine the possibility of growth potential of *R. eutropha,* it was grown in different carbon sources, of which fructose, lactic acid, sucrose and glucose yielded good growth and PHB. In order to incorporate cheaper nitrogen sources and growth factors in media, ammonium sulphate was substituted by ammonium nitrate, urea, and ammonium chloride. Urea featured the highest PHB accumulation of 3.84g/L after 60 h of growth.

Senthil Kumar and Prabhakaran (2006) investigated the effect of pH on the yield of PHB by *Alcaligenes eutrophus* MTCC1285 in different substrates. They studied PHB production at two pH levels viz., 6.9 and 8.0. The amount of PHB produced at pH 6.9 was 0.8, 0.5, 0.4 and at pH 8.0, they were 1.1, 0.65, 0.55 and 1.0 g/ml^{-1} in glucose, sago, thippi and molasses substrate based media, respectively.

2.5 CHEAPER SUBSTRATES FOR PHA PRODUCTION

The major factor limiting the commercial use of PHA is the cost of its production. The two significant areas increasing the production cost are the substrate used for the polymer production and the down stream processes. The use of cheaper carbon sources would bring down the polymer cost. Hobson *et al.* (1981) reported that anaerobic digestion of biological wastes could be used for production of PHB. The volatile fatty acids produced in anaerobic digestion process act as precursors for the formation of PHA. Sources of volaltile fatty acids (VFAs) include municipal wastes (Kalia and Joshi, 1995), banana pseudostem (Kalia *et al.,* 2000), damaged food grains, pea shells, apple pomace and palm oil mill effluent (Kalia *et al.,* 1992). Various bacteria like *Alaligenes eutrophus, Bacillus megaterium, P. oleovorans, Azotobacter, Beijerinckia, Rhizobium, Nocardia* utilized lower VFAs as substrates for PHA production (Kalia *et al.,* 2000).

Agricultural wastes like beet and cane molasses, malt extract, corn syrup, wheat bran (Page 1992), starch (Lillo and Valera, 1990) and dairy wastes like cheese whey (Yellore and Desai, 1998) with or without ammonium sulphate supplement have been used as raw materials for PHA production.

Seeking a less costly substrate, Bourque *et al.* (1995) investigated on the production of PHA by 118 Methylotrophic bacteria grown on a cheaper substrate like methanol. *Methylobacterium extorquens* was found to accumulate a high PHA content when grown on a mixture of methanol and valerate. A PHA content of 60-70% with 20% PHV was produced by the organism.

P. cepacia ATCC 17759 grown on xylose (10 g/l) produced a biomass of 2.6 g/l with 60 per cent (w/w) PHB in shake flasks under ammonium-limited conditions (Ramsay *et al*., 1995).

Saha *et al.* (2005) observed that *Azotobacter chroococcum* MAL-201, accumulated PHB. The polymer yield accounted to 69 per cent of cell dry weight when grown in N2-free stock dale medium containing 2 per cent (w/w) glucose. To make the polymer production cost effective, 12 wastes of different origin were tested for growth and polymer production. The candy factory waste was found to be the most suitable substrate for homopolymer production. The accumulated PHB accounted 17.8 and 40.58 per cent of cell dry weight under single step and two step cultivation conditions, respectively.

Yellore and Desai (1995) used cheese whey to minimize the cost of production of PHB using the newly isolated *Methylobacterium,* approximately 45% PHB (w/w) was produced.

R. eutropha NCIMB 11599 when grown on tapioca hydrolysate (90% glucose) as a potential cheap carbon source, PHB concentration of only 6.1 g/l could be obtained, probably due to the presence of toxic compounds, possibly cyanate (Kim and Chang 1995). PHB content of 0.33 g-PHB/g-dry cell has been reported when degraded alginic acid was used as the carbon source (Seki *et al.,* 1994). The lower PHB content was probably due to heterogenous hydrolysis of alginic acid.

Kim *et al.* (1997) studied the effects of different carbon sources on the growth and PHA production and reported that simultaneous supply of glucose and octanoic acid resulted in 35.9 g of PHA/l (65% of the cell dry weight) with the productivity of 0.92 g/l/h. From the preceding, it appeared that mixtures of cheap growth substrates and more expensive substrates for product formation provided a valuable means of lowering PHA production costs.

Sabat *et al.* (1998) showed that solid wastes could be used for the production of PHB. In their study, attempt was made to produce PHB under solid state fermentation by *Haloferax mediterranei*. Commercial cheaper substrate like wheat bran was used as the carbon source for the production of PHB. Under optimized temperature and pH conditions, in marine mineral salts medium, under incubation period of 72 hours, 6.14 g of PHB and 8.72 g of biomass were produced/kg of wheat bran used.

Liu *et al.* (1998) reported that recombinant *E. coli* strain was capable of production of PHB on molasses as the carbon source. The final cell dry weight, PHB content and PHB productivity were 39.5 g/l/h, 80 per cent (w/w) and 1g/l/ h, respectively.

Boraman *et al.* (1998) confirmed that PHB was produced by *R. eutropa* DSM 11348 when it was grown in a culture medium containing 20-30 g/l casein peptone casamino acids as the sole N source.

Residual oil from biotechnological rhamnose production was used as the carbon source for growth and for accumulation of PHA by *R. eutropha* H16 and *P. oleovorans* (Feuchtenbusch *et al.,* 2000). Approximately 20.25 per cent of the components of the residual oil were converted in to PHA.

Godbole *et al.* (2000), optimized, a two stage system for the production of PHB, using whey as the substrate for biomass production in the first stage, and lactic acid as the substrate for the accumulation of PHB in the second stage. Maximum PHB content of 74 per cent of cell dry weight was achieved. Results of this study indicated that it is possible to convert the carbonaceous materials present in whey in to environmentally friendly PHB polymer.

B. megaterium was grown on various carbon sources such as date syrup and beet molasses (Omar *et al.,* 2001). Best results with regard to growth and PHB production were obtained in these cheaper carbon sources.

Khardenavis *et al*. (2003) used activated sludge rom a food processing industry and waste water from different industries, as substrates for the production of bioplastics. Amongst the different waste water tried, anaerobic treatment waste water gave maximum production of bioplastics followed by deproteinized milk whey and soy whey, thereby achieving twin objectives of effective utilization of available resources for production of value added products and reduction in cost by solving the problem of waste disposal.

Sureshkumar *et al*. (2004) proposed the production of biodegradable plastics using excess sludge from a waste water treatment plant. In their study, activated sludge bacteria in a conventional waste water treatment system, were induced by controlling the C : N ratio to accumulate storage polymers. The polymer yield was increased to a maximum 33% of biomass (w/w) when the C/N ratio was increased from 24 to 144, whereas specific growth yield was decreased with increasing C/N ratio. The production and recovery of PHB from activated sludge could significantly reduce the cost of PHB production and at the same time, reduced the quantity of excess sludge produced in a waste water treatment system.

Oliveira *et al*. (2004) examined the use of solid-state fermentation as a low-cost technology for the production of poly (hydroxyalkanoates) (PHAs) by *Ralstonia eutropha*. Two agro industrial residues (bagasse and soy cake) were evaluated as culture media. The maximum PHB yield was 1.2 mg/g medium on soy cake in 36 h, and 0.7 mg/g medium on bagasse in 84 h. Addition of 2.5% (w/w) sugar cane molasses to soy cake increased PHB production to 4.9 mg/g medium in 60 h. Under these conditions, the PHB content of the dry biomass was 39% (w/w). The present results indicate that solid-state fermentation could be a promising alternative for producing biodegradable polymers at low cost.

Senthil kumar and Prabhakaran (2006) evaluated different bioeffluents such as Molasses, Sago and Thippi for PHB production by *Alcaligenes eutrophus* MTCC 1285. They obtained 1.0, 0.55, 0.65 grams of PHB/100 ml of Molasses, Thippi, and Sago used. The highest production was recorded in molasses based substrate medium.

2.6 PHA RECOVERY

PHA recovery processes, in addition to the costs of maintaining pure cultures and the high costs of organic substrates,is another factor that contributes to the high cost of PHA production. In the past two decades, several recovery processes have been investigated and studied in order to find the economic way to isolate and purify PHA.

Doi (1990) described a chloroform extraction method. PHA was extracted with hot chloroform in a soxhlet apparatus for over an hour. Then, PHA extracted was separated from lipids by precipitating with diethyl ether, hexane, methanol, or ethanol. Finally, PHA was redissolved in chloroform and further purified by precipitation with hexane.

Ramsay *et al*. (1994) examined the recovery of PHA from three different chlorinated solvents (chloroform, methylene chloride, and 1,2-dichloroethane). They obtained the best recovery and purity when biomass was pretreated with acetone. The optimum digestion time for all the three solvents were 15 minutes. Further digestion resulted in a degradation in the molecular weight of PHA. The degree of recovery when the biomass was pretreated with acetone were 70, 24, and 66 per cent, when refluxed for 15 minutes with chloroform, methylene chloride, and 1,2-dichloroethane respectively. Whereas the level of purity of these three solvents under these optimum conditions were 96, 95, and 93 per cent, respectively.

Ramsay *et al.* (1990) examined the PHA recovery process from *R. eutropha* using hypochlorite digestion with surfactant pretreatment. Two different surfactants were investigated: Triton X-100 and sodium dodecyl sulfate (SDS). Improvements in purity and molecular weight could be obtained by pretreating with surfactant prior to the extraction with sodium hypochlorite. They reported that the surfactant removed approximately 85% of the total protein and the additional protein (10%) was further removed by sodium hypochorite digestion.

Fidler and Dennis (1992) reported a system to recover PHB granules from *E. coli* by expressing T7 bacteriophage lysozyme gene. The lysozyme penetrated and disrupted the cells, and caused PHB granules to be released. The system developed by them used a separate plasmid and expressed it at a low level throughout the cell cycle. At the end of the accumulation phase, the cells were harvested and resuspended in the chelating agent, ethylene diamine tetra acetate. This activated the lysozyme to disrupt the cell structure and release PHA granules at the time that PHA accumulation reached the maximum. Triton X-100

was also added to assist the cell disruption. They reported that the efficiency of lysis was greater than 99%.

Hahn *et al.* (1994) recommended the method called, dispersion with sodium hypochlorite and chloroform. They claimed that this method removed most of the non-PHA cellular materials during sodium hypochlorite digestion, which facilitated the separation of PHA from the cells. In addition, digestion with hypochlorite reduced the viscosity of the chloroform phase. They also investigated on the optimum conditions for PHA recovery from *R. eutropha* using dispersions of sodium hypochlorite and chloroform. The optimum conditions from their experiments were reported to be 90 minutes digestion time with 30% sodium hypochlorite concentration and a chloroform-to-aqueous phase ratio of 1:1 (v/v). They obtained a degree of recovery of 91% and a level of purity of higher than 97%. The degradation rate increased as the concentration of sodium hypochlorite increased.

Due to the high cost of solvent extraction, the enzymatic digestion method was developed by ICI, UK. Steps of this process include thermal treatment (100-150°C) to lyze cells and denature nucleic acids, enzymatic digestion, and washing with anionic surfactant to solubilize non-PHA cellular materials. Finally, concentrated PHA from centrifugation was bleached with hydrogen peroxide.

Sodium hypochorite solubilizes non-PHA cellular materials and leaves PHA intact. Then, PHA could be separated from the solution by centrifugation. A severe degradation of polymers during sodium hypochorite digestion was frequently reported. Fifty percent reduction in the MW of the polymers were reported when the biomass was digested with sodium hypochorite (Lee, 1996).

According to Steinbuchel (1996), ICI used a mixture of various enzymes during the enzymatic digestion step. These enzymes were lysozyme, phospholipase, lecithinase, proteinase and others. These enzymes hydrolyzed most of the non-PHA cellular materials but PHA remained intact. Braunegg *et a*l. (1998) reported that ICI later used proteolytic enzymes, e.g., trypsin, pepsin, and papain, and a mixture of these enzymes to recover PHA.

2.7 PHA PRODUCTIVITY AND PHA YIELD

Choi and Lee (1999) reported that the best PHA productivity was obtained from the study of Wang and Lee (1997). They obtained PHA productivity of 4.94 g PHB/L/h from *A. latu*s. The cost of the carbon substrate was a major factor in the overall PHA production costs, i.e., 38% of the total operating cost based on the production scale of 100,000 ton/year.

2.8 DEGRADATION OF PHA

The property that distinguishes PHA from petroleum based plastics is their biodegradability. Biodegradation of PHA under aerobic conditions results in CO_2 and H_2O, whereas in anaerobic conditions, the degradation products are CO_2 and CH_4. PHA are compostble over a wide range of temperatures, even at a maximum of around 60^0C with moisture levels at 55 per cent. Studies have shown that 85 per cent of PHA were degraded in seven weeks (Johnstone, 1990; Flechter, 1993). PHA have been reported to degrade in aquatic environments (Lake Lugano Switzerland) within 254 days even at temperature not exceeding 6^0C (Jhonstone, 1990).

According to Doi (1990), the degradation of PHA by *R. eutropha* could occur simultaneously with its biosynthesis under nitrogen limitation. This observation is called “cyclic nature of PHA metabolism”. The author reported that the composition of polymer was changed from PHB homopolymer to PHB-49%PHV copolymer when the substrate was changed from butyric acid to pentanoic acid.

Brandl and Hanselmann (1991) reported that PHB differed in density from other conventional plastic materials. Because of high density, PHB does not float in an aquatic system. Therefore, once discarded, plastic materials made from PHB will sink and will be degraded in the surface sediment by biogeochemical mechanisms.

PHB, P(HB-HV) and other PHA are utilized by microorganisms as an energy source. P(HB-HV) is biodegraded in microbial active environments (Porier *et al.,* 1995; Lee, 1996). Microorganisms colonize on the surface of the polymer and secrete enzymes which degrade P(HB-HV) into HB and HV units. These units are then used by the cells as a carbon source for biomass growth. The rate of polymer degradation depends on a variety of factors including surface area, microbial activity of the disposal environment, pH, temperature, moisture and

presence of other nutrient materials. P(HB HV) is water insoluble and is not affected by moisture. It does not degrade under normal conditions of storage, and is stable indefinitely in air (Mergaert *et al.,* 1993).

The end products of PHA degradation in aerobic environments are CO_2 and H_2O, while methane is also produced in anaerobic conditions. The effect of different environments on the degradation rate of PHB and P(HB-HV) has been studied by several workers (Doi *et al.,* 1994 and Mergaert *et al.,* 1994)). Degradation occurs most rapidly in anaerobic sewage and slowest in sew water. Lee (1996) showed that P(HB-HV) completely degraded after 6, 75 and 350 weeks in anaerobic sewage, soil and sea water respectively.

PHAs are degraded upon exposure to soil, compost, or marine sediment. Biodegradation is dependent on a number of factors such as microbial activity of the environment and the exposed surface area, moisture, temperature and pH (Boopathy, 2000).

Kim *et al.,* (1997) observed that fungal biomass in soils generally exceeds the bacterial biomass and thus it is likely that fungi may play a considerable role in degrading polyesters, just as they predominantly perform the decomposition of organic matter in the soil ecosystem. However, in contrast to bacterial polyester degradation, which has been extensively investigated, the microbiological and environmental aspects of fungal degradation of polyesters are unclear.

Romen *et al,* (2004) isolated 18 gram-negative thermotolerant poly (3-hydroxybutyrate) (PHB) degrading bacteria (T_{max}60°C) from compost. Isolates produced clearing zones on opaque PHB agar, indicating the presence of extracellular PHB depolymerases.

Lee *et al.* (1994) studied the degradation of PHB by fungi from samples collected from various environments. PHB depolymerization was tested in plates containing a PHB-containing medium which were inoculated with isolates from the samples. The degradation activity was detected by the formation of a clear zone below and around the fungal colony. In total, 105 fungi were isolated from 15 natural habitats and 8 lichens, among which 41 strains showed PHB degradation. Most of these were deuteromycetes.

2.9 POTENTIAL APPLICATIONS OF BIODEGRADABLE PLASTICS PRODUCED FROM PHA

According to Lafferty *et al.* (1988), the possible applications of bacterial PHA is directly connected with their properties such as biological degradability, thermoplastic characteristics, piezoelectric properties, and depolymerization of PHB to monomeric D(-)-3-hydroxybutyric acid .

2.9.1 Medical and pharmaceutical applications

The degradation product of P(3HB), D(-)-3-hydroxybutyric acid, is a common intermediate metabolic compound in all higher organisms (Lafferty *et al*., 1988; Lee, 1996). Therefore, it is plausible that it is biocompatible to animal tissues and P(3HB) can be implanted in animal tissues without any toxic effect. Some possible applications of bacterial PHAs in the medical and pharmaceutical applications include: biodegradable carrier for long term dosage of drugs inside the body, surgical pins, sutures, and swabs, wound dressing, bone replacements and plates, blood vessel replacements, and stimulation of bone growth and healing by piezoelectric properties. The advantage of using biodegradable plastics during implantation is that it will be biodegraded, *i.e*., the need for surgical removal is not necessary.

Wang and Bakken (1998) reported that PHAs could particularly be used as biodegradable carriers for long term dosage of drugs, medicines and hormones. They were used as osteosynthetic materials in the stimulation of bone growth owing to their piezoelectric properties in bone plates, surgical sutures and blood vessel replacements.

Brandl *et al*. (1988) considered PHA as a source for the synthesis of chiral compounds (enantiomerically pure chemicals) and raw materials for the production of paints. PHA could be depolymerized to rich source of optically active pure bifunctional acids. PHB, for instance, is readily hydrolyzed to R-3- hydroxybutyric acid and used in the synthesis of Merck's antigaucoma drug 'truspot' in tandem with r-1,3-butanediol. It is also used in the synthesis of beta lactams.

2.9.2 Agricultural applications

PHAs are biodegraded in soil. Therefore, the use of PHAs in agriculture is very promising. They can be used as biodegradable carriers for long-term dosage of insecticides, herbicides, or fertilizers, seedling containers and plastic sheaths protecting saplings, biodegradable matrix for drug release in veterinary medicine, and tubing for crop irrigation. Here again, it is not necessary to remove biodegradable items at the end of the harvesting season (Lafferty *et al.*, 1988).

2.9.3 Food commodity packaging applications

According to Lafferty *et al.* (1988) PHB homopolymer and PHB-PHV copolymer have some properties, i.e., tensile strength and flexibility, similar to polyethylene and polystyrene. These properties of PHB films made it possible to use for food packaging.

Oeding and Schlegel (1973) reported that PHA have wide range of applications owing to novel features. Initially, PHAs were used in packaging films mainly in bags, containers and paper coatings. Similar applications as conventional commodity plastics include the disposable items such as razors, utensils, diapers, feminine hygiene products, cosmetic containers, shampoo bottles and cups.

2.10 COMMERCIAL PRODUCTION OF PHA

"Biopol", the commercially available PHA is a copolymer of P (3HB-CO3HB). The commercial production of biopol has seen a number of transformations with respect to producer organisms and their proprietorship. The initial production of biopol was started using *Azotobacter* sp. but the excessive uncontrolled production of polysaccharide (EPS) led to the use of methanol using organism, *Methylobacterium*. Due to slow growth and low polymer content, the commercial production was shifted to *Alcaligenes eutropus,* a glucose utilizing mutant from the original H16 strain (Byrom, 1992).

As a matter of fact, the commercial production of PHA was first started by ICI, UK. Later, the commercial production of Biopol was taken over by ZENECA, UK (Rotman, 1996) and subsequently by Monsanto USA, (Harbak, 1992). The annual production of Biopol was about 10,000 tones (Lee, 1996). The product is successfully being used for marketing of shampoo bottle (SANARA). Besides this, various containers, disposable razors and trays, for holding foods are available commercially.

2.10.1 Commercial status

Bacterial PHA were first commercialized way back in the early 1960s by an American company W. R. Grace. In 1970s, ICI Bioproducts, UK produced PHBV on an industrial scale from glucose utilizing mutant of *R. eutropha* with a glucose/propionic acid substrate mixture as the carbon source and under phosphate limiting conditions (Steinbüchel, 1991). The copolymer was marketed under the trade name 'Biopol' and used for the manufacture of biodegradable films and bottles. Pilot plant polymer was offered at US $ 30/kg and the material from a 5000 tones/year semi-commercial plant was projected to go down to US $ 8-10/kg. Fermentations with recombinant *E. coli* harboring *A. latus* PHA biosynthetic genes coupled with recovery method of simple alkali digestion, the production cost has been estimated to be US $ 3.32/kg PHB (Choi and Lee, 2000).

Another company involved in PHA production was Austrian Chemie Linz (Wilke 1999). Zeneca, earlier a part of ICI Bioproducts, UK sold its Biopol business in 1996 to Monsanto who continued the plant-based production approach to PHAs. In May 2001, Metabolix Inc. has purchased Monsanto's Biopol assets (http://www.metabolx.com/). Metabolix Inc., Cambridge, Massachusetts, founded in 1992, is actively involved in developing efficient technologies for PHA production (Williams and Peoples, 1996). In a world with shrinking petroleum reserves and increasing environmental issues, PHA is definitely a potential candidate that deserves further exploration.

2.10.2 Indian scenario

India is not lagging behind. Of late, SPC Biotech Pvt. Ltd., Hyderabad, in collaboration with Shanghai Institute of Industrial Microbiology, China and Vichy Biomaterials, France, has set up a manufacturing unit to make high and biodegradable polymers. The technology is based on the microbial fermentation of corn starch into lactic acid and vacuum distilling and polymerizing it into polylactic acid. It is the first project of its class in South East Asia. The end products have applications in Medical and Dental industry as absorbable

medical implants. The project has a capacity of 5,000 tonnes/year (www.spc.biotech.com). Table 1 furnishes the list of companies involved in production of biodegradable plastics.

2.11 ECONOMICS OF PHA PRODUCTION

It is a prerequisite to standardize all the fermentation conditions for the successful implementation of commercial PHA production systems. The price of the product ultimately depends on the substrate cost, PHA yield on substrate, and the efficiency of product formulation in the down stream processing (Lee, 1996). This implies high levels of PHA as a percentage of cell dry weight and high productivity in terms of gram of product per unit volume and time (de koning and witholt,1997; de koning *et al*., 1997). Commercial applications and wide use of PHA is hampered due to its price. The cost of PHA using the natural producer *A. eutropphus* is US$ 16/kg which is 18 times more expensive than polypropylene. With recombinant *E. coli* as a producer of PHA, price could be reduced to US $ 4 per kg, which is closer to other biodegradable plastic materials such as PLA and aliphatic polyesters. The commercially viable price should come to US $ 3-5 per kg (Lee, 1996).

Table 1 :Bacteria/raw materials used commercially for the production of biodegradable plastics

Sl. No.	Bacteria/raw material	Name of the company
1.	*Alcaligenes eutrophus* (H-16)	M/s. Zeneca Bio-Products, UK (formerly ICI Ltd.)
2.	*Alcaligenes latus*	M/s. Biotechnolgische Forschungs gelleschaft mbH (Austria) M/s. Petrochemia Danubia
3.	Unknown bacteria	M/s. Biocorp, USA M/s. Asahi Chemicals and Institute of Physical and Chemical Research, Japan
4.	Recombinant *E. coli*	M/s. Bioventures Alberta inc. Canada
5.	Starch	M/s. Warnr's Lambert USA M/s. Fertec, Italy, Ferruzie Technologia, Italy M/s. Biotec (Mellita) Emmerich Germany M/s. BASF ludwigshafen, Germany M/s. Bayer/Wolf warlsrodeleven, Germany M/s. Kusen, Germany M/s. Novamont Novara, Italy
6.	Cheaper sustartes	M/s. Polyferm Inc., Canada
7.	Transgenic plants	M/s Metabolix, Inc., USA M/s. Monsanto, USA M/s. ZENECA Seeds UK
8.	Corn starch	M/s. SPC Biotech Pvt. Ltd., India

- Reddy *et al.* (2003)

III. MATERIAL AND METHODS

Investigations were carried out at the Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad on the isolation of poly □-hydroxy butyrate (PHB) producing bacteria from different sources and their screening for maximum PHB production. The culture parameters were optimized for the selected efficient strains. The general procedure and special techniques adopted as well as the material used in the study are detailed in this chapter.

3.1 COLLECTION OF SAMPLES

Soil samples, waste water samples and activated sludge samples were collected from various sources as outlined in Table 2 and used for the isolation of bacteria.

3.2 ISOLATION OF PREDOMINANT BACTERIA FROM DIFFERENT SAPMLES

Various samples collected were serially diluted and plated on nutrient agar medium (Anon., 1957). The representative bacterial colonies were picked up, purified and preserved on nutrient agar slants till further use.

3.2.1 Heat treatment of soil samples

Mangrove soil samples were heat treated for 10-15 minutes at 80^0C in order to enrich the sporulating bacteria belonging to the genus *Bacillus*.

3.2.2 Reference strain

The reference strain for PHB production *Ralstonia eutropha* MTCC1285 was collected from the Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH) Chandigarh. All the experiments were carried out using this reference strain for comparison.

3.2.3 Rapid screening of native bacterial isolates for PHB production

All the bacterial isolates were qualitatively tested for PHB production following the viable colony method of screening using Sudan Black B dye (Juan *et al.,* 1998). For rapid screening of PHB producers, nutrient agar medium supplemented with 1 per cent glucose was sterilized by autoclaving at 121^0C for 20 minutes and cooled to 45^0C. The medium was poured into sterile petriplates and allowed for solidification. The plate was divided into 6 equal parts and in each part, a bacterial isolate was spotted. The plates were incubated at 30 ^{0}C for 24 hours. Ethanolic solution of (0.02%) Sudan Black B was spread over the colonies and the plates kept undisturbed for 30 minutes. They are washed with ethanal (96%) to remove the excess stain from the colonies. The dark blue coloured colonies were taken as positive for PHB production. All the positive isolates were assigned the code numbers based on their source of isolation.

3.2.4 Fluorescent staining method

Detection of PHB production in the isolates was also done following fluorescent staining method using acridine orange, as suggested by Senthilkumar and Prabhakaran (2006). Ten □l of 48 hr old culture of the isolate was transferred to an eppendorf tube containing 50 □l of acridine orange (“Himedia”) and incubated for 30 minutes at 30^0C. After the incubation period, the culture was centrifuged at 4000 rpm, for 5 min. The pellet was collected and resuspended in distilled water. A smear was prepared on a clean microscopic slide and observed in a fluorescent microscope at 460 nm. The appearance of yellow coloured granules inside the cell indicate PHB production.

3.2.5 Quantification of PHB production and selection of isolates

All the Sudan Black B positive isolates were subjected to quantification of PHB production as per the method of Jhon and Ralph (1961). The bacterial cells containing the polymer were pelleted at 10,000 rpm for 10 min. and the pellet washed with acetone ad ethanol to remove the unwanted materials. The pellet was resuspended in equal volume of 4 per cent sodium hypochlorite and incubated at room temperature for 30 min. The whole mixture was again centrifuged and the supernatant discarded. The cell pellet containing PHB was again washed with acetone and ethanol. Finally, the polymer granules were dissolved in

hot chloroform. The chloroform was filtered and to the filtrate, concentrated 10 ml hot H_2SO_4 was added. The addition of sulfuric acid converts the polymer into crotonic acid which is brown colored. The solution was cooled and the absorbance read at 235 nm against a sulfuric acid blank. By referring to the standard curve, the quantity of PHB produced was determined.

Based on the PHB yields, 10 promising bacterial isolates were selected for further studies.

3.2.5.1 Preparation of standard curve

Standard curve of PHB was prepared following the method of Law and Slepecky (1969). Pure PHB (Sigma, USA) was used to prepare the standard curve. It (200 mg) was dissolved in 10 ml of concentrated H_2SO_4 and heated for 10 min to convert PHB into crotonic acid, which gave 20 mg/ml of crotonic acid. From the above stock, working standard solution was prepared by diluting 5 ml of the stock (containing 100 mg of crotonic acid) to 10 ml with H_2SO_4, which gives the final concentration of 10 mg/ml (0.01 g/ml). This was used for the preparation of the standard curve.

3.2.6 Optimization of cultural parameters for maximum PHB production

Different factors affecting PHB production by the selected promising bacterial isolates were optimized *viz.*, carbon source, nitrogen source, pH and C/N ratio.

3.2.6.1 Effect of different carbon sources on PHB production

The selected bacterial isolates were grown in 250 ml conical flasks containing 100 ml MSM broth with different carbon sources *viz.,* glucose, fructose, sucrose, maltose and cellulose at 1 per cent level. The flasks were incubated at 30^0C on a rotary shaker (150 rpm) for 48 hours. The treatments were replicated three times. After incubation, PHB produced by the isolates were quantified spectrophotometerically following the method of John and Ralph (1961) as described in 3.2.5. Based on the PHB yields, the best carbon sucrose was arrived at.

3.2.6.2 Effect of different nitrogen sources on PHB production

The bacterial isolates were grown in 250 ml conical flasks containing 100 ml MSM broth with the best carbon source, and different 'N' sources were used *viz.,* ammonium sulphate, ammonium chloride, ammonium nitrate and yeast extract, all at 1.0 g/l concentration. After 48 h, PHB yields were quantified as done previously. Based on the yield data, the best N sources was carried at.

3.2.6.3 Effect of different concentrations of the best N source on PHB production

The bacterial isolates were grown in 250 ml conical flasks, containing 100 ml MSM broth having the best carbon source and different concentrations of the best N source *viz.,* 05, 1.0 and 1.5 g/L. After 48 hr, PHB yields were quantified as done previously. Based on the data, the optimum concentration of the best N source was arrived at.

3.2.5.4 Effect of pH on PHB production

The bacterial isolates were grown in 250 ml conical flasks containing 100 ml MSM broth containing the best carbon source and the best N source at the optimum concentration. Different pH of media were maintained *viz.,* 6.0, 7.0 and 8.0 and incubated. After 48 h, PHB produced were quantified as done previously. Based on the data, the best pH was arrived at.

3.2.6.5 Effect of different C/N ratios on PHB production

The bacterial isolates were grown in 250 ml conical flasks containing 100 ml MSM broth with different C:N ratios *viz.,* 10:1, 15:1, 20;1 and 25:1 using the best C and N sources and incubated on a rotary shaker (150 rpm) at 30^0C. After 48 h, PHB yields were quantified as done previously. Based on the data, the best C:N ratio was carried at.

3.2.7 Characterization of PHB producing bacterial isolates

The selected, most efficient PHB producing bacterial isolates were subjected to a set of morphological, physiological and biochemical tests for the purpose of identification.

I. Morphological tests

The two potent PHB accumulating strains DJC6 and BJC7 were examined for their colony morphology, pigmentation fluorescence, cell shape and gram reaction as per the standard procedures given by Anon (1957) and Barthalomew and Mittewer (1950).

1. Colony characterization

The colony characters *viz.,* shape, colour and polysaccharide production were observed on agar medium.

2. Simple staining

Twenty four hour old culture was smeared on a clean glass slide and heat fixed. It was then kept on the staining tray and five drops of safranin stain was applied for few seconds. Stain was poured off and smear was washed gently with slow running water. The slide was air dried and observed under oil immersion.

3. Gram staining

Twenty four hour old culture was smeared on a clean glass slide and heat fixed. The smear was covered with crystal violet for 30 seconds and washed off with 95 per cent ethyl alcohol. The slide was washed with distilled water and drained. Safranin was applied on smear for 30 seconds as counter stain, washed with distilled water and blot dried. The slide was observed under microscope for gram reaction.

4. Spore staining

Fourty eight hours old nutrient agar grown isolates were smeared on a glass slides, air dried and heat fixed. The smears were flooded with malachite green and steamed on water bath for 5 min. adding more stain on the smear from time. The slides were washed with water and counter stained with safranin for 30 seconds. The smear was washed with distilled water, blot dried and examined under oil-immersion objective.

II. Biochemical tests

Biochemical tests were carried out as per the method given by Cappuccino and Sherman (1992) with 24 hr old cultures.

1. Starch hydrolysis

The isolates were made a single streak on starch agar (Appendix-I) plate for 72-96 h at 25^0C in an inverted position. Grams iodine solution was flooded on the surface of the plates for 30 seconds. The plates were examined for the starch hydrolysis around the line of growth of each isolates i.e., for the color change of the medium. Clear zone surrounding the microbial colonies is a typical positive starch hydrolysis.

2. Gelatin liquefaction

The isolates were inoculated on gelatin agar deep tubes (Appendix-I) and gelatin agar medium plates at 37^0C, for 4-7 days. After incubation, the tubes were placed in a refrigerator for 15 minutes at 4^0C for 15 min and observed for liquefaction of gelatin.

3. Casein hydrolysis

Overnight grown cultures of the test isolates were spotted on skimmed milk agar (Appendix-I) plates and incubated at 28 ± 2^0C for 48 hours. The production of halo zone around the colony was taken as positive for the test.

4. Hydrogen sulfide test

Test cultures were stbbed into the tubes containing SIM (Appendix-I) agar and kept for in incubation at 37^0C for 24-48 hr. The tubes were examined for the presence or absence of black coloration along the lane of stab incubation.

5. Catalase test

Nutrient agar (Appendix-I) slants were inoculated with overnight grown test organisms and were incubated at 30^0C for 24 hr. After incubation, the tubes were flooded with one ml of three per cent hydrogen peroxide and observed for gas bubbles. The occurrence of gas bubbles was taken as positive for catalse test.

6. Urease test

The overnight grown cultures were inoculated to the test tubes containing sterilized urea broth (Appendix-I) and incubated for 24-48 hr at 28 ± 2^0C. The development of pink color was taken as positive for the test.

III. Physiological characterization

1. Growth at 4^0C

Twenty four h grown old cultures of the test organisms in nutrient broth were spotted on the trypticase soy agar plates and incubated for 24 of 48 ht at 4^0C and the observation of growth was recorded at the end of the incubation period.

2. Growth at 41^0C

Twenty four hours grown old cultures of the test organisms in nutrient broth were spotted on the trypticase soy agar plates and incubated for 24 of 48 ht at 4^0C and the observation of growth was recorded at the end of the incubation period.

3. Citrate utilization test

Citrate utilization test was performed to find out the ability of the bacterial isolates to utilize or ferment citrate as the sole source of carbon. It was done on the Simmon's Citrate Agars slants and a change in the colour of the medium from green to blue was positive for the test.

4. Indole production

To the pre sterilized SIM agar (Appendix-I) slants, the test cultures were inoculated. The tubes were incubated for 48 h at $28\pm2\ ^0$C. After incubation, each tube was added with ten drops of Kovac's reagent. The production of red colour was taken as positive for the indole production.

3.2.8 Use of cheaper substrates for PHB production

One of the limitations in the popularization of biodegradable plastics is the cost of production. Hence, in order to reduce the cost, different cheaper, locally available bioeffluents were used for PHB production by all the selected 10 promising bacterial strains.

Characterization of cheaper substrates

Whey was collected from M/s. KMF product dairy, Dharwad and waste water of a soft drink industry from M/s. Nectar Bevarage Pvt. Ltd., Dharwad. They were characterized for the following parameters.

pH

The pH of the cheaper substrate samples were determined by glass electrode using a digital pH meter (Systronics model 335).

Electrical conductivity

The electrical conductivity of cheaper substrate samples were determined using conductivity bridge (Digital conductivity bridge, "Systronics" model, 304).

Chemical oxygen demand (COD)

The chemical oxygen demand was estimated following the "Open reflux method" (Moroe *et al.,* 1949). The pre-acidified sample was refluxed in a strongly acid solution with a known excess volume of potassium dichromate. After digestion, the remaining untreated potassium dichromate was titrated with ferrous ammonium sulphate using ferrion indicator.

Estimation of reducing sugars

Reducing sugars in cheaper substrates was estimated by Nelson-Somogyi's method.

To one ml of 80 per cent diluted sample, one ml of freshly prepared alkaline copper reagent including blank was added. It was placed on a boiling water bath for 20 minutes and cooled under the tap without shaking to which one ml of arsenomolybdate reagent was added with immediate mixing till effervescence die. The volume was then made to 20 ml with distilled

water and per cent transmission was read against reagent blank which was set to 100 per cent T at 510 nm. Then mg of reducing sugar present per ml of sample was calculated with the help of the standard graph.

3.2.8.1 Use of dairy whey to produce PHB from isolates

The bacterial isolates were grown in 250 ml the flasks containing 100 ml sterilized whey for 48 h on a rotary shaker (150 rpm). After 48 h, PHB yields were quantified as done previously.

3.2.8.2 Use of soft drink industrial waste water for PHB production

The bacterial isolates were grown in 250 ml conical flasks containing 100 ml sterilized soft drink industrial waste water for 48 h on a rotary shaker (150 rpm). After 48 h, PHB yields were quantified as done previously.

3.2.9 Use of supplemented cheaper substrates for PHB production

The cheaper substrates supplemented with glucose were evaluated as substrates for PHB production.

3.2.9.1 Whey with glucose

The bacterial isolates were grown in 250 ml conical flaks containing sterilized whey amended with glucose (1%) for 48 hr on a rotary shaker (150 rpm) at 30^0C. The PHB yields were quantified as done previously.

3.2.9.2 Soft drink industrial waste water with glucose

The bacterial isolates were grown in 250 ml conical flasks containing sterilized waste water from a soft drink industry amended with glucose (1%) for 48 h on a rotary shaker (150 rpm) at 30^0C. The PHB yields were quantified as done previously.

3.3 SCALE UP STUDIES

Based on the performance of 10 promising strains, two potent strains were finally selected for the scale up studies. Scale up studies were conducted in 3 L bottle fermenters containing 2 L of sterilized MSM broth containing the best C and N sources and maintaining the optimum C:N ratio. The medium was bubbled with sterile air by connecting to an air compressor and the strains grown for 72 h. At regular intervals, samples were drawn and PHB yields determined.

3.4 STATISTICAL ANALYSIS

The data were analyzed using completely randomized two factorial design (Panse *et al*., 1985). Whenever, the treatment difference is found significant in ‘f’ test, CD was worked out at 1 per cent probability levels and the values furnished.

IV. EXPERIMENTAL RESULTS

Investigations were conduced to isolate bacteria from various environmental samples and screened them for accumulation of poly-□-hydroxybutyrate (PHB) within their cells. The efficient isolates were selected and their culture parameters for maximum PHB production were optimized. Different bioefluents were tested as cheaper substrates for PHB production by the promising isolates. The results obtained during the experimentation are presented in this chapter

4.1 ISOLATION OF REPRESENTATIVE BACTERIA

As many as 148 samples were collected from eight sources as listed in Table 2, bacteria from theses samples were isolated on nutrient agar amended with glucose (1%). In all, 680 representative bacteria were isolated, purified and maintained as pure cultures.

4.2 SCREENING OF THE ISOLATES FOR PHB PRODUCTION

All the 680 isolates were subjected for visual screening for PHB production using sudan black B. The colour of the sudan black B colonies were visually scored in comparison with that of the reference strain and BJC7 was tested under fluorescent microscope using acridine orange (Plate 1 and 2). The positive bacteria were assigned the code number depicting the place of their origin.

It was observed that out of 680 isolates as many as 125 were found to accumulate PHB (Table 3). It was interesting to note that all the 24 strains of the Departmental culture collection were positive for PHB production. Twenty isolates from the saline soils accumulated PHB. Fifteen were from Rhizophere soils, 13 from activated sludge of a dairy, 10 each from mangrove soils and soft drink industrial waste water, 11 from activated sludge of a food processing industry, nine from mangrove preheated soils and six each from acid soils and polluted soils.

A perusal of Table 4 indicate that the following 17 isolates were deeply black coloured with +++.

1. Mangrove isolates : KJC6, KJC7 and KJC10
2. Saline soil isolates : EJC2, EJC5 and EJC9
3. Soft drink industry waste water isolates : NJC3
4. Activated sludge (food processing industry) isolates : FJC4
5. Activated sludge (dairy) isolates : MJC3 and MJC10
6. Acid soil isolates : TJC3
7. Mangrove heat treated isolates : BJC5, BJC6 and BJC7
8. Polluted soil sample isolates : DJC5 and DJC6
9. Department collection strains : B25

Sixty nine isolates were medium black coloured with ++. And the rest were light black coloured with +.

Legend

I

A.1. : Ralstonia eutrapha +++

B.3,4 and 5 : Strongly stained isolated colonies with +++

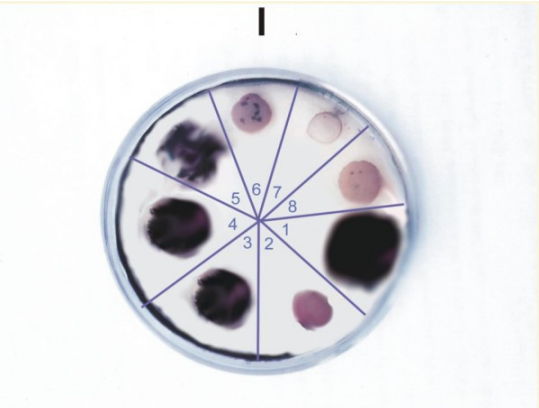
C.2, 6 and 8 : Medium stained isolated colonies with ++

D. 7 : Unstained isolated colony

II

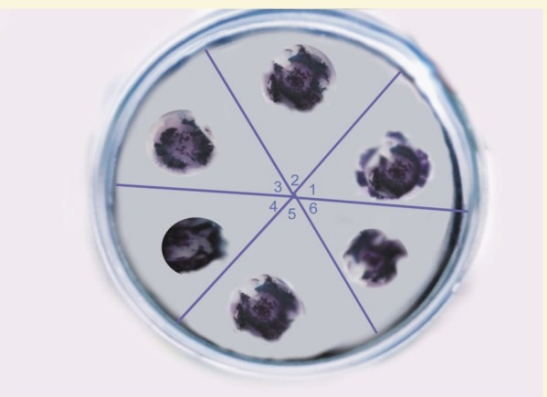
1. EJC2
2. BJC7
3. EJC5
4. NJC3
5. B25
6. FJC4
7. MJC10
8. DJC6
9. TJC1
10. BJC7

I



Scoring of Sudan Black B stained colonies

II



II

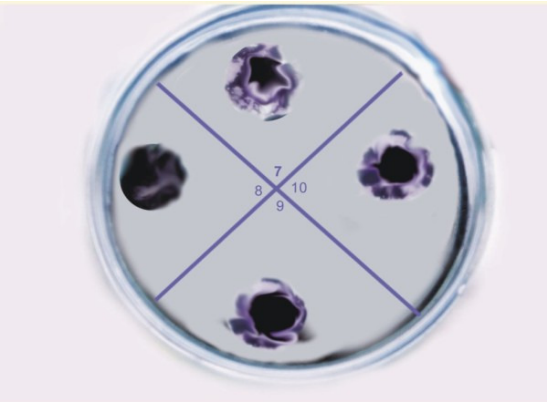


Plate 1 : Selected blue black coloured colonies after Sudan Black B staining for optimization of process parameters

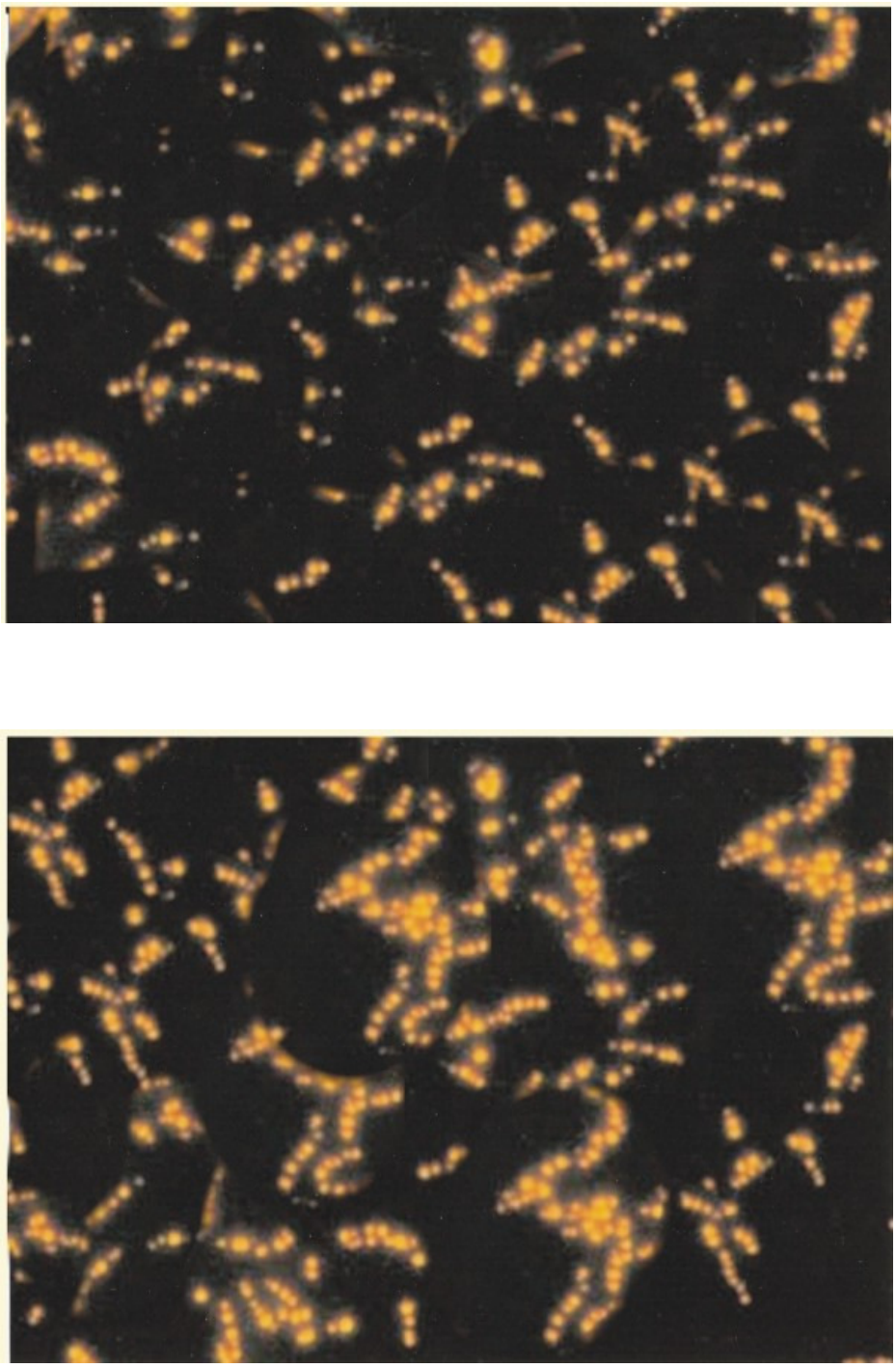


Plate 2 : Acridine orange stained BJC7 under a fluorescent microscope

Table 2 : Collection of samples from different sources for isolation of PHB synthesizing bacteria

Sl. No.	Sample type	Places of sampling
1.	Soils of Mangroves	Kumta, Honnavar (North Canara disrict)
2.	Activated sludge of food processing industry	Gerkins Export Industries Ltd. Narasapur, Bangalore
3.	Waste water of a soft drink industry	Nectar Beverage Pvt. Ltd., Dharwad
4.	Activated sludge of a dairy industry	KMF Product Dairy, Dharwad
5.	Rhizosphere soils	Yellapur, Mugad, Garag (Dharwad district)
6.	Acid soils	Taragoda, Byrumbe, Sirsi (North Canara district)
7.	Polluted soils	Ugar, MK Hubli (Belgaum district)
8.	Saline soils	Gangavati (Raichur district)
9.	Culture collection of the Department Agril. Microbiology	UAS, Dharwad

Table 3: Origin and description of the samples used for the isolation of PHB producing bacteria

Sl. No.	Sample description	No. of samples tested	No. of representative bacteria isolated	No. of Sudan Black B positive strains
1	Mangrove soils (Kumata, Honnavar)	6	82	10
2	Saline soils (pH 7.5-10; EC 12.502 to 1.0374 dS/m)	30	100	21
3	Activated sludge of a food processing industry	6	60	11
4	Soft drink industrial waste water	6	62	10
5	Activated sludge of a dairy industry	4	68	13
6	Rhizosphere soils (Dharwad)	30	180	15
7	Acid soils (pH 5.2-5.5)	14	32	6
8	Mangrove soils heat treated	6	42	9
9	Polluted soils (MK Hubli)	12	30	6
10	Departmental collections	-	24	24
	Total	**148**	**680**	**125**

4.3 QUANTITATIVE SCREENING OF THE ISOLATES FOR PHB PRODUCTION

One hundred twenty five isolates obtained from the preliminary screening following the viable colony staining technique were subjected to quantification of PHB production following the technique of John and Ralph (1961). The organisms were grown in LB broth supplemented with 2 per cent glucose (Sujata *et al.,* 2005) were used for the quantification.

Table 4 : Viable colony staining of PHB producing bacteria and their scoring

Sl. No.	Code no. of the isolate	Scoring of black colour of the colony	Sl. No.	Code no. of the isolate	Scoring of black colour of the colony
I	**Mangrove isolates**		27	EJC17	++
1	KJC1	+	28	EJC18	++
2	KJC2	+	29	EJC19	++
3	KJC3	++	30	EJC20	++
4	KJC4	++	31	EJC21	++
5	KJC5	++	**III**	**Soft drink industrial waste water isolates**	
6	KJC6	+++	32	NJC1	++
7	KJC7	+++	33	NJC2	++
8	KJC8	++	34	NJC3	+++
9	KJC9	++	35	NJC4	++
10	KJC10	+++	36	NJC5	++
II	**Saline soil isolates**		37	NJC6	++
11	EJC1	+	38	NJC7	++
12	EJC2	+++	39	NJC8	+
13	EJC3	+	40	NJC9	++
14	EJC4	+	41	NJC10	++
15	EJC5	+++	**IV**	**Activated sludge of food processing industry isolates**	
16	EJC6	++	42	FJC1	+
17	EJC7	++	43	FJC2	++
18	EJC8	+	44	FJC3	++
19	EJC9	+++	45	FJC4	+++
20	EJC10	++	46	FJC5	+
21	EJC11	++	47	FJC6	++
22	EJC12	++	48	FJC7	++
23	EJC13	++	49	FJC8	++
24	EJC14	+	50	FJC9	+
25	EJC15	+	51	FJC10	+
26	EJC16	++	52	FJC11	++

Contd…

V	Activated sludge dairy isolates		VIII	Mangrove heat treated soil isolates	
53	MJC1	+	92	BJC6	+++
54	MJC2	+	93	BJC7	+++
55	MJC3	+++	94	BJC8	++
56	MJC4	++	95	BJC9	++
57	MJC5	++	**IX**	**Polluted soil isolates**	
58	MJC6	++	96	DJC1	++
59	MJC7	++	97	DJC2	++
60	MJC8	++	98	DJC3	++
61	MJC9	++	99	DJC4	++
62	MJC10	+++	100	DJC5	+++
63	MJC11	++	101	DJC6	+++
64	MJC12	+	**X**	**Departmental collections**	
65	MJC13	+		*Rhizobium*	
VI	**Rhizosphere soil isolates**		102	SB120	+
66	SJC1	+	103	NC92	+
67	SJC2	+	104	GR2	+
68	ZJC1	+		*Azospirillum*	
69	ZJC2	++	105	ACD20	++
70	ZJC3	++	106	ACD15	++
71	CJC1	+		PGPR isolates	++
72	CJC2	+	107	427	++
73	GJC1	+	108	B25	+++
74	GJC2	++	109	B15	+
75	RJC1	++	110	B23	+
76	RJC2	+	111	B21	+
77	RJC3	+	112	139 (2)	+
78	WJC1	++	113	327	+
79	WJC2	+	114	361	+
80	WJC3	+	115	331 (2)	+
VII	**Acid soil isolates**			PPFM	
81	BYJC1	++	116	ML1	+
82	BYJC 2	++	117	ML2	++
83	BYJC3	++	118	ML4	++
84	TJC1	++	119	ML5	++
85	TJC2	++	120	ML6	++
86	TJC3	+++	121	ML10	++
87	BJC1	++	122	ML15	++
88	BJC2	++	123	ML18	++
89	BJC3	++	124	ML21	++
90	BJC4	++	125	ML29	+
91	BJC5	+++	126	Standard *R. eutropha*	+++

Table 5 : PHB production by Sudan Black B positive isolates

Sl. No.	Code no. of the isolate	PHB yield (g/100 ml)	Sl. No.	Code no. of the isolate	PHB yield (g/100 ml)
I	**Mangrove isolates**		27	EJC17	0.049
1	KJC1	0.020	28	EJC18	0.055
2	KJC2	0.018	29	EJC19	0.050
3	KJC3	0.030	30	EJC20	0.049
4	KJC4	0.055	31	EJC21	0.042
5	KJC5	0.044	**III**	**Soft drink industrial waste water isolates**	
6	KJC6	0.079	32	NJC1	0.060
7	KJC7	0.089	33	NJC2	0.071
8	KJC8	0.0740	34	NJC3	0.095
9	KJC9	0.069	35	NJC4	0.059
10	KJC10	0.060	36	NJC5	0.049
II	**Saline soil isolates**		37	NJC6	0.050
11	EJC1	0.030	38	NJC7	0.043
12	EJC2	0.100	39	NJC8	0.020
13	EJC3	0.029	40	NJC9	0.045
14	EJC4	0.090	41	NJC10	0.050
15	EJC5	0.098	**IV**	**Activated sludge of food processing industry isolates**	
16	EJC6	0.060	42	FJC1	0.020
17	EJC7	0.058	43	FJC2	0.040
18	EJC8	0.025	44	FJC3	0.038
19	EJC9	0.090	45	FJC4	0.089
20	EJC10	0.070	46	FJC5	0.020
21	EJC11	0.068	47	FJC6	0.039
22	EJC12	0.071	48	FJC7	0.042
23	EJC13	0.070	49	FJC8	0.046
24	EJC14	0.022	50	FJC9	0.018
25	EJC15	0.030	51	FJC10	0.021
26	EJC16	0.060	52	FJC11	0.045

Contd…

V	Activated sludge dairy isolates		VIII	Mangrove heat treated soil isolates	
53	MJC1	0.023	87	BJC1	0.042
54	MJC2	0.020	88	BJC2	0.044
55	MJC3	0.080	89	BJC3	0.038
56	MJC4	0.030	90	BJC4	0.040
57	MJC5	0.040	91	BJC5	0.079
58	MJC6	0.038	92	BJC6	0.080
59	MJC7	0.045	93	BJC7	0.110
60	MJC8	0.039	94	BJC8	0.043
61	MJC9	0.045	95	BJC9	0.044
62	MJC10	0.098	**IX**	**Polluted soil isolates**	
63	MJC11	0.044	96	DJC1	0.042
64	MJC12	0.021	97	DJC2	0.039
65	MJC13	0.023	98	DJC3	0.043
VI	**Rhizosphere soil isolates**		99	DJC4	0.056
66	SJC1	0.010	100	DJC5	0.080
67	SJC2	0.014	101	DJC6	0.160
68	ZJC1	0.019	**X**	**Departmental collections**	
69	ZJC2	0.030		*Rhizobium*	
70	ZJC3	0.035	102	SB120	0.020
71	CJC1	0.010	103	NC92	0.029
72	CJC2	0.020	104	GR2	0.024
73	GJC1	0.020		*Azospirillum*	
74	GJC2	0.034	105	ACD20	0.040
75	RJC1	0.033	106	ACD15	0.039
76	RJC2	0.020		PGPR isolates	
77	RJC3	0.018	107	427	0.040
78	WJC1	0.040	108	B25	0.080
79	WJC2	0.021	109	B15	0.014
80	WJC3	0.010	110	B23	0.040
VII	**Acid soil isolates**		111	B21	0.030
81	BYJC1	0.040	112	139 (2)	0.039
82	BYJC 2	0.050	113	327	0.019
83	BYJC3	0.048	114	361	0.018
84	TJC1	0.088	115	331 (2)	0.014
85	TJC2	0.050		PPFM	
86	TJC3	0.040	116	ML1	0.020
117	ML2	0.040	122	ML15	0.024

Contd…

118	ML4	0.044	123	ML18	0.035
119	ML5	0.041	124	ML21	0.039
120	ML6	0.028	125	ML29	0.039
121	ML10	0.043	126	Standard *Ralstonia eutropha*	0.160

4.4 SELECTION OF PROMISING BACTERIAL ISOLATES

Based on the PHB yields, 10 promising isolates were selected covering all the sources of the isolates (Table 6 and Plate 3). These were EJC2, EJC5, KJC7, NJC3, FJC4, MJC10, TJC1, BJC7, B25 and DJC6 which produced PHB yields of 0.100, 0.098, 0.089, 0.095, 0.089, 0.098, 0.088, 0.110, 0.080 and 0.160 g/100 ml, respectively.

4.5 EFFECT OF DIFFERENT CARBON SOURCES ON PHB YIELD

The data in Table 7 and Fig. 1 depicts the effects of different carbon sources on PHB yield. The results interpreted significantly differences for the isolates, carbon sources and their interactions as well.

Amongst the different PHB isolates, DJC6 was significantly superior when compared to all other isolates including the reference strain. The isolate BJC7 was the next best eventhough the mean PHB yield was significantly lower than the reference strain. The rest of the isolates were found to be ineffective.

Among the different carbon sources tested to evaluate their effects on PHB yield, glucose was found to be the best carbon source. It yielded a mean PHB of 0.262 g/100 ml. This was followed by fructose with a mean PHB of 0.202 g/100 ml.

The interaction effects of the isolate and carbon sources were also found to be significant. The isolate DJC6 on glucose as the carbon source (1%) recorded the highest PHB yield of 0.500 g/100 ml which is superior to the yield of PHB (0.490 g/100ml) produced by *Ralstonia eutropha*. The isolate DJC6 was found to produce significantly higher yield on fructose also (0.410 g/100 ml). Other sugars tested such as maltose and cellulose did not yield any PHB.

Table 6 : Promising bacterial isolates selected based on PHB production

Sl. No.	Isolates	Yield (g/100 ml)	Source
1	EJC2	0.100	Saline soil
2	EJC5	0.098	Saline soil
3	KJC7	0.089	Mangrove soil
4	NJC3	0.095	Soft drink industrial waste water
5	FJC4	0.089	Activated sludge (food processing industry)
6	MJC10	0.098	Activated sludge (dairy industry)
7	TJC1	0.088	Acid soil
8	BJC7	0.110	Mangrove heat treated soil
9	B25	0.080	Department
10	DJC6	0.160	Polluted soil
11	Reference strain *R. eutropha*	0.160	IMTECH, Chandigarh

LEGEND

1. EJC2
2. BJC7
3. EJC5
4. NJC3
5. B25
6. FJC4
7. MJC10
8. DJC6
9. DJC6
10. BJC7

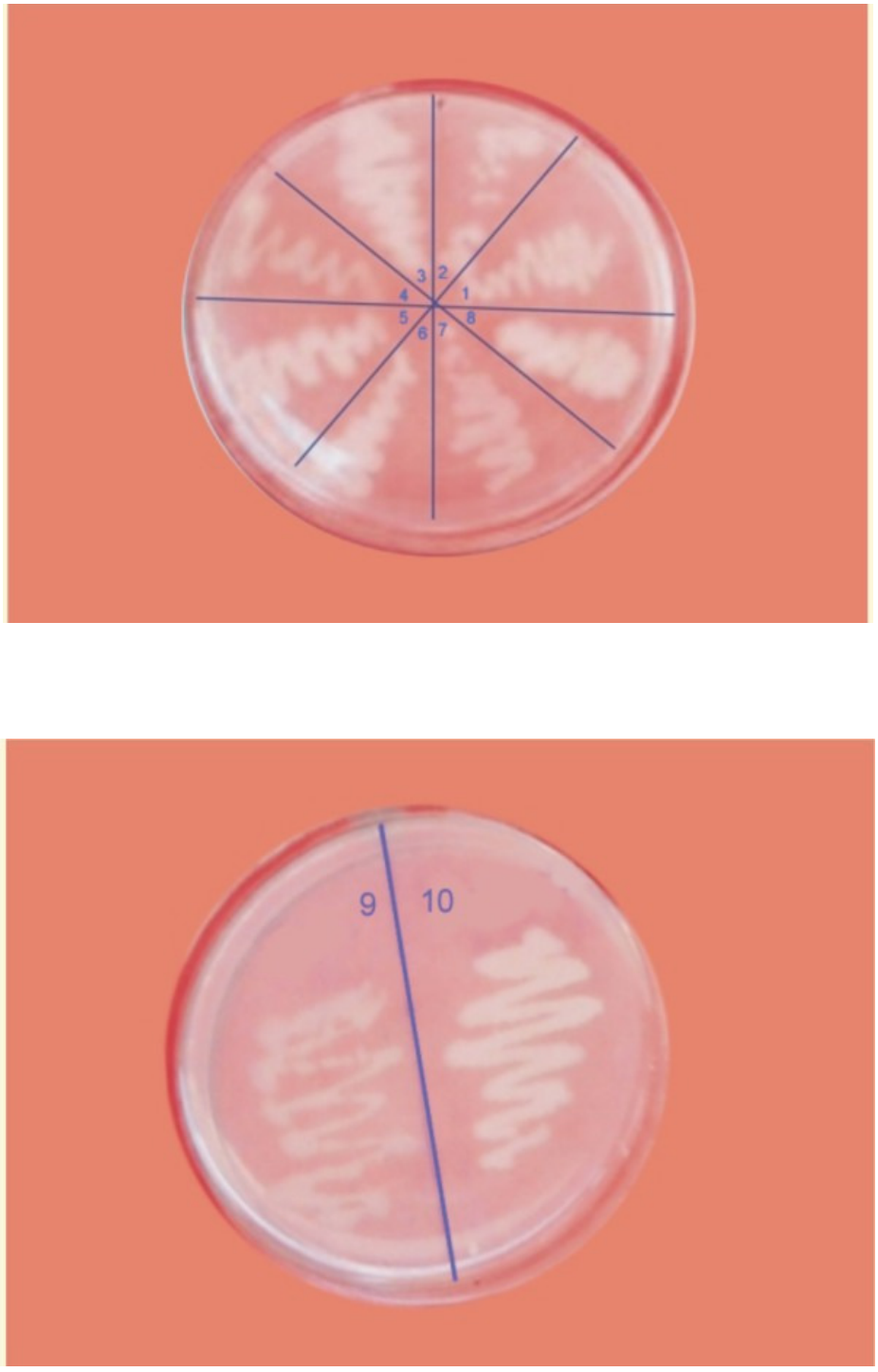


Plate 3 : Selected PHB synthesizing bacterial isolates

Table 7 : Effect of different carbon sources on yield of PHB (g/100 ml) produced by selected bacterial isolates

Sl. No.	Isolates	Carbon sources (1%)					Mean
		Glucose	Fructose	Sucrose	Maltose	Cellulose	
1	EJC2	0.350	0.270	0.150	0.000	0.000	0.154
2	EJC5	0.220	0.190	0.100	0.000	0.000	0.102
3	KJC7	0.200	0.150	0.080	0.000	0.000	0.0860
4	NJC3	0.140	0.100	0.050	0.000	0.000	0.058
5	FJC4	0.150	0.100	0.040	0.000	0.000	0.058
6	MJC10	0.100	0.090	0.020	0.000	0.000	0.042
7	TJC1	0.230	0.200	0.080	0.000	0.000	0.102
8	BJC7	0.400	0.290	0.100	0.000	0.000	0.158
9	B25	0.100	0.040	0.013	0.000	0.000	0.031
10	DJC6	0.500	0.410	0.160	0.000	0.000	0.214
11	*R. eutropha*	0.490	0.380	0.160	0.000	0.000	0.206
	Mean	0.262	0.202	0.087	0.000	0.000	-

	SE m±	CD at 1%
Isolates (A)	0.002	0.007
Carbon sources (B)	0.013	0.035
A x B	0.0044	0.016

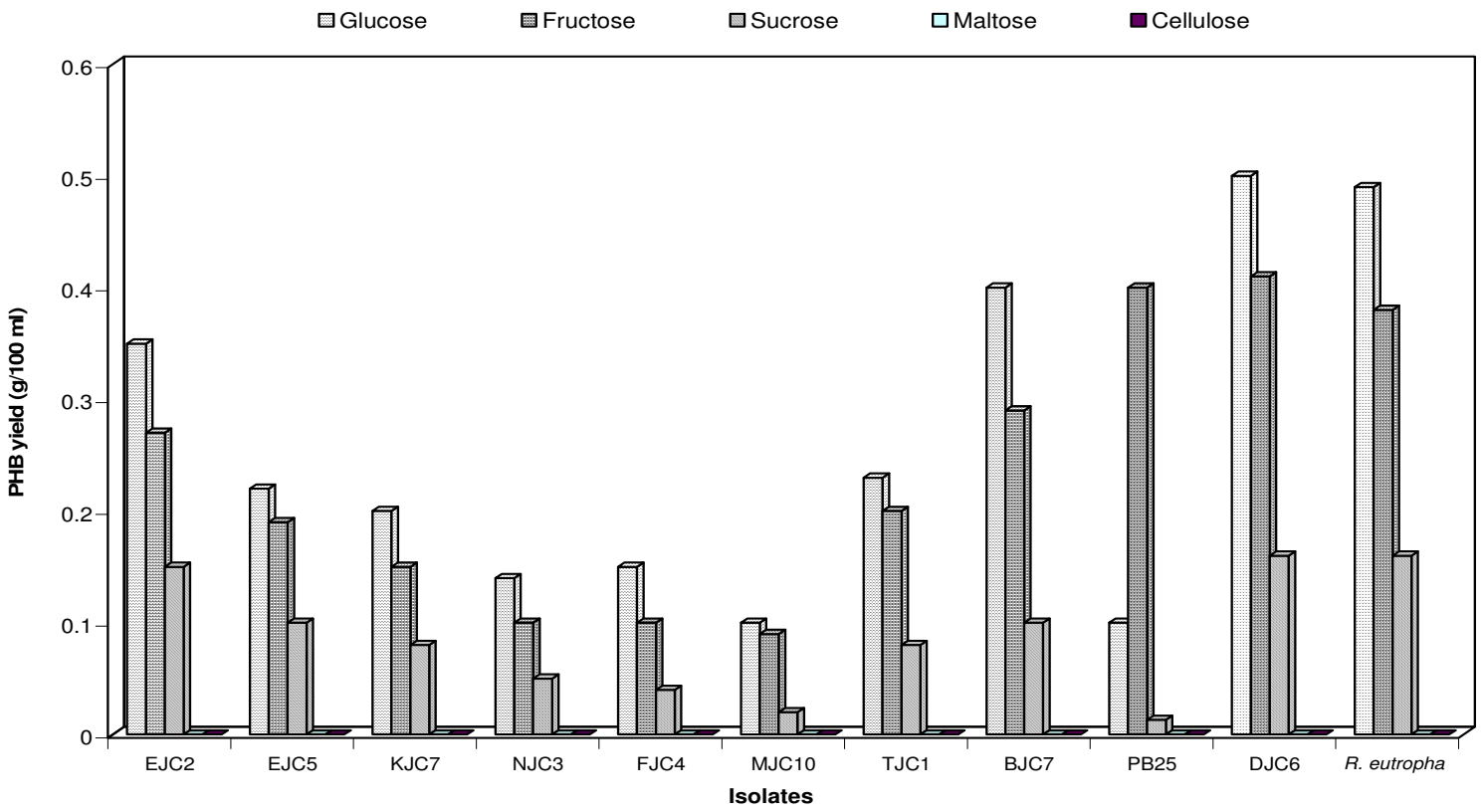


Fig. 1 : PHB production by the selected bacterial isolates as influenced by different carbon sources

Fig. 1 : PHB production by the selected bacterial isolates as influenced by different carbon sources

4.6 EFFECT OF DIFFERENT N SOURCES ON PHB YIELD BY THE SELECTED ISOLATES

Data pertaining to PHB yields produced by the selected isolates in presence of different N sources and the best carbon source (glucose 1%) are presented in Table 8 and Fig. 2. There were significant differences for the isolates, N sources and their interactions.

Amongst the isolates, DJC6 found to be a significantly higher PHB producer compared to all other isolates including the reference strain *R. eutropha*. It produced a mean PHB of 0.320 g/100 ml. This was followed by BJC7 and EJC2. B25 produced the least mean PHB yield of (0.076 g/100 ml).

Amongst different N sources, ammonium sulpahte was found to be the best N source. It produced a mean PHB of 0.377 g/100 ml. The next promising N sources were ammonium chloride with 0.236 g/100 ml and ammonium nitrate with 0.102 g/100 ml PHB yields. Yeast extract was found to be the least supporter of PHB production.

The interaction effects of isolates and N sources were also found to be significant. DJC6 on ammonium sulphate produced the highest PHB of 0.680 g/100 ml, which is significantly higher compared to PHB that is produced by *R. eutropha* (0.590 g/100 ml).

The PHB production by DJC6 with Ammonium chloride and ammonium nitrate and yeast extract were found statistically on par with those produced by R. *eutropha*.

The rest of the isolates and their interaction effects were found to be significantly lower than those of *R. eutropha*.

Table 8 : PHB yields (g/100 ml) produced by selected bacterial isolates as influenced by different N sources

Sl. No.	Isolates	N sources (0.1%)				Mean
		Ammonium sulphate	Ammonium chloride	Ammonium nitrate	Yeast extract	
1	EJC2	0.450	0.380	0.150	0.006	0.260
2	EJC5	0.320	0.200	0.140	0.007	0.167
3	KJC7	0.290	0.190	0.090	0.001	0.143
4	NJC3	0.230	0.100	0.050	0.002	0.096
5	FJC4	0.190	0.080	0.020	0.001	0.073
6	MJC10	0.200	0.180	0.070	0.004	0.113
7	TJC1	0.420	0.300	0.100	0.005	0.206
8	BJC7	0.580	0.290	0.090	0.004	0.241
9	B25	0.200	0.090	0.013	0.003	0.076
10	DJC6	0.680	0.390	0.200	0.008	0.320
11	*R. eutropha*	0.590	0.400	0.197	0.008	0.299
	Mean	0.377	0.236	0.102	0.009	-

	SE m±	CD at 1%
Isolates (A)	0.0031	0.012
Carbon sources (B)	0.0019	0.007
A x B	0.0063	0.023

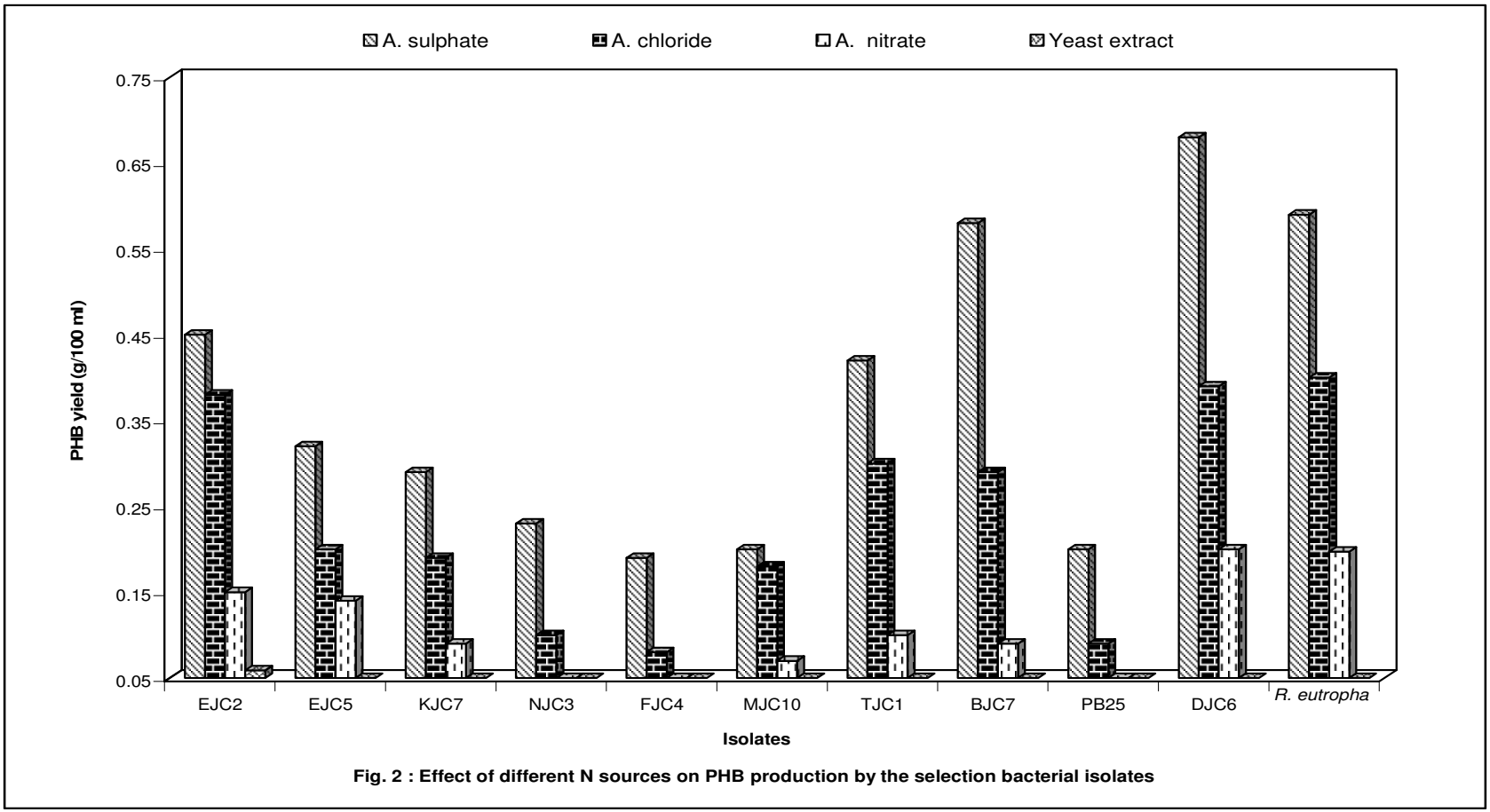


Fig. 2 : Effect of different N sources on PHB production by the selection bacterial isolates

Fig. 2 : Effect of different N sources on PHB production by the selection bacterial isolate

4.7 INFLUENCE OF DIFFERENT CONCENTRATIONS OF AMMONIUM SULPHATE (THE BEST N SOURCE) ON PHB YIELD BY THE SELECTED ISOLATES

PHB yields produced by the selected isolates when grown on different concentrations of the best N source *i.e.,* ammonium sulphate in presence of the best carbon source (glucose 1%) are furnished in Table 9 and Fig. 3. Amongst the different selected isolates DJC6, was found to be significantly superior compared to all isolates including the reference strain. It produced a mean PHB yield of 0.520 g/100 ml. BJC7 and EJC2 were the next leading PHB accumulators. However, the data were statistically inferior when compared to the reference strain.

Out of three concentrations of ammonium sulphate, it was found that of ammonium sulphate, at 1.0 g/l supported the highest PHB production (0.377 g/100 ml) when compared to other levels. In general, there was an increasing PHB production with an increase in ammonium sulphate concentration from 0.5 g/l to 1.0 g/l.

Amongst the interaction, it was found that the isolate DJC6 was found to produce the highest PHB yield of 0.680 g/100 ml at 1.0 g/l concentration which was significantly higher than the PHB produced by *R. eutropha* (0.590 g/100 ml). In fact, DJC6 was the highest PHB producer at all levels of ammonium sulphate, and B25 isolate was, similarly to be the least PHB producer at all levels.

Table 9 : Influence of different concentrations of ammonium sulphate on PHB production (g/100 ml) by the selected isolates

Sl. No.	Isolates	Different concentrations of ammonium sulphate (g/l)			Mean
		0.5	1.0	1.5	
1	EJC2	0.360	0.450	0.300	0.370
2	EJC5	0.280	0.320	0.210	0.270
3	KJC7	0.230	0.290	0.190	0.237
4	NJC3	0.160	0.230	0.100	0.163
5	FJC4	0.170	0.190	0.130	0.163
6	MJC10	0.140	0.200	0.090	0.143
7	TJC1	0.300	0.420	0.180	0.300
8	BJC7	0.470	0.580	0.240	0.430
9	B25	0.140	0.200	0.080	0.140
10	DJC6	0.580	0.680	0.300	0.520
11	*R. eutropha*	0.507	0.590	0.290	0.462
	Mean	0.303	0.377	0.192	-
		SE m±		**CD at 1%**	
Isolates (A)		0.0034		0.013	
Carbon sources (B)		0.0018		0.0060	
A x B		0.0059		0.0220	

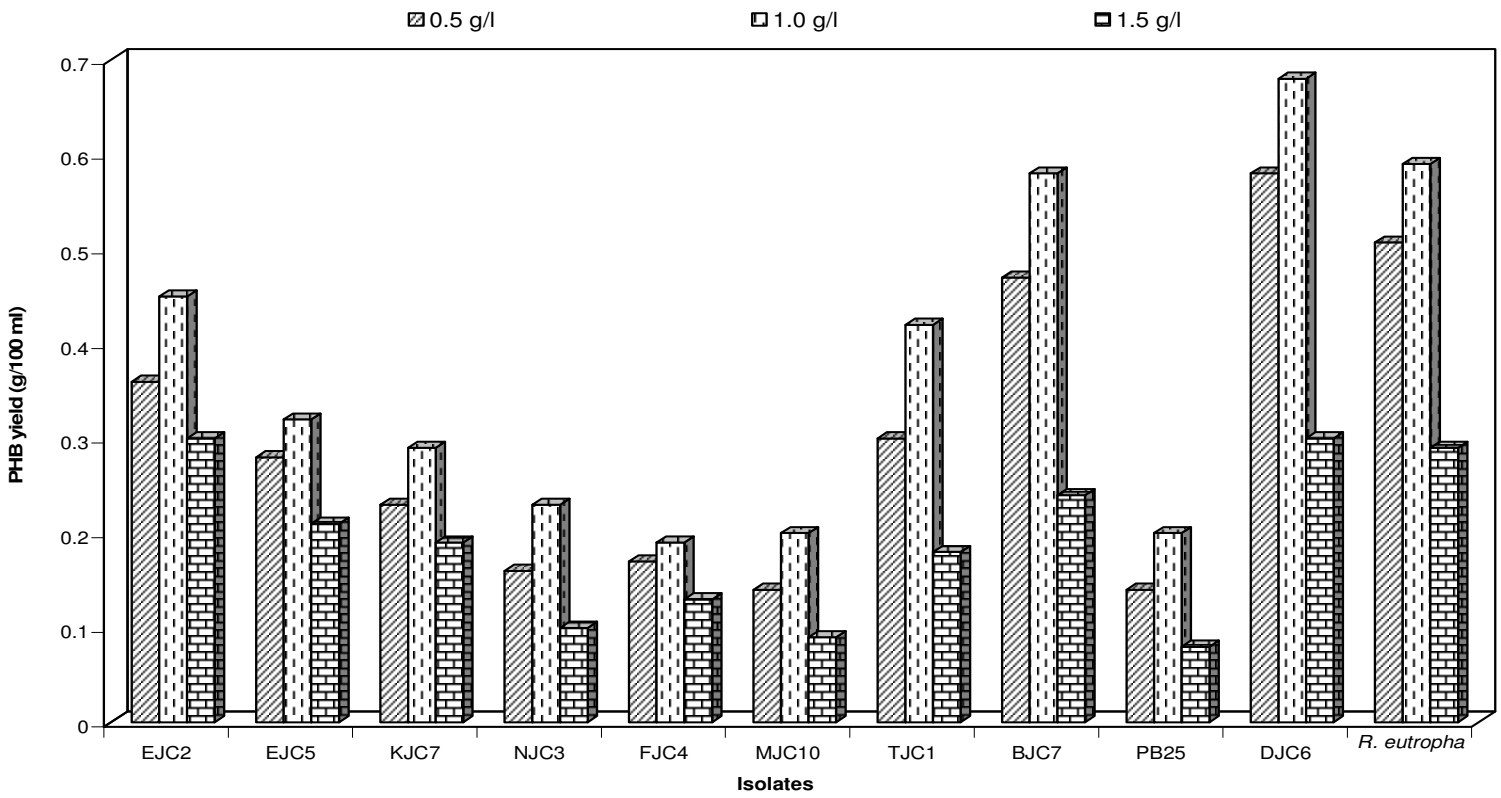


Fig. 3 : Influence of different concentrations of ammonium sulphate on PHB production by the selected bacterial isolates

Fig. 3 : Influence of different concentrations of ammonium sulphate on PHB production by the selected bacterial isolates

Table 10 : Effect of different C:N ratios on PHB yields (g/100 ml) by the selected bacterial isolates

Sl. No.	Isolates	Different C:N ratios				Mean
		10.:1	15:1	20:1	25:1	
1	EJC2	0.350	0.480	0.750	0.730	0.578
2	EJC5	0.220	0.39	0.700	0.690	0.500
3	KJC7	0.200	0.300	0.500	0.480	0.370
4	NJC3	0.140	0.280	0.450	0.430	0.325
5	FJC4	0.150	0.250	0.350	0.330	0.270
6	MJC10	0.100	0.190	0.400	0.380	0.267
7	TJC1	0.230	0.350	0.500	0.490	0.393
8	BJC7	0.400	0.590	0.890	0.840	0.680
9	B25	0.100	0.200	0.300	0.350	0.238
10	DJC6	0.500	0.827	1.100	1.080	0.877
11	*R. eutropha*	0.490	0.750	1.033	1.100	0.843
	Mean	0.262	0.419	0.634	0.627	-

	SE m±	CD at 1%
Isolates (A)	0.0098	0.036
Carbon sources (B)	0.0059	0.021
A x B	0.0195	0.072

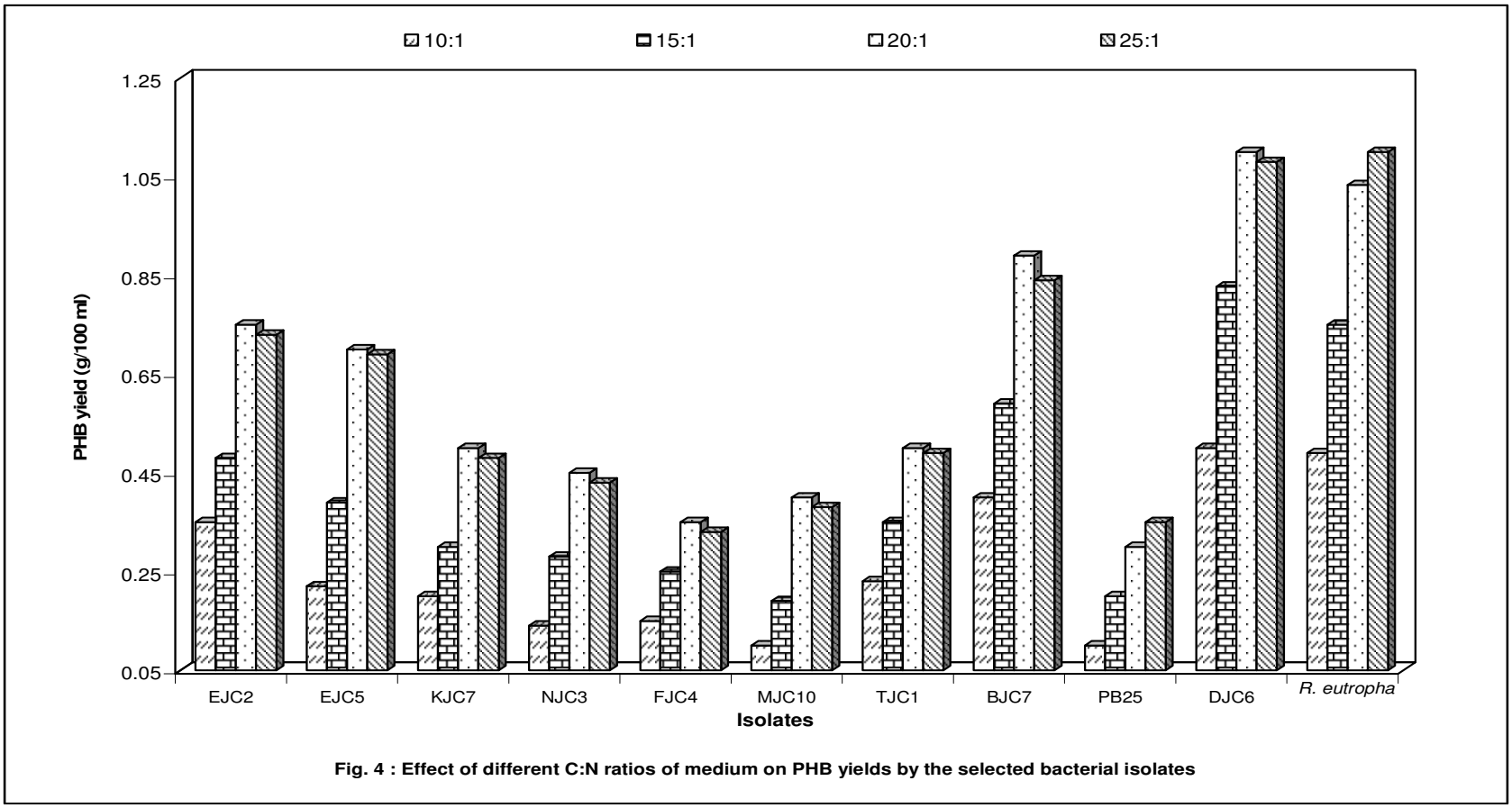


Fig. 4 : Effect of different C:N ratios of medium on PHB yields by the selected bacterial isolates

Fig. 4 : Effect of different C:N ratios of medium on PHB yields by the selected bacterial isolates

4.8 EFFECT OF DIFFERENT C:N RATIOS ON PHB YIELD BY THE SELECTED ISOLATES

Different C:N ratios were maintained using the best carbon and nitrogen sources in the mineral salts medium and their effects on PHB production were studied. The data are presented in Table 10 and Fig. 4.

Out of the isolates tested, DJC6 was found to be significantly higher PHB producer mean PHB of 0.877 g/100 ml. BJC7 and EJC2 were found to be the next promising isolates although statistically inferior when compared to the reference strain. The least PHB was accumulated by B25 (0.238 g/100 ml).

Amongst the different C:N ratios tested, 20:1 was found to be best C:N ratio supporting the highest PHB production (0.634 g/100 ml). In all the isolates, there was an increasing PHB production with an increase in C:N ratios upto 20:1 and tapering thereof. Interaction of DJC6 with different C:N ratio were found to be significantly higher than *R. eutropha*. The highest yield of PHB (1.100 g/100 ml) was by DJC6 at 20:1. This was followed by *R. eutropha* with 1.033 g/100 ml.

The reference strain was found to produce the highest PHB content at 25:1 C:N ratio which was 1.100 g/100 ml. At, 20:1, the next promising strains were BJC7 (0.890 g/100 ml) and EJC2 (0.75 g/100 ml). B25 recorded the lowest PHB content of 0.300 g/100 ml at 20:1 C:N ratio compared to all other isolates.

4.9 EFFECT OF DIFFERENT pH LEVELS ON PHB YIELD

Different pHs were maintained in the media prepared using the best carbon and nitrogen sources. Their effects on PHB production was evaluated. Data are presented in Table 11 and Fig. 5.

Out of the different pHs of media tested, 7.0 pH was found to be optimum for maximum PHB production by all the isolates.

No PHB production was observed at pH 6.0 by any of the isolate. At pH 7.0, the highest PHB of 1.100 g/100 ml was produced by DJC6 which was significantly higher than all the isolates including *R. eutropha.* The reference strain produced the second highest of 1.033 g/100 ml. The least PHB of 0.300 g/100 ml was accumulated by B25.

At pH 8.0, all the isolates were found to produce lower yields showing that pH 6.0 and pH 8 were not suitable for PHB accumulation. DJC6 was again the best isolate with mean PHB production of 0.567 g/100 ml. This was followed by *R. eutropha* (0.541 g/100 ml) and BJC7 (0.430 g/100 nl) pH 7.0 was found as the best pH for all the isolate. It resulted in the mean PHB production of 0.631, while it was 0.349 g/100 ml at pH 8.0.

Table 11 : Effect of different pH levels on PHB production (g/100 ml) by the selected isolates

Sl. No.	Isolates	pH			Mean
		6.0	7.0	8.0	
1	EJC2	0.000	0.750	0.410	0.387
2	EJC5	0.000	0.700	0.420	0.373
3	KJC7	0.000	0.500	0.400	0.300
4	NJC3	0.000	0.450	0.280	0.243
5	FJC4	0.000	0.350	0.200	0.183
6	MJC10	0.000	0.400	0.190	0.197
7	TJC1	0.000	0.500	0.250	0.250
8	BJC7	0.000	0.890	0.400	0.430
9	B25	0.000	0.300	0.100	0.133
10	DJC6	0.000	1.100	0.600	0.567
11	*R. eutropha*	0.000	1.033	0.590	0.541
	Mean	0.000	0.631	0.349	-
		SE m±		**CD at 1%**	
Isolates (A)		0.0086		0.0320	
Carbon sources (B)		0.0045		0.0170	
A x B		0.0149		0.0560	

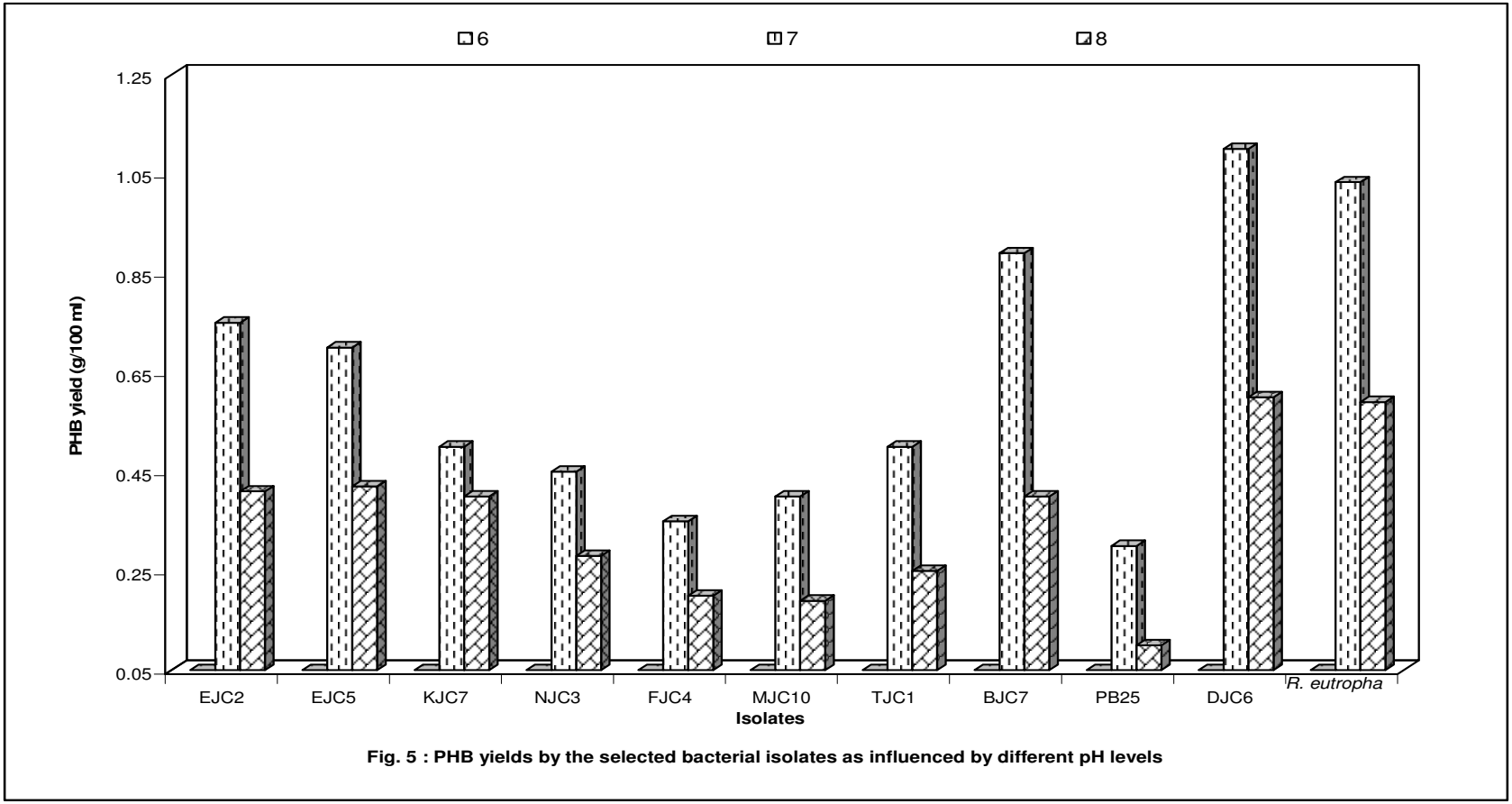


Fig. 5 : PHB yields by the selected bacterial isolates as influenced by different pH levels

Fig. 5 : PHB yields by the selected bacterial isolates as influenced by different pH levels

4.10 PHB YIELDS PRODUCED BY THE SELECTED ISOLATES ON CHEAPER SUBSTRATES

Bioeffluents such as dairy whey and soft drink industrial waste water were examined as cheaper substrates for PHB producton. Data revealed that the yields on these wastes were significantly lower than those produced using the optimized medium (Table 12 ; Fig. 6 ; Plates 4 and 5).

The mean PHB production on whey was 0.226 g/100 ml. In the optimized medium, it was as high as 0.634 g/100 ml. DJC6 produced the higher mean PHB yield of 0.587 g/100 ml which was significantly higher than that of *R. eutropha* which produced 0.548 g/100 ml. This was followed by BJC7 with an yield of 0.490 g/100 ml. The least was by B25 (0.150 g/100 ml).

DJC6 was found as the superior strain in utilizing sugars in whey medium. It has resulted in the highest PHB yield of 0.350 g/100 ml. *R. eutropa* and BJC7 strains were found to be next promising strains on whey. B25 and FJC4 yielded the least PHB of 0.100 g each. In soft drink industrial waste water also, DJC6 performed the best with 0.310 g/100 ml. This was followed by *R. eutropha* and BJC7 with 0.290 g/100 ml and 0.280 g/100 ml respectively.

4.11 PHB YIELDS BY THE SELECTED ISOLATES ON THE CHEAPER SUBSTRATES AMENDED WITH GLUCOSE

Cheaper substrates *viz.,* whey and soft drink industrial waste water were amended with glucose at 1 per cent level and selected bacterial isolates were inoculated and PHB production by them was tested. Data are given in Table 13 and Fig. 7 and Plate 6 and 7. Even when amended, both the wastes did not support high PHB production.

The optimized medium yielded the mean PHB of 0.634 g/100 ml, while whey and soft drink industrial waste water yielded 0.327 and 0.272 g/100 ml, respectively.

Amongst the isolates, DJC6 performed the best with a mean PHB of 0.667 g/100 ml. Which was significantly higher than all the isolates. Whereas, *R. eutropha* produced 0.624 g/100 ml. The least was produced by B25 (0.250 g/100 ml).

DJC6 in the amended whey medium produced significantly higher PHB yield of 0.500 g/100 ml. Whereas, *R. eutropha* and BJC7 were the next promising strains producing 0.450 g/100 ml each. The least was again by B25 (0.140 g/100 ml). In the amended soft drink industrial waste water also, DJC6 out performed *R. eutropha* with 0.400 g/100 ml PHB production, although statistically non-significant.

In the optimized medium, DJC6 resulted in a PHB yield of 1.100 g/100 ml which was significantly higher than that of *R. eutropha* (1.033 g/100 ml). The next best isolate was BJC7 which produced a PHB of 0.890 g/100 ml. The least was again by B25 with 0.300 g/100 ml of PHB production.

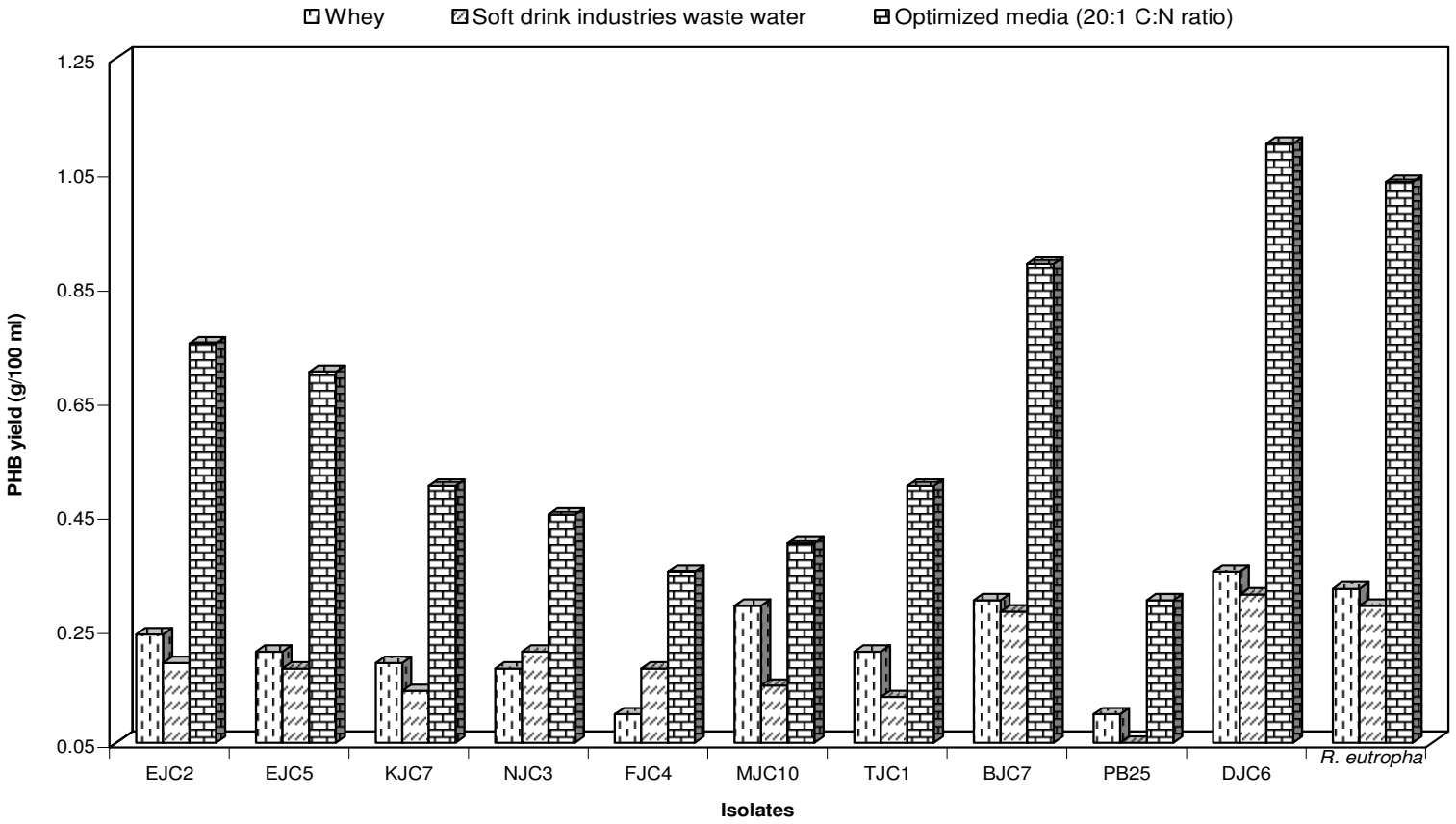


Fig. 6 : PHB yields (g/100 ml) produced by the selected bacterial isolates on cheaper substrates

Fig. 6 : PHB yields (g/100 ml) produced by the selected bacterial isolates on cheaper substrates

Table 12 : PHB yields (g/100 ml) produced by the selected bacterial isolates on cheaper substrates

Sl. No.	Isolates	Whey	Soft drink industrial waste water	Optimized medium	Mean
1	EJC2	0.240	0.190	0.750	0.393
2	EJC5	0.210	0.180	0.700	0.366
3	KJC7	0.190	0.141	0.500	0.277
4	NJC3	0.180	0.210	0.450	0.280
5	FJC4	0.100	0.180	0.350	0.210
6	MJC10	0.290	0.150	0.400	0.280
7	TJC1	0.210	0.130	0.500	0.280
8	BJC7	0.300	0.280	0.890	0.490
9	B25	0.100	0.050	0.300	0.150
10	DJC6	0.350	0.310	1.100	0.587
11	*R. eutropha*	0.320	0.290	1.033	0.548
	Mean	0.226	0.192	0.634	-
		SE m±		**CD at 1%**	
Isolates (A)		0.008		0.032	
Carbon sources (B)		0.0046		0.017	
A x B		0.0153		0.057	

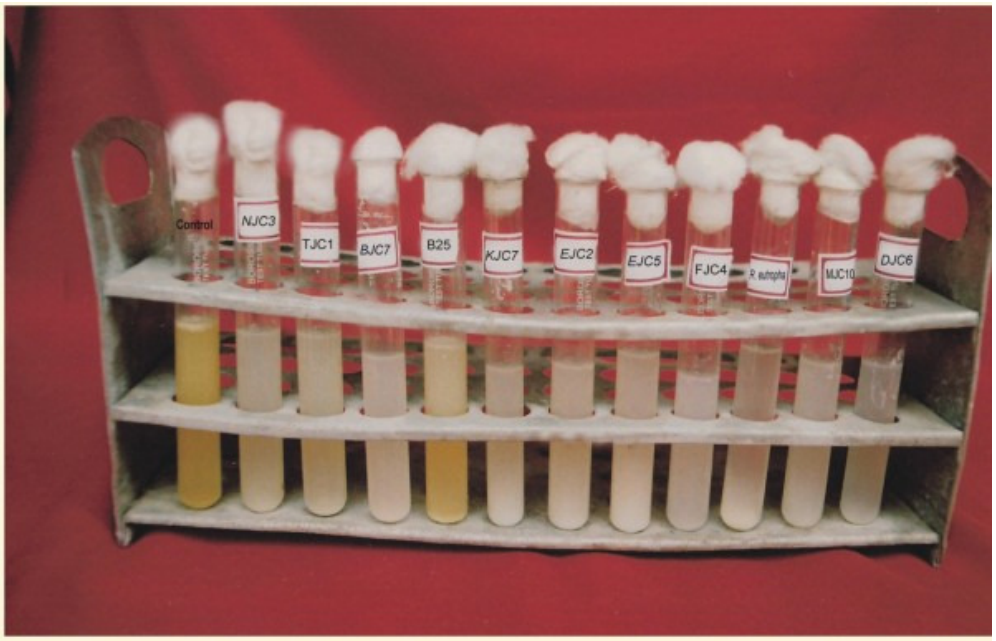


Plate 4 : Selected PHB synthesizing isolates growing on whey

Plate 4: Selected PHB synthesizing isolates growing on whey

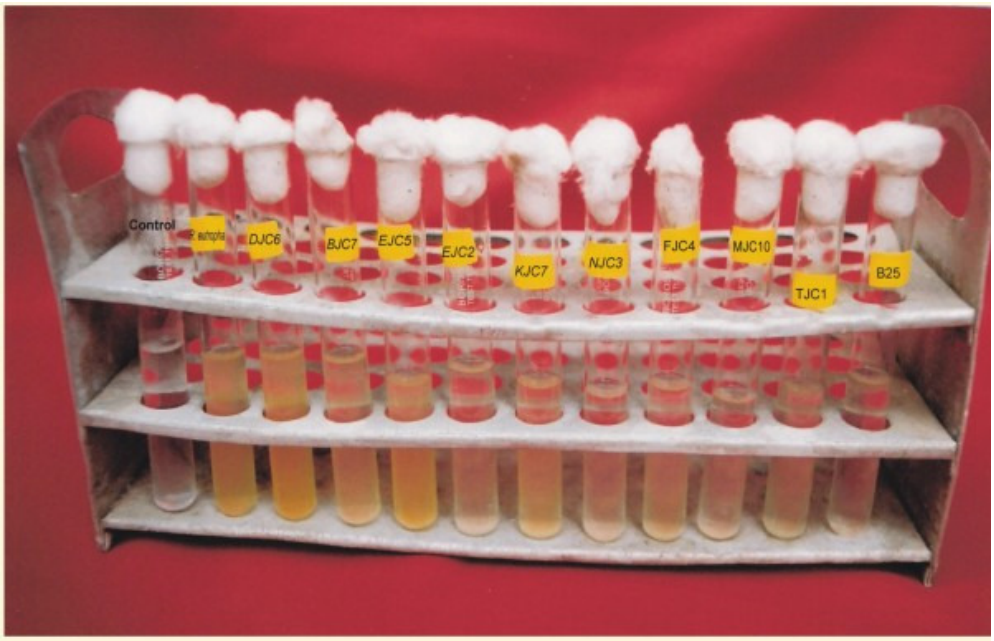


Plate 5 : Selected PHB synthesizing isolates growing on soft drink industrial waste water

Plate 5 : Selected PHB synthesizing isolates growing on soft drink industrial waste water

Table 13 : PHB yields (g/100 ml) produced by the selected bacterial isolates on cheaper substrates amended with glucose (1%)

Sl. No.	Isolates	Whey	Soft drink industries waste water	Optimized media (20:1 C:N ratio)	Mean
1	EJC2	0.350	0.220	0.750	0.440
2	EJC5	0.310	0.200	0.700	0.400
3	KJC7	0.280	0.190	0.500	0.323
4	NJC3	0.250	0.340	0.450	0.347
5	FJC4	0.230	0.320	0.350	0.300
6	MJC10	0.360	0.230	0.400	0.330
7	TJC1	0.250	0.200	0.500	0.317
8	BJC7	0.450	0.370	0.890	0.570
9	B25	0.140	0.310	0.300	0.250
10	DJC6	0.500	0.400	1.100	0.667
11	*R. eutropha*	0.450	0.390	1.033	0.624
	Mean	0.327	0.272	0.634	-
		SE m±		**CD at 1%**	
Isolates (A)		0.0090		0.033	
Carbon sources (B)		0.0047		0.018	
A x B		0.0156		0.058	

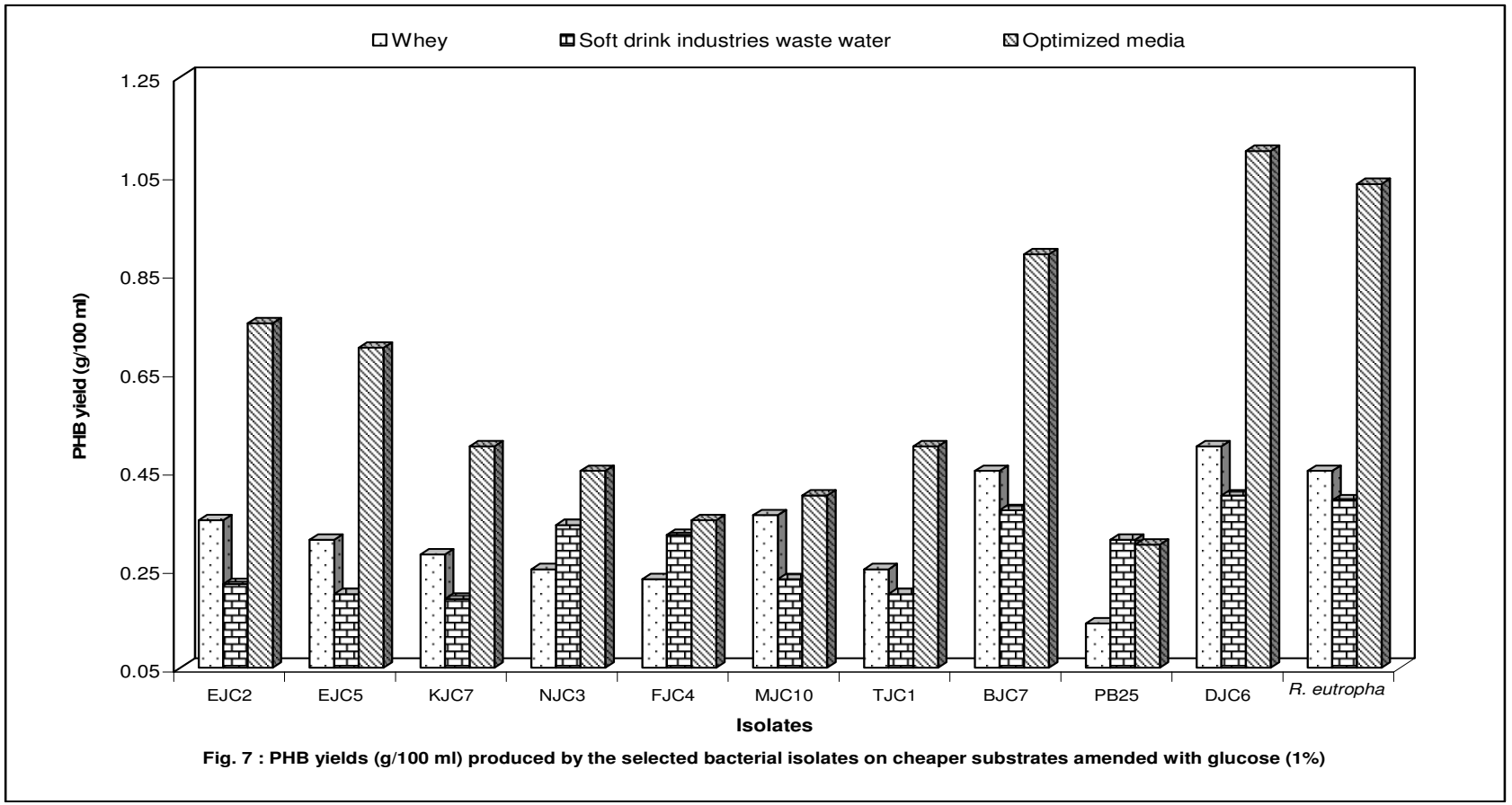


Fig. 7 : PHB yields (g/100 ml) produced by the selected bacterial isolates on cheaper substrates amended with glucose (1%)

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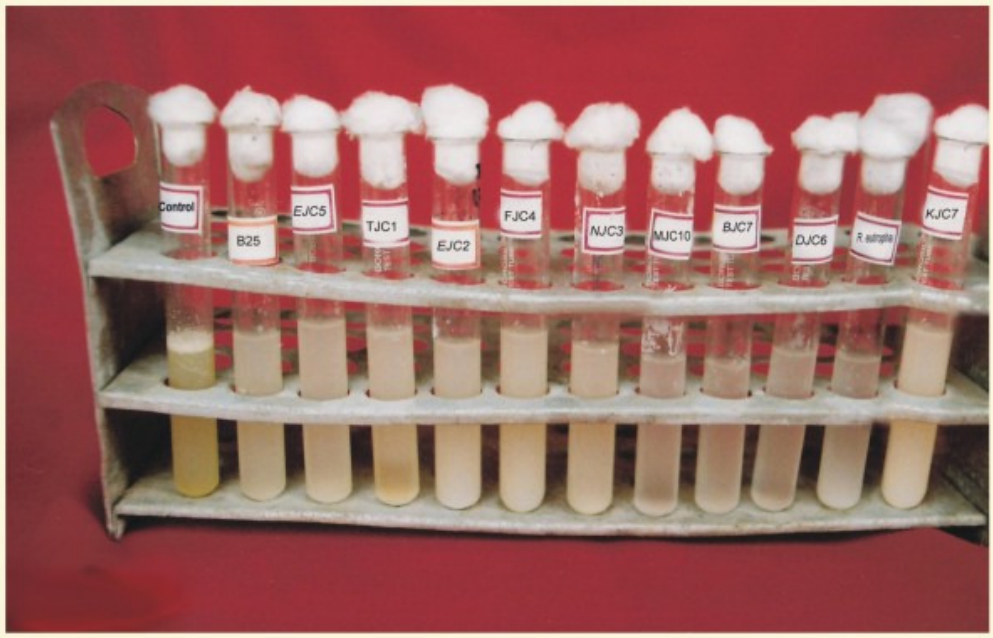

Plate 6 : Selected PHB synthesizing isolates growing on glucose amended whey

Plate 6 : Selected PHB synthesizing isolates growing on glucose amended whey

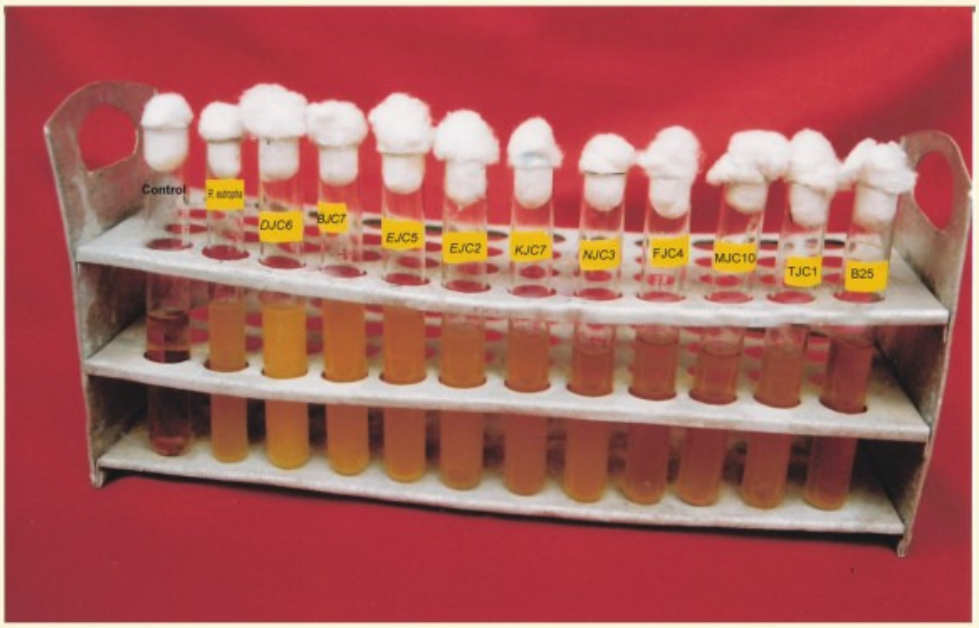

Plate 7 : Selected PHB synthesizing isolates growing on glucose amended soft drink industrial waste water

Plate 7 : Selected PHB synthesizing isolates growing on glucose amended soft drink industrial waste water

4.12 CHANGES IN THE CHEMICAL COMPOSITION OF CHEAPER SUBSTRATES AFTER GROWTH OF PHB SYNTHESIZING BACTERIA

After cultivation of *Pseudomonas* DJC6 in the cheaper substrates for 48 h, the chemical composition in terms of reducing sugars and COD were determined. It was interesting to note that all the reducing sugars in both the wastes were completely utilized by the strain (Table 14). In whey, there was a reduction in COD by about 43 per cent, in case of soft drink industrial waste water, COD reduction was about 55 per cent.

4.13 IDENTIFICATION OF THE MOST EFFICIENT PHB PRODUCING BACTERIA

Since DJC6 and DJC7 were found to be the most efficient PHB producing bacterial isolates, they were selected for further studies including characterization. They were tentatively identified as *Pseudomonas* sp. and *Bacillus* sp. respectively based on the morphological, physiological and biochemical tests (Table 15).

4.14 SCALE UP STUDIES USING THE EFFICIENT STRAINS IN OPTIMIZED MEDIUM

Out of 10 selected promising isolates, two (BJC6 and BJC7) were finally selected based on their performance under optimized conditions. Using these strains, scale up studies were conducted in 3 liter bottle fermenters containing 2 liter optimized medium. PHB accumulation by these strains was monitored every 12 h. PHB yield data are presented in Table 16 ; Fig 8 and Plate 8.

In all the strains, PHB started accumulating. Maximum accumulation was found at 48 h and remained stable thereafter. There was no PHB accumulation during the first 12 hr.

Out of three strains tested, the highest PHB production of 1.10 g/100 ml was obtained by DJC6. This was followed by the reference strain (1.033 g/100 ml) and the least was by BJC7 (0.089 g/100 ml).

Table 14 : Changes in the composition of cheaper substrates due to cultivation of *Pseudomonas* DJC6

Sl. No.	Cheaper substrates	pH	EC	Characteristics				
				Reducing sugars (%)		COD (ppm)		COD reduction (%)
				Initial	Final	Initial	Final	
1	Whey	7.01	2.10 dS/m	0.85	0.00	14000	8000	42.85
2	Soft drink industry waste water	7.06	1.80 dS/m	0.43	0.00	11000	5000	54.55

Table 15 : Morphological and biochemical characterization of the efficient PHB producing bacteria

Sl. No.	strain code	Morphological tests						Biochemical tests							Physiological tests			Carbon source utilization				Probable genus
		Colony colour	Pigment	Shape	Gram reaction	Fluorescence	Motility	Gelatinliquefaction	Starch hydrolysis	Casin hydrolysis	Citrate utilization	Indole production	Catalase test	Urease tests	Spore formation	Growth at 4^0C	Growth at 4^0C	Glucose	Fructose	Sucrose	Lactose	
1	BJC7	White	-	Rod	+	-	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	*Bacillus*
2	DJC6	Yellow	+	Rod	-	-	+	+	-	+	+	-	+	-	-	+	+	+	+	+	+	*Pseudomonas*

Table 16 : Scale up studies on PHB production (g/100 ml) by the most efficient bacterial isolates under optimized conditions

Sl. No.	Isolates	Incubation time (h)						
		0	12	24	36	48	60	72
1	*Pseudomonas* DJC6	0.0	0.0	0.070	0.390	1.100	1.100	1.100
2	*Bacillus* BJC7	0.0	0.0	0.050	0.280	0.890	0.890	0.890
3	*R. eutropha*	0.0	0.0	0.068	0.390	1.003	1.033	1.033

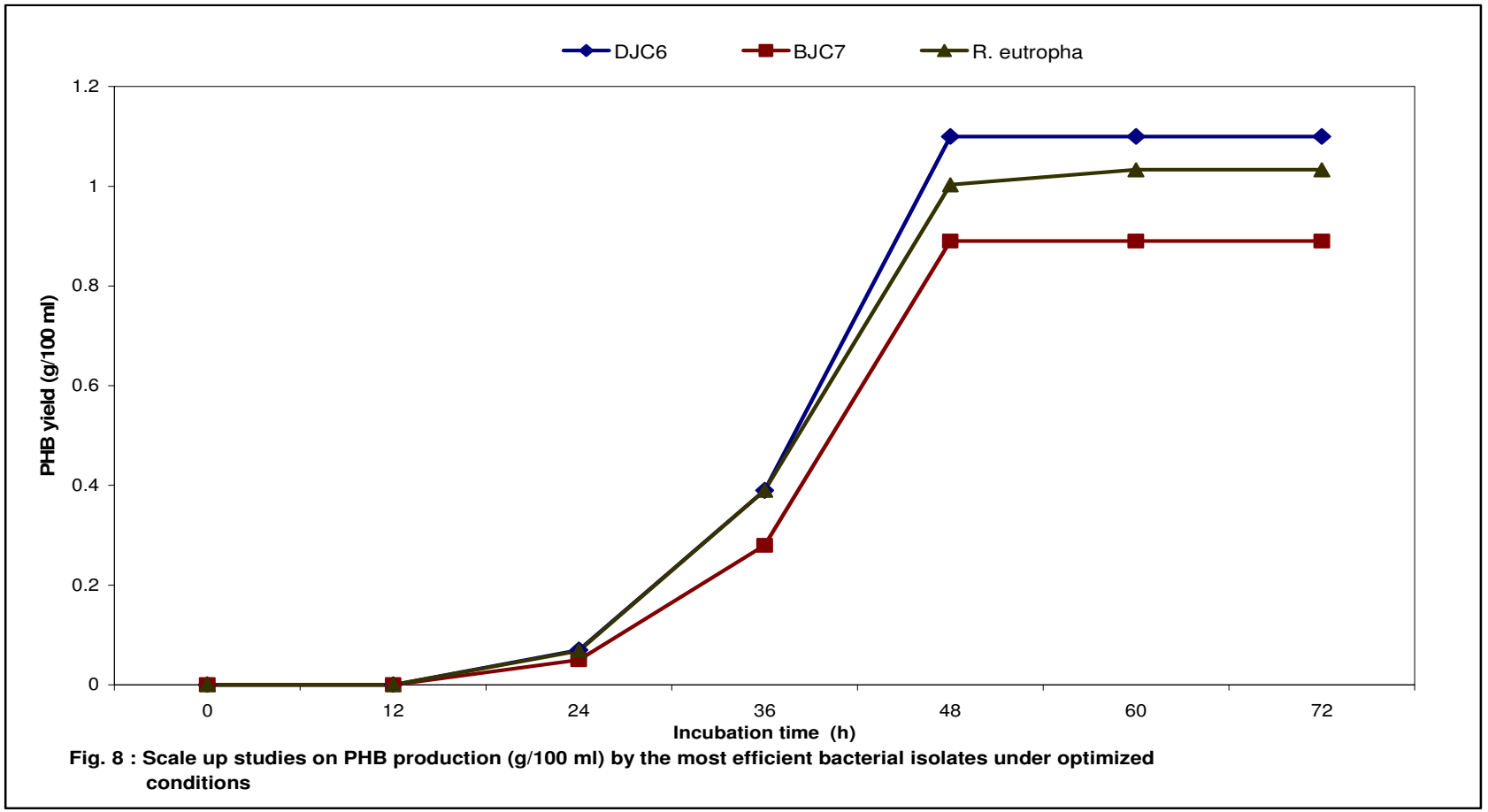

Fig. 8 : Scale up studies on PHB production (g/100 ml) by the most efficient bacterial isolates under optimized conditions

Fig. 8 : Scale up studies on PHB production (g/100 ml) by the most efficient bacterial isolates under optimized conditions

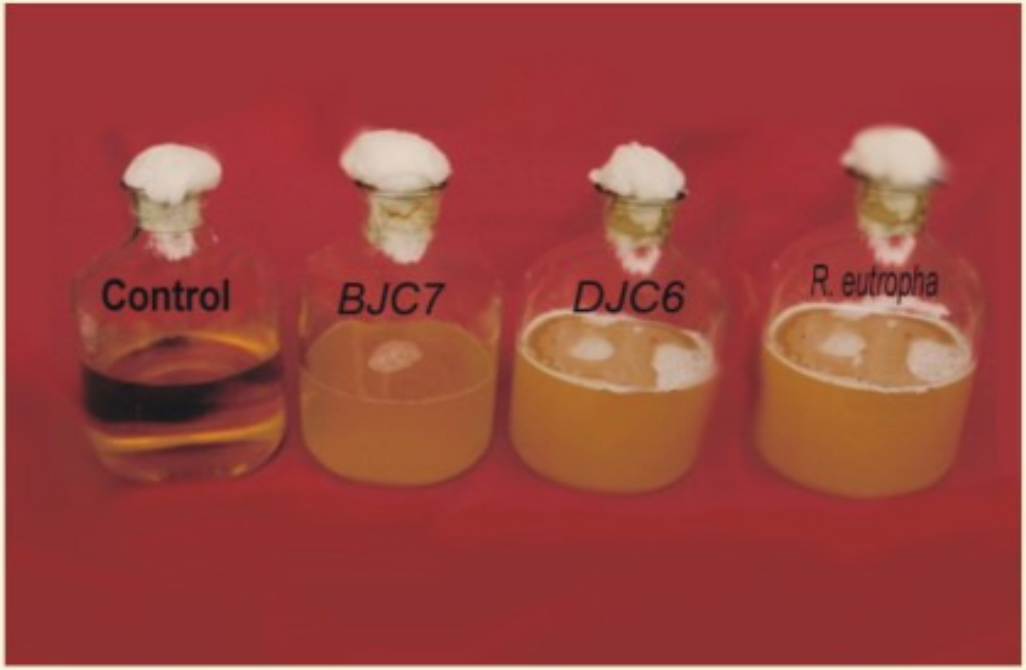

Plate 8 : Scale up studies on PHB production by efficient bacterial isolates

Plate 8 : Scale up studies on PHB production by efficient bacterial isolates

V. DISCUSSION

Plastics have become an important part of modern life and are used in different sectors of operations like packaging, building materials, consumer products and many more. Each year, about 100 million tonnes of plastics are produced world wide. Demand for plastics in India reached about 4.3 million tonnes in 2001-2002 and is expected to increase to about 8 million tonnes in 2006-07. Currently, however, the per capita consumption of plastics in India is only about 2 kg compared to 30-40 kg in developed countries (Kalia *et al.,* 2000). The present market, in India, is about Rs. 25,000 crores.

Most of the plastics and synthetic polymers are produced from petrochemicals. Because of their persistence in the environment, several communities are more sensitive to the impact of discarded plastics on the environment including deleterious effects on wild life and on the aesthetic qualities of cities and forests. Plastic bags or sheets do not allow water and air to percolate into earth causing reduction in fertility status of soil, depletion of underground water sources and damage to animal life. In seas too, plastic rubbish choke and entangle the marine mammals. In cities, they choke drains leading to submergence of roads especially during rainy season.

The increased cost of solid waste disposal as well as potential hazards from incineration of wastes such as dioxin emission from PVC makes synthetic plastic waste management a problem (Ojumun *et al.,* 2004).

Consequently hence, for the past two decades, there have been a growing public and scientific interest in the development and use of biodegradable polymers as an ecologically useful alternative to plastics. Biodegradable plastics are made from renewable resources and do not lead to depletion of finite resources. Polyhydroxyalkanoates (PHA) synthesized by at least 75 different genera of microorganisms are attracted as biodegradable plastics. They are accumulated intracellularly, as high as 90 per cent of cell dry weight under conditions of nutrient stress and act as a source of carbon and energy (Madison and Huisman, 1999).

Hence, in this investigation, attempts were made to isolate PHB accumulating bacteria from diverse sources and to select the efficient strains. The process parameters for maximum PHB production were also optimized.

5.1 SAMPLE COLLECTION FROM DIVERSE SOURCES AND ISOLATION OF PHB PRODUCING BACTERIA

Different samples were collected from different ecological areas and were used for isolating PHB producers (Table 1). Bacteria were isolated on nutrient agar medium supplemented with 1 per cent glucose (Anon., 1957), purified and preserved on nutrient agar slants till further use. All the 680 preserved isolates were tested for PHB production following the viable colony screening method (Juan *et al.,* 1998) based on the intensity of staining. The black stained isolates were ranked in terms of + symbol. The poorly stained colonies were indicated with + symbol, medium stained colonies as ++ symbol and strongly stained colonies as +++ symbol. All the isolates were given a code to represent the place of origin as done by Cristina *et al.* (1999). They also isolated bacteria from sugarcane ecosystem and screened for the PHB accumulators following viable colony staining method using Sudan Black B stain. They obtained the Sudan Black B positive colonies stained strongly (+++) on the media supplemented with 1 per cent glucose but did not observe any stained colonies which were grown on media supplemented with cellulose.

The relative occurrence of PHB accumulating microbes from a variety of samples were studied. A striking prevalence was observed in the activated sludge samples of KMF dairy, waste water samples of a soft drink industry, activated sludge samples of a food processing industry and from mangrove soil samples. PHB producers found in other samples were comparatively lower considering the number of samples collected. Similar results were also observed by Sujatha *et al.* (2005). They obtained higher PHB producers from tannery effluent and sewage sludge samples compared to garden and field soil samples.

In all, 680 bacterial isolates were isolated based on colony characteristics, out of which 125 were PHB positive. Out of these, 39 were moderate accumulators with ++ and 16 were strong accumulators with +++ and the rest 70 were poor accumulators with +.

5.2 QUANTIFICATION OF PHB PRODUCTION

All the 125 Sudan Black B positive isolates were subjected to quantitative PHB production spectrophotometrically following the method of John and Ralph (1961). In this method PHB is converted to crotonic acid which is brown coloured. One gram of PHB is equallent to 1 gram of crotonic acid (Law and Slepecky, 1969). By referring to the standard curve PHB production was quantified. For quantification, isolates were grown in Luria Bertani borth supplemented with glucose @ 2 per cent for 48 hr and used. Similarly, Sujatha *et al.* (2005) also used LB broth containing glucose 2 per cent as the medium, which favoured PHB accumulation due to higher C:N ratio.

It was interesting to note that PHB production varied from 0.010 to 0.160 g/100 ml of cell culture. Out of 125 isolates, 10 promising isolates were selected based on PHB yields. For optimization of cultural parameters. They were : EJC2, EJC5, KJC7, NJC3, FJC4, MJC10, TJC1, BJC7, B25 and DJC6. They were selected to include at least an isolate from each source tested. Out of 10 isolates, DJC6 was the highest PHB producer with 0.160 g/100 ml. Sujatha *et al.* (2005) also selected two isolates out of 65, based on PHB yields.

5.3 OPTIMIZATION OF PROCESS PARAMETERS

Optimization of fermentation conditions has long been used to enhance yields and productivities of many bioprocesses. Hence, in order to maximize PHB production by the selected isolates, various factors such as carbon source, nitrogen source, pH and C:N ratio were optimized.

5.3.1 Effect of carbon source on PHB production

Different carbon sources like glucose, fructose, sucrose, maltose and cellulose (1%) were amended to mineral salts medium and the bacterial isolates grown in them. Glucose was found to be the best carbon source. It yielded the mean PHB of 0.262 g/100 ml. The best isolate DJC6 has yielded the maximum PHB content of 0.500 g/100 ml which was significantly higher than all the isolates including the reference strain. This was followed by fructose (0.202/100 ml) and sucrose (0.087 g/100 ml). But, cellulose and maltose did not support any PHB accumulation. The superiority of the glucose in increasing PHB is in line with Choi *et al.* (1994). They studied the biosynthesis of PHB by *Hydrogenophaga pseudoflava* from various carbon substrates. D-glucose (1%) yielded the highest PHB content of 67.30 per cent.

Working with different carbon sources in MSM broth, Khanna and Srivastav (2005) observed higher PHB yield on fructose by *A. eutrophus.* They reported that glucose and fructose, being monosaccharides were readily utilized by bacteria and, hence, have supported growth and subsequently PHB production. The complex molecules like starch and lactose were not utilized. In our experiment also, the isolates did not produce PHB on maltose and cellulose indicating that the isolates do not possess enzymes involved in the degradation of cellulose and maltose into glucose. As the complexity of the carbon source increased PHB yield also decreased.

5.3.2 Effect of different N sources on PHB yield

To study the effects of N and to select the best N source for maximum PHB production, different nitrogen sources like ammonium sulphate, ammonium chloride and yeast extract were included in the mineral salts medium (1 g/l), with the best carbon source (glucose, 1%).

It is evident from the data that ammonium sulphate was the best supporter of PHB production (0.377 g/100 ml), followed by ammonium chloride (0.236 g/100 ml). These results are in agreement with the results obtained by Khanna and Srivastav (2005) who also observed the highest PHB production (2.260 g/100 ml) by *R. eutropha* on MSM medium supplemented with ammonium sulphate. Mulchandani *et al.* (1989) and Raje and Srivastava (1989) also worked on the accumulation of PHB by *A. eutrophus* with different salts of of ammonium. The highest PHB was obtained in ammonium sulphate followed by ammonium chloride.

5.3.3 Effect of different concentrations of the best N source (ammonium sulphate)

The presence of higher concentration of N in the medium is inhibitory for the accumulation of PHB. Hence, the concentration of the best N source needs to be optimized. Different concentrations of ammonium sulphate were amended to the MSM broth containing glucose (1%) as the best carbon source. From the studies, it was revealed that ammonium sulphate at 1.00 g/l was the optimum level for all the isolates including the reference strain with the mean value of 0.377 g/l. When ammonium sulphate concentration was increased from 0.50 g/l to 1.00 g/l, the PHB accumulation was also increased. But, at 1.5 g/l the PHB accumulation was decreased. This may be due to the non creation of N stress condition required for accumulation of PHB.

The effectiveness of ammonium sulphate @ 1g/l in enhancing PHB production is in accordance with the Khanna and Srivastav (2005) who found that this level produced the highest PHB by *R. eutropha.*

5.3.4 Effect of different C:N ratios on PHB yield

Under normal conditions, bacteria synthesize their body materials like proteins and grow. But, during nutrient limiting conditions, bacteria may shift their protein synthesis to PHB synthesis for survival. To exploit this phenomenon, experiments were carried out to study the accumulation of PHB with nitrogen limiting conditions. The C:N ratios were adjusted to 10:1, 15:1, 20:1 and 25:1 using glucose and ammonium sulphate as C and N sources respectively. The PHB yield analysis showed that there was the highest accumulation of PHB at 20:1 C:N ratio, by all the isolates. The most efficient isolate DJC6 was found to produce the PHB yield of 1.100 g/100 ml which is significantly higher than the PHB produced by the reference strain (1.033 g/100 ml).

The reference strain has produced a PHB of 1.100 g/100 ml at 25:1 C:N ratio, but our native strain DJC6 has produced this yield at 20:1. That means, our strain requires less of glucose when compared to the reference strain leading to lesser cost of production at the industrial scale.

At 25:1 C:N ratio, PHB yields were significantly lower by all the isolates when compared to those at 20:1. As the carbon content increased in the media keeping N as constant, upto certain limit (i.e., 20:1) PHB accumulation was increased and there off (25:1), it showed a decline. This was probably due to the substrate inhibition. Similar observations were made by other scientists. Belfors *et al.* (1995) reported that glucose and ammonium ions were inhibitory at certain levels, which affect the specific growth rate and PHB production. Inhibition by ammonium ions Heinzle and Lafferty (1980) and substrate inhibition by carbon source (Lee and Yoo, 1991) on PHB production have been reported.

5.3.4 Effect of pH on PHB yield

The effect of pH of the medium on PHB production was assessed. The data revealed that pH 7.0 was found to be optimum for all the isolates. pH 7.0 has recorded the mean PHB of 0.634 g/100 ml compared to pH 8.0 (0.349 g/100 ml). The isolate DJC6 was found to produce a PHB yield of 1.10 g/100 ml at pH 7.0 on the optimized medium, which is significantly higher than the PHB produced by the reference strain (1.033 g/100 ml). pH 7.0, being neutral, is the most favorable pH for bacterial growth and hence, would have contributed to higher PHB production.

This was in agreement with Aslim *et al.* (2002) who observed that the PHB in *Rhizobium* strain grown on yeast extract mannitol broth adjusted to pH 7.0, the amount of PHB in strain was 0.01 to 0.5 g/l and the percentage of PHB in these cells was between 1.38 and 40 per cent of cell dry weight.

Tavernler *et al.* (1997) also investigated the effect of different nitrogen, carbon and different pH levels on exopolysaccharide and PHB production in two strains of *Rhizobium meliloti.* They reported that these two strains showed higher PHB content at pH 7.0.

5.4 CHEAPER SUBSTRATES AND PHB PRODUCTION

Currently, the cost of production of PHB is higher mainly due to the factors such as the substrate, capital and recovery costs thereby making the use of these (PHB) materials in commercial production difficult. At present, the industrial production of PHBs are carried out from glucose as the substrate. If cheaper materials can be used to produce PHB, the substrate cost can be reduced, and the use of PHBs can be more feasible.

Cheese whey is a cheaper and easily available by product of the dairy industry. It is known to contain large amounts of fermentable sugars, mainly lactose, which causes much problem on its disposal, in our study, whey was used to produce PHB from the selected isolates. All the isolates were able to produce PHB on whey. The isolate DJC6 produced a PHB of 0.350 g/100 ml which is significantly higher compared to 0.320 g/100 ml by the reference strain. Results of this study indicated that it is possible to convert carbonaceous material present in the whey, in to environmentally friendly PHB polymer.

The PHB yields on whey were significantly higher than those on soft industrial waste water. But, the PHB yield on whey were very low, when compared to those on optimized media. In the optimized media, the mean PHB was 0.634 g/100 ml. While on whey, it was 0.226 g/100 ml. Yellore and Desai also attempted to produce PHB from whey using *Methylobacterial* sp. They obtained an yield of 0.98 g/l on whey. They further reported that PHB yield could be increased by amending whey with glucose. In our study also when we supplemented whey with 1 per cent glucose, the PHB accumulation was improved. The mean PHB content of 0.226 g/100 ml was enhanced to 0.327 g/100 ml due to glucose amendment. All the strains showed increased PHB content when grown on amended whey. Here also, DJC6 was found to produce significantly higher yield of 0.500 g/100 ml on amended whey when compared to *R. eutropha*.

Godabole *et al* (2000) studied PHB production using whey as the substrate for biomass production in the first stage and lactic acid as the substrate for accumulation of PHB in the second stage, maximum PHB content of 74 per cent of cell dry weight was observed.

Another substrate, waste water from a soft drink industry was used to produce PHB. In this waste water, all the isolates were found to produce PHB, but yields were lower than those produced in whey and optimized medium. DJC6 was again the highest PHB producer (0.310 g/100 ml) than the reference strain (0.290 g/100 ml). When soft drink industry waste water was supplemented with 1 per cent glucose, PHB content was increased in all the treatments but, still they were significantly lower than those on amended whey.

The isolate DJC6 produced a PHB yield of 0.400 g/100 ml. This is higher than the PHB yield produced by the reference strain (0.390 g/100 ml). Sureshkumar *et al.* (2004) proposed the production of PHB from activated sludge of waste water treatment plant of food processing industry waste water. They suggested that production and recovery of PHB from activated sludge could significantly reduce the cost of PHB and at the same time reduce the quality of excess sludge produced in waste water treatment system that required further treatment.

Pseudomonas DJC6 not only produced PHB on bioeffluents but also decreased their COD levels. By decreasing COD the strain has reduced the pollution levels of the bioeffluents on the environment. The strain has thus, achieved twin objectives of effective utilization of available natural resources for production of value added product (PHB) and reduction in cost of waste water treatment.

A perusal of Tables 12 and 13 indicate that DJC6 and BJC7 are more efficient in PHB production either in whey or soft drink industrial waste water or optimized medium. Hence, these two strains were selected for further studies after characterization and identification. These strains DJC6 and BJC7 were tentatively identified upto generic level as *Pseudomonas* and *Bacillus* respectively. DJC6 is actually from distillery spent wash polluted soil of Ugar. It is well known that *Pseudomonas* is a highly versatile organism in nature with myriad activities. It represents, the most abundant genus of the bacteria in many polluted soils (Leach *et al.,* 1994). Sujatha *et al* (2005) also isolated two PHB producing bacteria from tannery effluents and identified both of them as *Pseudomonas*. As many as 55 species of *Pseudomonas* have been characterized as PHB producers (Timm and Steinbuchel, 1990). *Bacillus magterium* as a PHB producer has been documented as early as 1926 by lemoigne (1926).

Substantial research has been carried out PHB production by different species of *Bacillus* and *Pseudomonas* by many workers (Yuksekdaj *et al.,* 2004 ; Yilmaz *et al.,* 2005 ; Ayub *et al.,* 2004).

5.5 SCALE UP STUDIES ON PHB PRODUCTION

Out of 10 promising bacterial isolates, two efficient strains (*Pseudomonas* DJC6 and *Bacillus* BCJ7) were further selected and used in a three liter lab scale bioreactor under optimized conditions. The strains were cultivated for 72 hr on mineral salts medium containing the best carbon and nitrogen sources, maintaining a C:N ratio of 20:1 and a pH of 7.00. Their PHB yields were monitored over a period of time and compared with those produced by the reference strain.

In all the strains, PHB accumulation was increased with time upto 48 h and remained stable thereafter, showing that 48 h as the optimum time of incubation for maximum PHB synthesis. *Pseudomonas* DJC6 accumulated higher PHB of 1.100 g/100 ml which is on par with *R. eutropha* with 1.033 g/100 ml.

Thus the study has come out with an efficient natural PHB synthesizing bacterial strain *Pseudomonas* DJC6. Which is superior to the reference strain *R. eutropha.* Under the optimized conditions, in three litre bottle fermentors. The strain produced 1.10 g/100 ml in 48 h. The strain could produce PHB on cheaper substrates like whey, besides reducing the COD by about 43 per cent.

VI. SUMMARY

In the present study, an attempt was made to isolate efficient PHB producing bacteria from samples collected from various sources. The cultural parameters were optimized for the promising isolates and scale up studies were conducted for the efficient isolates to study the consistency in their production. With a view to reduce the cost of PHB production, two cheaper substrates were evaluated for production of PHB.

The salient features of the findings are outlined below.

1. A total of eight samples were collected from different ecological areas and 656 bacteria were isolated and 24 bacteria were collected from the Departmental culture collection. All these were subjected for the detection of PHB accumulation by employing viable colony staining method using Sudan Black B stain.
2. Out of 656 isolates, 101 were found to be PHB positive and out of 24 collected bacterial isolates, 24 were found to be PHB positive.
3. All the 125 PHB positive isolates were subjected to quantitative estimation of PHB production. PHB yields varied from 0.010 to 0.160 g/100 ml.
4. Ten promising bacterial isolates were selected based on their PHB yields. They were EJC2, EJC5, KJC7, NJC3, FJC4, MJC10, TJC1, BJC7, B25 and DJC6.
5. The culture parameters were optimized for all the 10 isolates, Glucose was found to be the best carbon source for maximum PHB production by all the isolates.
6. Ammonium sulphate supplementation at 0.1 per cent was found to be optimum.
7. Maintaining the C:N ratio of 20:1 using the best C and N source was found to be optimum.
8. Maintaining the pH of the medium at 7.0 was found optimum.
9. Whey and waste water of a soft drink industry were tested as cheaper substrates. Although they supported PHB production the yields were rather low when compared to those on standard media. However, the yields could be improved to some extent, by supplementation with glucose at 1 per cent level.
10. DJC6 not only produced PHB on whey but also reduced its COD by 43 per cent, thus functioning as a bioremediating agent.
11. Out of ten natural promising isolates DJC6 and BJC7 were found to be the most efficient PHB producers. Under optimized conditions, DJC6 produced a PHB yield of 1.100 g/100 ml while BJC7 produced 0.890 g/100 ml.
12. DJC6 performed better than the reference strain *R. eutropha* in PHB production.
13. DJC6 and BJC7 were tentatively identified as *Bacillus* spp. and *Pseudomonas* spp. respectively, based on the morphological and biochemical tests.
14. Scale up studies in 3 litre bottle fermenters were conducted under the optimized conditions. *Pseudomonas* sp. DJC6 produced a PHB yield of 1.100 g/100 ml which is marginally higher than that of *R. eutropha* 1.033 g/100 ml.

VII. REFERENCE

ANDERSON, A. J. AND DAWES, E. A., 1990, Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiology Review*, 54 : 450-472.

ANONYMOUS, 1957, Manual of microbiological methods Mc Graw Hill Book Company inc., New York, p. 127.

ARKIN A. H. HAZER B. AND BORCAKLI, M., 2000, Chlorination of poly (3-hydroxyalkanoates) containing unsaturated side chains. *Macromolecules,* 33 :3219-3223.

ASENJO, J. A., SCHMIDT, S. A. ANDERSON, P. R. AND ANDREWS, B. A., 1995, Effect of single nutrient limitation on poly-β-hydroxybutyrate molecular weight distribution in *Alcaligenes eutrophus. Biotechnology and Bioengineering*, 46: 497-502.

ASLIM, L. B., YUKSEKDAG, N. Z. AND BEYATLI, Y., 2002, Production of poly-□-hydroxybutyrate PHB by some *Rhizobium* bacteria. *Turkish Journal of Bacteriology*, 26: 215-219.

AYUB, N. D., PETTINARI, M. J., RUIZ, J. A., AND LOPEZ, N. J., 2004, A polyhydrobutyrate – porducig *Pseudomonas* sp. isolated from Antarctic environments with high stress resistance. *Current Microbiology*, 49 : 170-174.

BAILEY, W. R. AND SCHOTT, E. G., 1970, Diagnostic microbiology, A Laboratory Manual.

BALLARD, B. G. H., HOLMES, P. A., SENIOR, P. J., 1987, Formation of polymers of poly-□-hydroxybutyric acid in bacterial cells and a comparison of the morphology of growth with the formation of polyethylene in the solid state. *Recent Advance Mechanical Synthesis Aspects of Polymer*, pp. 293-214.

BARTHALOMEW, J. W. AND MITTEWER, T., 1950, A simplified bacterial strain. *Stain Technology*, 25 : 153.

BELFARS, L., PERRIER, M., RAMSAY, B. R., JOLICOEUR, M. AND CHAVARI, C., 1995, Multi inhibition kinetic model for the growth of *Alcagenes eutrophus. Canadian Journal of Microbiology*, 41 : 249-256.

BOOPATHY, R., 2000, Factor limiting bioremediation technologies. *Bioresource Technology,* 74 : 63-67.

BORAMANN, E. J., LEISNNER, M., ROTH, M., BEER, B. AND METZNER, K., 1998, Production of polyhydroxybutyrate by *Ralstonia eutropha* from protein hydrolysates. *Applied Environmental Microbiology*, 50 : 604-607.

BOURQUE, D., OUELLETTE, B., ANDRE, G. AND GROLEAU, D., 1992, Production of poly-β-hydroxybutyrate from methanol: Characterization of a new isolate of *Methylobacterium extorquen*s. *Applied Microbial Biotechnology*, 37 : 7-12.

BOURQUE, D., POMERLEAU, Y. AND GROLEAU, D., 1995, High cell-density production of poly-β-hydroxybutyrate (PHB) from methanol by *Methylobacterium extorquen*s: Production of high-molecular-mass PHB. *Applied Microbial Biotechnology*, 44 : 367-376.

BRANDAL, H., GROSS, R. A., LENG, R. W. AND FULLER, R. C., 1988, *Pseudomonas olevorans* as a source of poly-□-hydroxybutyrate for potential applications as biodegradable polyesters. *Applied Environmental Microbiology*, 54 : 1977-1982.

BRANDL, H. AND HANSELMANN, K. W., 1991, Evaluation and application of dialysis pore water samples for microbiological studies at sediment water inter faces. *Aquatic Science*, 53 : 55-73.

BRANDL, H., GROSS, R. A., LENZ, R. W. AND FULLER, R. C., 1988, *Pseudomonas olevorans* was a source of poly (□-hydroxy alkanoates) for potential applications as biodegradable polyesters. *Applied Environmental Microbiology*, 54 : 1977-1982.

BRAUNEGG, G., LEFEBVRE, G., RENNER, G., ZEISER, A., HAGGE, H. AND LOIDL-LANTHALER, K., 1998, Kinetics as tool for PHA production and optimization, 41 : 239-2478.

BURDON, K. L., STOKES, J. C. AND KIMBROUGH, C. E., 1942a, Studies of the common aerobic spore-forming *Bacilli* staining for fat with Sudan Black B- stain. *Journal of Bacteriology,* 43 : 717-724

BYROM, D., 1990, Industrial production of Copolymer from *Alcaligenes eutrophu*s. In: Novel Biodegradable Microbial Polymers, (ed.), Dawes, E. A. Kluwer Academic Publishers, The Netherlands, pp. 113-117.

BYROM, D., 1992. Production of Poly-β-hydroxybutyrate: Poly-β-hydroxyvalerate Copolymers. *FEMS Microbiological Reviews,* 103 : 247-250.

CAPPUCCINO, T. G. AND SHERMAN, N., 1992, Microbiology, a laboratory manual. The Benjamin/cummings publishing, Company Inc., California.

CHOI, J. AND LEE, S. Y., 1999, Factors affecting the economics of poly-hydroxyalkanoates production by bacterial fermentation. *Applied Microbial Biotechnology*, 51 : 13-21.

CHOI, J. AND LEE, S. Y., 2000, Economic consideration on the production of poly(□-hydroxybutyrate-co-3-hydroxyvalerate) by bacterial fermentation. *Applied Microbial Biotechnology*, 53 : 646-649.

CHOI, M. H., SONG, J. J., YOON, S. C., 1994, Biosynthesis of copolyesters by *Hydrogenophaga pseudoflava* from various lactones. *Biomacromolecules*, 1 : 60-67.

COOL, G. T. AND CANNON, M. C., 1999, poly-□-hydroxybutyrate inclusion body – associated proteins and coding region in *Bacillus megaterum*. *Journal of Bacteriology*, 181 : 589-592.

CRISTINA, T. S., DE LIMA, GRISI, B. M. AND BONATA, C. M., 1999, Bacteria isolated from a sugarcane agro ecosystem : their potential production of PHA and resistance to antibiotics. *Revista Microbiology*, 30 (3) : 50-55.

DANIEL, K., SAUL, B., WDOUARD, J. AND YAACOV, O., 2002, Identification and isolation of genes involved in poly(-□-hydroxybutyrate) biosynthesis in *Azospirillum brasilense* and characterization of PHB mutant. *Applied Environmental Microbiology*, 68 : 2943-2949.

DANIEL, M., CHOI, J. H., KIM, J. H. AND LEBEAULT, J. M., 1992, Effect of nutrient deficiency on accumulation and relative molecular weight of poly-β-hydroxybutyric acid by *Methylotrophic* Bacterium, *Pseudomonas* 135. *Applied Microbial Biotechnology*, 37 : 702-706.

DAWES, E. A., 1985, Starvation, survival and energy reserves. In: Bacteria in their natural environment, (eds), Fletcher, M. and G. D. Floodgate, Academic Press, Florida, pp.43-79.

De KONING, G. J. M., KELLERHALS, M., VAN MEURS, C. AND WITHOLT, B., 1997, A process for the recovery of poly(hydroxyalkanoates) from *Pesudomonads* 2. process development and economic evaluation. *Bioprocess Engineering*, 17 : 15-21.

De KONING, G.. J. M., WITHHOLT, B., 1997, A process for the recovery of poly(-3-hydroxyalkanoates) from *Pseudomonads* 1. solubalization. *Bioprocess Engineering*, 17 : 15-21.

DOI Y., KUNIOKA, M. NAKAMURA, Y. AND SOGA, K., 1987, Biosynthesis of Copolyesters in *Alcaligenes eutrophus* H16 from 13 C-Lebeled Acetate and Propionate. *Macromolecules*, 20 : 2988-2991.

DOI, Y., 1990, *Microbial Polyester*s. VCH Publishers, Inc. New York, USA.

DOI, Y., SEGWA, A., NAKAUMURA, S. AND JUNIOKA, M. T., 1990, Production of biodegradable co-polyesters by *Alcaligenes eutrophus*. In : *New Biosynthetic Biodegradable Polymers of Industrial Interest from Micro-organisms*, pp. 37-48.

FEUCHTENBUSCH, B., WULLBARNADT, D. AND STEINBYCHEL, A., 2000, Production of polyhydroxybutyrate acids by *Ralstoni eutropha* and *Pseudomonas olevorans*

from oil remaining from biotechnological rhamnose production. *Applied Microbial Biotechnology*, 53 : 167-172.

FIDLER, S. AND DENNIS, D., 1992, Polyhydroxyalkanoate production in recombinant. *E. coli. FEMS Microbiology Review*, 103 : 231-236.

FINDLAY, R. H. AND WHITE, D. C., 1983, Polymeric poly-□-hydroxybutyrates from environmental samples and *Bacillus megaterium*. *Applied Environmental Microbiology*, 45 : 71-78.

FLECHTER, A., 1993, In : plastics from bacteria and for bacteria : PHA as natural, biodegradable polyesters. Springer Verlag, New York, pp. 77-93.

FRAZIER, W. C., MARTH, E. H. AND DIEBEL, R. H., 1967, Laboratory manual for food microbiology. Burgess Publishing Company.

FUECHTENBUSCH, B. AND STEINBUECHEL, A., 1999, A biosynthesis of polyhydroxyalkanoates from low rank coal liquefaction products by *Pseudomonas oleovorans. Applied Environmental Microbiology*, 52 : 91-95.

GALAGO, N., ROZA, C., SANCHEZ, R., FUNG, T., VAZQUEZ, A. AND TOMAS, J. S., 2000, Charactreization and application of poly(□-hydroxybutyrates) family as composite biomaterials. *Polymer Technology*, 19 : 485-492.

GARCIA B., OLIVERA, E. R., MINMBRES, B., VALVERDE, M. F., LIBRADA, M., CANEDO, M. L., PRIETO, M. A., GARCIA, J. L., MARTINEZ, M. AND LUENGO, J.M., 1999, Novel biodegradable aromatic plastics from bacterial source: Genetic and biochemical studies on a route of the phenyl acetyl-CoA catabolism. *Journal of Biological Chemistry,* 274 : 29228-29241.

GHATNEKAR, M. S., PAI, J. S. AND GANESH, M., 2002, Production and recovery of poly-□-hydroxybutyrate from *Methylobacterium* sp. V49. *Journal of Chemical Technology and Biotechnology*, 77: 444-448.

GODBOLE, S., DAGINAWALA, H. F. AND CHAKRABARTI, T., 2000, Effect of carbon and nitrogen sources on production of poly-□-hydoxybutyrate from whey by *Alcaligenes eutrophus.* In : *59th Annual Conference of Association of Microbologists of India*, p. 59.

HAHN, S. K., CHANG, Y. K. AND LEE, S. Y., 1994, Recovery and characterization of poly(3-hydroxybutyric acid) synthesized in *Alcaligenes eutrophus* and recombinant *Escherichia coli*. *Applied Environmental Microbiology*, 61 : 34-39.

HARTMAN T. L., 1940, The use of Sudan Black B as a bacterial fat stain. *Staining Technology,* 15 : 23-28.

HEIN, S., TRAN, H. AND STEINBUCHEL, 1998, A *Synechocystis* sp. PCC6803 possess a two component polyhydroxyalkanoic acid synthase gene similar to that of anoxygenic purple sulfur bacteria. *Archieves of Microbiology*, 170: 162-170.

HEINZLE, E. AND LAFFERTY, R. M., 1980, A kinetic model for the growth and synthesis of poly-□-hydroxybutyric acid (PBH) in *Alcaligenes eutrophus* H16. *European Journal of Applied Microbial Biotechnology*, 11 : 8-16.

HOBSON, P. N., BOUSEFIELD, S. AND SUMMERS, R., 1981, Methane production from agricultural and Domestic wastes. Energy from wastes series. *Applied Science Publishers*, London.

HOLMES, P. A., 1985, Application of PHB- a Microbially Produced Biodegradable Thermoplastics, *Physical Technology,* 16 : 32-36.

HARBAK E. M AND WILLIS, D. K., 1992, The *lemA* gene required for pathogenecity of *Pseudomonas syringae* pv. Syringae on bean is a member of a family of two-component regulators. *Journal of Bacteriology,* 174 : 3011-3020.

HRABAK, O., 1992, Industrial production of Poly-β-hydroxybutyrate. *FEMS Microbial Reviews*, 103 : 251-256.

HUISMAN, G. W., LEEUW, De. AND EGGINK, G., 1989, Synthesis of poly-□-hydroxybutyrates is a common feature of fluorescent *Pseudomonas*. *Applied Environmental Microbiology*, 55: 1949-1954.

JACKSON, D. E. AND SRIENC, F., 1994, Novel methods to synthesize Polyhydroxyalkanoates. *Biochemical Engineering Annals of the New York Academy of Sciences*, 8 (745):134-148.

JI-HOE, K. AND LEE, J. K., 1997, Cloning, nucleotide sequence and expression of gene coding for poly-3-hydoxybutyate acid (PHB) of *Rhodobacter sphaeroides*. *Journal of Microbial Biotechnology*, 7 : 229-232.

JOHN, H. L., RALPH, A. S., 1961, Assay of poly-□-hydroxybutyric acid. *Journal of bacteriology*, 82 : 33-36.

JOHNSTONE, B., 1990, A throw away answer. *Far Eastern Economical Review*, 147 (6) : 62-63.

JUAN, M. L., GONZALEZ, L. W. AND WALKER, G. C., 1998, *A Novel Screening Method for Isolating Exopolysaccharide deficient Mutants. Applied and Environmental Microbiology,* 64 : 4600-4602.

KALIA, V. C. AND JOSHI, A. P., 1995, Conversion of waste biomass (pea-shells) into hydrogen methane through anaerobic digestion. *Bioresource Technology*, 53: 165-168.

KALIA, V. C., KUMAR, A., JAIN, S. R. AND JOSHI, A. R., 1992, Biomethonation of plant materials. *Bioresource Technology*, 41: 209-212.

KALIA, V. C., RAIZADA, N. AND SONAKYA, V., 2000, Bioplastics. *Journal of Scientific and Industrial Reserach*, 59 : 433-445.

KHANNA, S. A. K. AND SRIVASTAVA, 2005, Statistical media optimization studies for growth and PHB production by *Ralstonia eutropha*. *Process of Biochemistry*, 4 : 2173-2183.

KHARDENAVIS, A., SURESHKUMAR AND CHAKRABARTI, J., 2003, Biodegradable plastics production from industrial waste water by activated sludge. In : *44th Annual Conference of Association of Microbologists of India*, p. 107.

KIDWELL, J., VALENTIN, H. E. AND DENNIS, D., 1995, Regulated expression of the *Alcaligenes eutrophus pha* biosynthesis genes in *Escherichia coli. Applied Environmental Microbiology*, 61 : 1391-1398.

KIM, B. S., LEE, S. C., LEE, S.Y., CHANG, H. N., CHANG, Y. K. AND WOO, S. I., 1994, Production of poly(3-hydroxybutyric acid) by *Ralstonia eutropha* with glucose concentration control. *Biotechnology and Bioengineering,* 43 : 892-898.

KIM, G. J., LEE, I. Y., YOON, S. C., SHIN, Y. C. AND PARK, Y. H., 1997, Enhanced yield and a high production of medium-chain-length poly(3-hydroxyalkanoates) in a two-step fed-batch cultivation of *Pseudomonas putida* by combined use of glucose and octanoate. *Enzyme Microbial Technology,* 20 : 500-505.

KOLIBUCHUK, D., MILLER, A. AND DENNIS, D., 1999, Cloning, molecular analysis and expression of the polyhydroxy alkanoic acid synthase (*phaC*) gene from *Chromobacterium violaceum*. *Applied Environmental Microbiology*, 65 : 3561-3565.

KRANZ R. G., GABBERT, K. K. AND MADIGAN, M. T., 1997, Positive selection systems for discovery of novel polyester biosynthesis genes based on fatty acid detoxification. *Applied Environmental Microbiology,* 63 (8) :3010-3013

LAFFERTY, R. M., KORSATKO, B. AND KORSTAKO, W., 1988, Microbial production of poly-□-hydroxybutyric acid. In : Rehm H. J., Reed, G., editors. *Biotechnology Special microbial Processes*, pp. 136-176.

LAW, J. AND SLEPECKY, R. A., 1969, Assay of poly-□-hydroxybutyric acid. *Journal of Bacteriology*, 82 : 52-55.

LEACH, C. K., MIDDLEBEEK, E. AND MINBEEK, G., 1994, Waste pollution and the use of landfill. In : *Biotechnological Innovations in Energy and Environmental Management*, Eds. Leach, C. K. and MCE Van Dam-Mieras, DeMountfort University, Keicerter, UK, pp. 29-60.

LEE, S. Y., 1996, Bacterial Polyhydroxyalkanoates. *Biotechnology and Bioengineering*, 49 : 1-14.

LEE, S. Y., LEE, K. M., CHANG, H. N. AND SREINBUCHEL, A., 1994, Comparison of recombinant *Escherichia coli* strains for synthesis and accumulation of poly (3-hydroxybytyric acid) and morphological changes. *Biotechnology and Bioengineering*, 44: 1337-1347.

LEE, Y. U. AND YOO, Y. J., 1991, Kinetics for the growth of *Alcaligenes eutrophus* and the biosynthesis of poly-□-hydoxybutyrate. *Korean Journal of Applied Microbial Biotechnology*, 19 : 186-192.

LEES, S. Y., AND CHANG, H. N., 1995, Production of poly-□-hydroxybutyrate by recombinant *Escherichia coli* strains : 1995, genetic and fermentation studies. *Canadian Journal of Microbiology*, 4 : 207-215.

LEMOIGNE, M., 1926, Produits de doshydration et de polymorization de acid □-oxybutyrique. *Bull. Soc. Chim. Biol.,* 8 : 770

LEWIS I. M., 1941, The cytology of bacteria. *Bacterialogical Reviews,* 5 :181-236.

LILLO, J. G. AND VALERA, R. F., 1990, Effects of culture conditions on poly-□-hydroxybutyric acid production by *Haloferax meditteranei*. *Applied Environmental Microbiology*, 56 : 2157-2521.

LIU, F., LI, W., RIDGWAY, D. AND GU, T., 1998, Production of poly-□-hydroxybutyrate on molasses by recombinant *Escherichia coli*. *Biotechnology Letters*, 20 : 345-348.

LOKESH, B. E., SHAMALA, T. R. AND CHANDRASHEKHAR, A., 2005, Utilization of organic acids and sugars for PHA co-polymer biosynthesis by a native soil isolate, *Sphingomonas* spp. In : *46th Annual Conference of Association of Microbologists of India*, p. 48.

MADISON, L. L. AND HUISMAN, G. W., 1999, Metabolic engineering of poly-□-hydroxyalkanoates from DNA to plastic. *Microbial Molecular Biology Reviews*, 63 : 21-53.

MAHISHI, L. H., TRIPATHI, G. AND RAWAL, S. K., 2003, Poly-□-hydroxybutyrate (PHB) synthesis by recombinant *Escherichia coli* harbouring, *Streptomyces aureoficiens* PHB biosynthesis genes : Effects of various carbon and nitrogen sources. *Microbiological Research*, 158 : 19-27.

MERGAERT, J., ANDERSON, C., WOULERS, A. AND SWINGS, T., 1994, Microbial degradation of poly (3-hydroxybutyrate) and poly (3-hydroxybutyrate co-3-hydroxyvalarate) in compost. *Journal of Environmental Polymer Degradation*, 2: 177-183.

MERGERT, J., WEWEBB, A., ANDERSON, C., WOULERS, A. AND SWING, S. J., 1993, Microbial degradation of poly(3-hydroxybutyrate), PHB by bacteria and purification of a novel PHB depolyerase from *Comamonas* sp. *Journal of Environmental Polymer Degradation*,1 : 53-63.

MOORE, W. A., KRONER, R. C. AND RUCHHOFT, C. C., 1949, Dichromate reflux method for determination of oxygen consumed. *Annals of Chemistry*, 21 : 953.

MUKHOPADHYAY, M. AND PAUL, A. K., 2003, Poly(3-hydroxybutyric acid) Accumulation by some indigenous purple non-sulphur bacteria. In : *44th Annual Conference of Association of Microbologists of India*, p. 178.

MULCHANDANI, A., LUONG, J. H. T. AND GROOM C., 1989, Substrate inhibition kinetics for microbial growth and synthesis of poly-□hydroxybutyric acid by *Alcaligenes eutrophus* ATCC 17697. *Applied Microbial Biotechnology,* 30 : 11-17.

NAWRATH, C., PRIOR, Y. AND SOMERVILLE, C., 1994, Targetting of the poly-□-hydroxybutyric acid biosynthetic pathway to the plastids of *Arabidopsis thaliana* results in high levels of polymer accumulation. *Proceedings of National Academic Science*, 91 : 12760-12764.

OEDING, V., SCHLEGEL, H, G., 1973, □-ketothiolase from *Hydrogenomona eutropha* H16 and a significance is in the degradation of poly-□-hydroxybutyrate metabolism. *Journal of Biochemistry*, 134 : 239-248.

OJUMUU, T. V., YU, J. AND SOLAMON, B. O., 2004, Production of polyhydroxyalkanoates a bacterial biodegradable polymer. *African Journal of Biotechnology*, 3 : 18-24.

OKON, Y. AND ITZIGSOHN, R., 1992, Poly-□-hydroxybutyrate metabolism in *Azospirillum brasilense* nad the ecological role of PHB in the rhizosphere. *FEMS Microbiology Review*, 103 : 131-140.

OLIVEIRA, F. C., FREIRE, D.M.G. AND CASTILHO, L.R., 2004, Production of poly(3-hydroxybutyrate) by solid-state fermentation with *Ralstonia eutropha. Bioresource Technology*, 25 : 65-70.

OMAR, S., RAYER, S., EQUAAB, A., VISS, R. AND STEINBUCHEL, A., 2001, Optimziation of cell growth and poly-□-hydroxybutyrate accumulation on date syrup by *Bacillus megaterium* strain. *Biotechnology Letters*, 23 : 1119-11123.

OSTLE, A. G. AND HOLT, J. G., 1982, Nile Blue A as a Fluorescent Stain for Poly-3-Hydroxybutyrate. *Applied Environmental Microbiology,* 44 (1) : 238-241

PAGE, W. J., 1992, Production of Polyhydroxyalkanoates by *Azotobacter vinelandii* Strain UWD in Beet Molasses Culture. *FEMS Microbiol Reviews,* 103 : 149-158.

PAGE, W. J., 1995, Bacterial Polyhydroxyalkanoates, Natural Biodegradable Plastics with a Great Future. *Canadian Journal of Microbiology,* 41 (1) :1-3.

PAL, S. AND PAUL, A. K., 2000, Accumulation of biodegradable plastics by *Azotobacter*. In : *59th Annual Conference of Association of Microbologists of India*, p. 58.

PANSE, V. S. AND SUKHATME, P. V., 1985, Statistical methods for Agricultural Workers. ICAR, New Delhi, pp. 152-155.

PARSHAD, J., SUNEJA, S., KUKEJA, K. AND LAKSHMINARAYANA, K., 2001, Poly-□-hydroxybutyrate production by *Azotobacter chroococcum. Foliar Microbiology*, 46 (4) : 315-320.

PEFFER, J. T., 1992, Recycling solid waste management. *Engineering*, pp. 72-84.

PORIER, Y., NAWARATH, C., SOMERVILLE, C., 1995, Production of polyhydroxyalkanotes, a family of biodegradable plastics and elastomers in bacterial and plant. *Biotechnology,* 13: 142-150.

POUL, E., MULARD, D., BLANC, P., FAGES, J., HOMA, G. AND PARELLEUX, A., 1990, Effects of partial 'O' pressure and agitation on growth kinetics of *Azospirillum lipoferum* under fermentor conditions. *Applied Environmental Microbiology*, 56 : 3235-3239.

POVOLO S. R., TOMBOLINI, A. MOREA, A. ANDERSON, A. J. CASELLA, S. AND NUTI, M. P., 1994, Isolation and characterization of mutants of *Rhizobium meliloti* unable to synthesize poly-□-hydroxybutyrate. *Canadian Journal of Microbiology,* 40 : 823-829.

RAJE, P. AND SRIVASTAV, A. K., 1998, Updated mathematical model and fed batch strategies for poly-□-hydroxybutyrate (PHB) production by *Alcaligenes eutrophus*. *Bioresource Technology*, 64 : 185-192.

RAMASAY, J. A., BERGER, E., VOYER, R., CHARAVARIC, C. AND RAMASAY, B. T., 1994, Extraction of poly-3-hydroxybutyrate using chlorinated solvents. *Biotechnology and Bioengineering*, 8: 589-594.

RAMSAY, J. A., BERGER, E., RAMSAY, B. A. AND CHAVARIE, C., 1990, Recovery of poly-□-hydroxyalkanoic acid granules by a surfactant hypochlorite treatment. *Biotechnological technique*, 4 : 221-226.

RAMSAY, J. A., HASSAN, A. M. C. AND RAMSAY, B. A., 1995, Hemicellulose as a potential substrate for production of (poly-□-hydroxybutyrates). *Canadian Journal of Microbiology*, 41: 262-266.

RAWATE, T. AND MAVINKURVE, S., 2001, Biodegradable plastics. *Bacterial* polyhydroxyalakonate. *Indian Journal of Microbiology*, 41 : 233-245.

RAWTE, T. AND MAVINKURVE, S., 1998, Polyhydroxybutyrates from mangrove flora Annual Conference of Association of Microbiologists of India: Mangalore, p. 241.

REDDY, C. S. R., GHAI, R., RASHMI AND KALIA, V. C., 2003, Polyhydoxyalkanoates an over view. *Bioresource Technology*, 87 : 137-146.

RENNER, G., HAAGE, G. AND BRAUNEGG, G., 1996, Production of short-side-chain polyhydroxyalkanoates by various bacteria from the rRNA superfamily III. *Applied Microbiology and Biotechnology,* 46 (3) :268 - 272

ROMEN, F. S., REINHARDT AND JENDROSSET, 2004, Thermo tolerant poly-3-hydroxybutyrate degrading bacteria from hot compost and characterization of the PHB depolymerase of *Schlegelella s*p. KB1a. *Archieves Microbiology*, 182 (2,3) : 157-164.

ROTMAN, D., 1996, Monsanto takes a chance on Zeneca's Biopol Technology. *Chemical Week*, 158 : 21.

RYU, H. W., HAHN, S. K., CHANG, Y. K. AND CHANG, H. N., 1997, Production of poly(3-hydroxybutyrate) by high cell density fed-batch culture of *Alcaligenes eutrophus* with phosphate limitation. *Biotechnology and Bioengineering,* 55 : 27-32.

SABAT, S., DESHPANDE, M. K. AND KHANDWEKAR, P. N., 1998, Microbial production of poly-3-hydroxybutyrate-a biopolymer. *Journal of Scientific and Industrial Research*, 57 : 654-657.

SAFAK, S., MERLAN, N., ASLIM, B. AND BEYATTI, Y., 2002, A study on the production of poly-□-hydroxybutyrate by some Eukaryotic micro organisms. *Turkish Electronic Journal of Biotechnology*, pp. 11-17.

SAHA, S. P. AND PAUL, A. K., 2003, Production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by *Azotobacter chroococcum* MAL-201. In : *44th Annual Conference of Association of Microbologists of India*, p. 180.

SAHA, S. P., PATRA A. AND PAUL, A. K., 2005, Production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by *Azotobacter chroococcum* MAL-201. In : *44th Annual Conference of Association of Microbologists of India*, p. 80.

SAMBROOK, J., FRITSCH, E. F. AND MANITIS, J., 1989, Molecular cloning : A laboratory manual, 2nd edition (Cold Spring Harbor Laboratory Press. CSH, New York), pp. 21-74.

SCHLEGEL, H., LAFFERTY, R. AND KRAUSS, I., 1970. The isolation of mutant not accumulating poly-3hydroxybutyric acid. *Archieves of Microbiology*, 71 : 283-294.

SCHOLZ, C., 2000, Poly(□-hydroxyalkanoates) as potential biochemical material : an overview. In : (Ed.) Scholz, C., Gross, R. A. polymers from renewable resources – biopolymers and biocatalysis. *ACS Series*, 764 : 328-334.

SEKI, H., KARITA, M. AND SUZUKI, S., 1994, The production of poly-β-hydroxybutyrate by hydrogen bacteria, *Alcaligenes eutrophus* H16 from alginic acid. *Journal of Chemical Engineering*, 27 : 423-425.

SENIOR, P. J. AND DAWES, E. A., 1971, Poly-β-hydroxybutyrate biosynthesis and the regulation of glucose metabolism in *Azotobacter beijerinckii*. *Journal of Biochemistry,* 125 : 55-66.

SENIOR, P. J. AND DAWES, E. A., 1973, The regulation of poly-β-hydroxybutyrate metabolism in *Azotobacter beijerinckii*. *Journal of Biochemistry,* 134 : 225-238.

SENTHIL KUMAR, B. AND PRABHAKARAN, G., 2006, Production of PHB (bio plastics) using bio-effluents as substrates by *Alcaligens eutropha*. *Indian Journal of Biotechnology*, 5 : 76-79.

SHEU, D. S., WANG, Y. T. AND LEE, C.Y., 2000, Rapid detection of polyhydroxyalkanoate accumulating bacteria isolated from the environment by colony PCR. Microbiology, 146 : 2019-2025.

SHIMIZU, H., SONOO, S., SHIOYA, S., SRYA, K., 1992, Production of poly-□-hydroxybutyric acid (PHB) by *Alcaligenes eutrophus* H16 in Fed-Batch culture. In : Furusaki *et al.* (Eds.), Biochemical Engineering for 2001, Springer-Verlag, Tokyo, pp. 195-197.

SHIMIZU, H., TAMURA, S., ISHIHARA, Y., SHIOYA, S. AND SUGA, K., 1994, Control of molecular weight distribution and mole fraction in Poly(-D(-)-3-hydroxyalkanoates) (PHA) production by *Alcaligenes eutrophus.* In:

Biodegradable Plastics and Polymers, (eds) Doi, Y. and K. Fukuda, Elsevier Science B.V., New York, p.365-372.

SILLMAN, C. E. AND CASIDA, L. E., 1986, Cyst formation versus poly-□-hydroxybutyric acid accumulation in *Azotobacter*. *Soil Biology and Biochemistry*, 18 : 23-28.

SODIAN, R., SPERLING, J. S., MARTIN, D. P., EGOZY, A., STOC, K. U., MAYER, J. E. AND VACNTI, J. P., 2000, Fabrication of a trileaflet heart value scaffold from a polyhydroxy alkanoate biopolyester for use in tissue enigeering. *Tissue Engineering,* 6 : 183-188.

SOLAIMAN, D. K. Y. ASHBY, R. D. AND FOGLIA, T. A., 1992, Rapid and specific identification of medium-chain length polyhydroxyalkanoate synthase gene by polymerase chain reaction. *Applied Microbiology and Biotechnology*, 37 : 7-12.

SPIEKERMAN, P., REHM, B. H. A., KALSCHEUER, R., BAUMEISTER, D. AND QTEINBUCHEL, A., 1999, A sensitive viable colony staining method using Nile Red for direct screening of bacteria that accumulate polyhydroxyalkonic acid and other lipid storage compounds. *Archives of Microbiology*, 171 : 73-80.

STEINBUCHEL, A. AND HEIN, S., 2001, Biochemical and molecular basis of polyhydroxyalkanoic acids in microorganisms. *Advances in Biochemical Engineering and Biotechnology*, 71: 81-122.

STEINBUCHEL, A. AND PIEPER, U., 1992, Production of a copolyester of 3-hydroxybutyric acid and 3-hydroxyvaleric acid from single unrelated carbon sources by a mutant of *Alcaligenes eutrophu*s. *Applied Microbial Biotechnology*, 37 : 1-6.

STEINBUCHEL, A. AND VALENTIN, H., 1995, Diversity of bacterial Polyhydroxyalkanoic acids. *FEMS Microbiology Letters,* 128 : 219-228.

STEINBUCHEL, A., 1991, Polyhydroxyalkanoic acids. In: Biomaterials: novel materials from biological sources. (Ed) D. Byrom. Stockton, New York. pp. 124-213.

STEINBUCHEL, A., 1996, PHB and other Polyhydroxyalkanoic Acids. In: Biotechnology, (eds.) Rehm H. J. and G. Reed. VCH, New York, pp. 403-464.

STENBUCHEL, A., SCHEGEL, H. G., 1991, Physiology and molecular genetics of poly(□-hydroxy-alkanoic acid) synthesis in *Alcaligenes eutrophus*. *Molecular Microbiology*, 5: 535-542.

STEVENSON, L. K. AND SOCOLOFSKY, M. D., 1973, Role of poly-□-hydroxybutyric acid in cyst formation by *Azotobacter*. *Antonie Van Leewen hoek*, 39 : 341-350.

STOCKDALE, H., RIBBONS, D. W. AND DAWES, E. A., 1968, Occurrence of poly-□-hydroxybutrrate in an Aztobacteriaceae. *Journal of Bacteriology,* 95 : 1798-1803.

SUBAT, S., DESHPANDE, M. K. AND KHANDWEKAR, P. V., 1998, Microbial production of poly-□-hydroxybutyrate a biopolymer. *Journal of Scientific and Industrial Research*, 57 : 654-657.

SUDESH, K., ABE, H. AND DOI, Y., 2000, Structure and properties of polyhydroxylkanoates, biological polyesters. *Polymer Science*, 25 : 1503-1533.

SUDESH, K., JAGUCHI, K. AND DOI, Y., 2001, Can *cyanobacteria* be a potential PHA producer? *Riken Review*, 4 : 75-77.

SUJATHA, K, MAHALAKSHMI, A. AND SHENBAGARATHAI, 2005, A study on accumulation of PHB in native pseudomonas isolates LDC-5 and LDC-25. *Indian Journal of Biotechnology*, 4 : 216-221.

SURESHKUMAR, M., MUDALIAR, S. N., REDDY, K. M. K. AND CHAKRABARTI, J., 2004, Production of biodegradable plastics from activated sludge generated from a food processing industrial waste water. *Bioresource Technology*, 95 : 327-330.

SUZUKI, T., YAMANE, T. AND SHIMIZU, S., 1986. Mass production of poly-β-hydroxybutyric acid by fully automatic fed-batch culture of Methylotroph. *Applied Microbial Biotechnology,* 23 : 322-329.

TAJIMA, K., IGARI, T., NISHIMURA, D., NAKAMURA, M., SATOH, Y. AND MUNEKATA, M., 2003, Isolation and characterization of *Bacillus* sp. INT005 accumulating

polyhydroxyalkanoate (PHA) from gas field soil. *Journal of Bioscience and Bioengineering,* 95 (1): 77-81.

TAVERNLER, D., PORTAIS, J. C., SANCEDO, J. E. N., COURTOIS, T., COURTIOS, B. AND BARBOTIN, T. N., 1997, Exopolysacchoride and poly-□-hydroxybutyrate co-production in two *Rhizobium meliloti* strains. *Applied and Environmental Microbiology*, 63 : 21-25.

THOMPSON, J. P. AND SKERMAN, V. B. D., 1979, Azotobacteriaceae : The taxanomy and Ecology of Aerobic nitrogen fixing bacteria. *Academic Press*, New York.

TIMM, A. AND STEINBACHEL, A., 1990, Formation of polyesters consisting of medium chain length 3-hydroxy alkanoic acids from glycolate by *Pseudomonas aeuroginosa* and other fluorescent *Pseudomonades. Applied and Environmental Microbiology*, pp. 3360-3367.

TOHYAMA, M., TAKAGI, S., SHIMIZU, K., 2000, Effect of controlling lactate concentration and periodic change in DO concentration on fermentation characteristic of a mixed culture of *Lactobacillus delbrueckii* and *Ralstonia eutropha* for PHB production. *Journal of Bioscience and Bioengineering,* 89 : 322-328.

UMEDA, F., KITANO, Y, MURAKING, K., YOGI, K., MIVRA, Y. AND MIZOGUCHI, T., 1998, Cloning and sequence analysis of the poly(3-hydroxyalkanoic acid) synthesis genes of *Pseudomonas acidophila*. *Applied Biochemistry and Biotechnology*, 70 : 341-352.

WANG, F. AND LEE, S. Y., 1997, Poly(3-hydroxybutyrates) Production with High Polymer Content by Fed-Batch Culture of *Alcaligenes latus* Under Nitrogen Limitation. *Applied Environmental Microbiology,* 63 : 3703-3706.

WANG, J. G. AND BAKKEN, L. R., 1998, Screening of soil bacteria for poly-□-hydroxybutyric acid production and its role in the survival of starvation. *Microbiology and Bioengineering*, 35 : 94-101.

WILKE, D., 1999, Chemical from biotechnology. Molecular plant genetics will challenge the chemical and the fermentation industry. *Applied Microbial Biotechnology*, 52 : 135-145.

WILLIAMS, S. F. AND PEOPLE, O. P., 1996, Biodegradable plastics from plants. *Chemtech*, 26 : 38-44.

YAMANE, T., 1996, Yield of poly-D(-)-hydroxybutyrate from various carbon sources : a theoretical study. *Biotechnology and Bioengineering*, 41: 165-170.

YELLORE, V. AND DESAI, A., 1998, Production of poly-□-hydroxybutyrate from lactose and whey by *Methylobacterium* sp. 2P24. *Applied Microbiology*, 26 : 391-394.

YELLORE, V. S. AND DESAI, A. J., 1995, Production of biodegradable polyesters from whey. *Applied and Environmental Microbiology*, pp. 11-13.

YILMAZ, M., SORAN, H., B. AND YAVUZ. 2005. Determination of poly-□-hydroxybutyrate (PHB) production by some *Bacillus* spp. *World Journal of Microbiol Biotechnology,* 21 (4) : 565-566(2)

YUKSEKDAG, Z. N., ASLIM, B., BEYADLTI, X. AND MERCAN, N., 2004, Effect of carbon and nitrogen sources and incubation time on poly-□-hydroxybutyrate (PHB) synthesis by *Bacillus subtilis* 25 and *Bacillus megaterium* 12. *African Journal of Biotechnology*, 3 (1) : 63-66.

ZHANG, H., OBIAS, V., GONYER, K. AND DENNIS, D., 1994, Production of polyhydroxalkanoates in sucrose-utilizing recombinant *Escherichia coli* and *Klebsiella* strains. *Applied Environmental Microbiology*, 60 ; 1198-1205.

ZHANG, S. YASOUT, LENZ, R. W. AND GOODWIN, S., 2000, Kinetic and mechanistic characterization of the polyhydroxybutyrate synthase from *Ralstonia eutropha*. *Biomacromolecules*, 1 : 244-251.

APPENDIX – I

I. Composition of media used

1. Nutrient agar (Anon., 1957)

Peptone	: 5 g
Beef extract	: 3 g
Sodium chloride	: 5 g
Agar	: 18 g
Distilled water	: 1000 ml
pH	: 6.8-7.2

2. Skimmed milk agar (Baily and Schott, 1970)

Skim milk powder	: 1 g
Peptone	: 5 g
Distilled water	: 1000 ml
pH	: 7.2
Agar	: 18 g

3. SIM agar (Cappuccino and Sherman, 1992)

Peptone	: 30 g
Beef extract	: 3 g
Perrous ammonium sulphate	: 0.2 g
Thiosulphate	: 0.025 g
Distilled water	: 100 ml
pH	: 7.3
Agar	: 18.00 g

4. Urea broth (Frazier et al., 1967)

Yeast extract	: 0.1 g
KH2PO4	: 9.1 g
Ka2HPO4	: 9.5 g
Urea	: 20.0 g
Phenol red	: 0.01 g
Distilled water	: 100 ml
pH	: 6.8

5. Nutrient gelatin (Cappuccino and Sherman, 1992)

Peptone	: 5.0 g
Beef extract	: 3.0 g
Gelatin	: 120 g
Distilled water	: 1000 ml
pH	: 6.8

6. Tributyrin agar

Peptone	: 5.0 g
Beef extract	: 3.0 g
Tributyrin	: 10.0 ml
Distilled water	: 1000 ml
pH	: 7.2
Agar	: 18.0

7. Starch agar (Bailey and Schott, 1970)

Peptone	: 5.0 g
Beef extract	: 3.0 g
Starch	: 10.0 g
Distilled water	: 10 ml
pH	: 7.2
Agar	: 18.0 g

8. Luria brota

Yeast extract	: 5 g
Trypton	: 10 g
Sodium chloride	: 5 g
Distilled water	: 1000 ml

9. Mineral salt medium (Suresh Kumar et al., 2004)

MgSo4. 7H2O	: 0.2 g
CaCl2.2H2O	: 10 mg
Ferrous ammonium citrate	: 6 mg
KH2PO4	: 0.83 g
Trace elemental solution	: 1 ml
Distilled water	: 1000 ml

10. Trace elemental solution

H3BO3	: 0.3 g
CoCl2 6H2O	: 0.2 g
ZnSO4 7H2O	: 0.1 g
MnCl2 4H2O	: 30 mg
NaMoO4 2H2O	: 30 mg
NiCl2 6H2O	: 20 mg
CuSO4 5H2O	: 10 mg
Distilled water	: 1000 ml

APPENDIX – II

II. Stains used

1. Sudan Black B (Juvan et al., 1998)

    0.02 per cent solution of Sudan Black B in 96 per cent ethanol

2. Acridine orange

    50 µl of acridine irange

ISOLATION, SCREENING AND SELECTION OF EFFICIENT POLY-β-HYDROXYBUTYRATE (PHB) SYNTHESIZING BACTERIA

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ABSTRACT

In the present study, an attempt was made to isolate efficient PHB producing bacteria from different environmental samples. A total of eight samples were collected and 656 bacteria were isolated and 24 bacteria were collected from the Departmental culture collection. Out of these, 101 were found to be PHB positive based on viable colony staining method using Sudan Black B. All the 125 PHB positive isolates were subjected to quantitative estimation of PHB production. PHB yields varied from 0.010 to 0.160 g/100 ml. Ten promising bacterial isolates were selected based on their PHB yields. They were EJC2, EJC5, KJC7, NJC3, FJC4, MJC10, TJC1, BJC7, B25 and DJC6. The culture parameters were optimized for all the 10 isolates, Glucose was found to be the best carbon source for maximum PHB production by all the isolates. Ammonium sulphate supplementation at 0.1 per cent was found to be optimum. Maintaining the C:N ratio of 20:1 using the best C and N source was found to be optimum. Maintaining the pH of the medium at 7.0 was found optimum.

In order to reduce the cost of PHB production,whey and waste water of a soft drink industry were tested as cheaper substrates. Although they supported PHB production, the yields were rather low when compared to those on standard media. However, the yields could be improved to some extent, by supplementation with glucose at 1 per cent level. But, the advantage is that the biowastes can not only be disposed off but also value added product like PHB can be obtained. DJC6 reduced COD of whey by 43 per cent, thus functioning as a bioremediating agent. Out of ten natural promising isolates, DJC6 and BJC7 were found to be the most efficient PHB producers. Under optimized conditions, DJC6 produced a PHB yield of 1.100 g/100 ml while BJC7 produced 0.85`90 g/100 ml. DJC6 performed better than the reference strain *R. eutropha* in PHB production. DJC6 and BJC7 were tentatively identified as *Bacillus* spp. and *Pseudomonas* spp. respectively, based on the morphological and biochemical tests. Scale up studies in 3 litre bottle fermenters were conducted under the optimized conditions. *Pseudomonas* sp. DJC6 produced a PHB yield of 1.100 g/100 ml which is marginally higher than 1.033 g/100 ml produced by *R. eutropha*.