SUMMARY AND CONCLUSIONS

Plastics, occupy a unique position in the world of materials as they are durable and play a key role, in the manufacture of materials pertaining to transportation, communication, entertainment, health care products, food packaging etc. They possess many attractive properties, such as lightweight, durability and flexibility, they are produced from cost effective raw materials and hence they meet a large share of the material needs of man. The highly recalcitrant nature has lead to their presence in the environment after disposal and hence is regarded as environmental hazard. The rapid increase in production and consumption of plastics has resulted in plastic waste accumulation leading to serious pollution problems. As an alternative to this, biodegradable polymers offer the best solution to the environmental hazard posed by the conventional plastics. In the recent past, there has been growing public awareness and scientific interest regarding the use and development of biodegradable polymer materials as ecologically useful alternatives to synthetic plastics. In addition to biodegradability, such a polymer material must posses the physical and chemical properties of synthetic plastics. In this context polyhydroxyalkanoate (PHA) produced by bacteria has been identified as environmental friendly biological plastic of the future.

PHAs are structurally simple macromolecules that are synthesized by various bacteria as carbon and energy reserve of the cells. These intracellular inclusions are formed under stressed growth conditions, which occur in the presence of excess of carbon source on one hand, and a limiting nutrient condition on the other. The nutrient condition may be limitation of nitrogen, potassium, iron, magnesium, manganese, phosphate, sulphate, oxygen etc. Besides its importance as a source of energy, the intracellular presence of this polymer seems to play a significant role in the survival of microorganisms under several environmental stress conditions. Several hydroxyalkanoates units are synthesized as homopolymer or heteropolymer units by bacteria. These are broadly classified as short chain length PHA (scl-PHA; 4-5 carbon
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atoms) and medium chain length PHA (mcl-PHA; 6-14 carbon atoms). The physical and mechanical properties depend on the monomer composition. Copolymers possess better thermo mechanical properties compared to homopolymers. PHAs are used for medical and industrial applications. They are used for manufacture of surgical sutures, drug delivery systems, biodegradable carrier for herbicides, packaging containers, bags, films etc. PHA producing microorganisms include various genera that are taxonomically placed in different groups. This includes species of Azotobacter, Chromobacterium, Methylobacterium, Micrococcus, Pseudomonas, etc. Microorganisms accumulate PHA from 1-80% of their cell dry weight. Ralstonia eutropha is known to synthesize up to 80% PHA under specific growth conditions. Polyhydroxybutyrate (PHB), which is a commonly found homopolymer of PHA, was first identified during 1926 in Bacillus megaterium. The members of the genus Bacillus that produce PHA include B. megateium, B. cereus, B. anthracis, B. holodurans, B. thuringiensis etc. In the present work different Bacillus spp were isolated from soil samples collected from various parts of the country. The isolates were screened for PHA production by sudan black staining. PHA production studies were carried out in shake flask culture in PHA production medium. Among 38 different Bacillus spp tested for PHA production, Bacillus sp 256 produced highest amount of the polymer (55% of biomass) and hence it was selected for further studies. The selected isolate was characterized by morphological, biochemical and molecular methods. Bacillus 256 was gram positive, rod shaped and non motile. The bacterium produced endospores at apical position and showed positive reaction for catalase, nitrate reduction, and oxidase tests. It could not hydrolyse starch, gelatin and casein. The results of the morphological studies suggested that the isolated bacterium belonged to the genus Bacillus. The strain showed similarities with B. endophyticus because: it had ellipsoidal spore situated at terminal position, nonmotile, absence of anaerobic growth, Voges-Proskauer negative, oxidase positive, not able to hydrolyze casein, gelatin and starch; acid production from arabinose, glucose, manitol, maltose, mannose, rhamnose, xylose; etc. The strain produced pale pink pigmentation on PHA agar slants and the pigment produced was not diffusible. On nutrient agar the colonies
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were slimy; the cells were resistant to ampicillin and also grew in the presence of lysozyme. Some of the morphological or biochemical characters can be confused with that of *B. licheniformis* hence the bacterium was further examined by 16S rRNA to determine its relationship at the genomic level. One portion of 16SrRNA gene was amplified from the genomic DNA of *Bacillus sp* 256 by PCR. The DNA fragment was cloned and sequenced. The DNA sequence was found to be very conserved and it was analysed using various online softwares. The sequence was aligned with about 80 published and unpublished *Bacillus* related 16S rRNA gene sequences. Sequence of 16SrRNA gene was similar to that of *Bacillus endophyticus* (99%), *Bacillus sp* 19490 (99%), *Bacillus sp* GB02-16/18/20 (97%), *Bacillus sp* MSSRF (96%) etc. Only recently, *B. endophyticus* has been isolated and characterised from the inner tissues of cotton plants. Therefore the identification of this bacterium based on morphological and biochemical studies and comparision of the data with earlier report would indicate that it is similar to *B. licheniformis* in sevral aspects. The literature data on phylogenetic tree indicates that *B. licheniformis* is closer to *B. endophyticus* in the evolutionary position. Based on these factors the isolated culture has been tentatively identified as *B. endophyticus*. Our study is the first report to show that this endophytic bacterium exists in the soil. This is similar to other endophytic bacteria that have been isolated as free living forms from the soil. Importance of *Bacillus* in food fermentation has been known since long time. The genus is industrially important for the production of extracellular amylases, proteases. The genus also includes several species that are pest control agents. In the present study it has been shown that *B. endophyticus* can exist outside as a free living form in the soil and can produce industrially important polymer such as polyhydroxyalkanoate. The amount of PHA produced is relatively high (55% of biomass) and it appeared encouraging to study various aspects of PHA production and characterize the PHA synthesis genes using this new strain.

Production of PHA has gained industrial importance for its use as biodegradable polymer applications or as biocompatible plastics. Homopolymers or heteropolymers
may be obtained depending on the co carbon substrates used in the fermentation and the capacity of the microorganisms to metabolize the substrate for polymer production. *Bacillus spp* produce PHA due to limitation of potassium and phosphorous in the medium. In order to increase the PHA yield it is essential to optimize the nutrient and growth conditions. Different carbon and nitrogen sources were tested for PHA production by *Bacillus sp* 256. Among various N sources studied, (NH)2H PO was found to be a better nitrogen source for PHA production (biomass 1.6 g/l and PHA 69% of biomass). Amongst the carbon sources, sucrose gave higher biomass (1.6 g/l) and PHA yield (55% of biomass). The bacterium was also cultivated in media containing molasses, cornstarch, and corn steep liquor. Maximum production of PHA (55%) was obtained in the medium containing hydrolysed cornstarch, while biomass was highest in molasses medium (2.6 g/l). Amongst the plant oils used as co carbon substrates, non-edible plant oil such as pongemia oil resulted in optimal yield of PHA in the biomass (65%). Fermentor cultivation in medium containing a) sucrose as the sole carbon source, b) sucrose as main carbon source and pongemia oil as co-carbon substrate, c) sucrose as main carbon source and saponified pongemia oil as co carbon substrate resulted in high biomass (4g/l) and accumulation of PHA (2 g/l) in pongemia oil containing medium. The results also showed that the medium containing saponified pongemia oil was a better medium for growth (4 g/l) and accumulation of PHA (2.5 g/l) by *Bacillus sp*. When only sucrose was used as carbon substrate nearly 60% degradation occurred due to prolonged fermentation (72 h) compared to 20% degradation in pongemia oil containing growth medium. This shows that supplementation of pongemia oil also prevented degradation of PHA once maximal production was achieved, which indicates that such fermentation conditions can be used for this strain for commercial process. The formation and breakdown of PHA in the cells appear to be important in defining nutritional status of the microbial cells. In *Bacillus spp.* it serves as an endogenous source of carbon and energy for cell activities and spore formation. The co substrate absorption may lead to availability of energy in the form of fatty acids to the cells which may lead to delayed degradation of PHA polymer. Hydroxyacyl coenzyme A thioesters required for quantitative and qualitative productivity
of PHA are supplied by fatty acid biosynthesis and degradation pathway. The extra metabolic flux of fatty acid degradation appears to have channelised towards enhanced copolymer production in oil fed cells, which was absent in sucrose fed cells. The overall results indicate that plant oils may be better suited for the stabilized production of PHA copolymer by *Bacillus spp*.

FTIR, GC and NMR were used to characterize the PHA obtained from the cells. FTIR is one of the powerful and rapid tools to obtain information on polymer structure. The spectrum indicated a transmittance band at 1752 cm\(^{-1}\), which is attributed to the stretching vibration of the \(\text{C}=\text{O}\) group (ester carbonyl) in the PHA polyester. Accompanying bands of the \(\text{C}-\text{O-}\text{C}\) groups appeared in the spectral region from 1150 cm\(^{-1}\) to 1300 cm\(^{-1}\). Transmittance regions from 2800 to 3100 cm\(^{-1}\) correspond to the stretching vibration of \(\text{C-H}\) bonds of the methyl (\(\text{CH}_3\)), and methylene (\(\text{CH}_2\)) groups. Other characteristic bands present for scl-PHA were 2977, 2934, 1282 (\(\text{CH}_3\) bend), 1100, 1058 (\(\text{C-O}\)) 979 and 515.

Gas chromatography is a very efficient method for quantitative estimation as well as characterization of PHA. The results indicated that PHA extracted from media containing rice bran oil, pongemia oil and oleic acid as co substrates was a copolymer of P(HB\(-\text{co-HV}\)). The butyrate methyl ester eluted at 9.5 min and valerate methyl ester at 12.81 min. GC also showed that the copolymer (hydroxyvalerate) was about 4 and 6-mol\% of the total polymer content. Molar concentration of P(HB\(-\text{co-HV}\)) was maximum in the presence of saponified pongemia oil (80:20 mol\%) compared to unsaponified oil (94:6). Homopolymer of PHB was obtained from the cells fed with only sucrose.

Nuclear Magnetic Spectroscopy (NMR) is a powerful technique used for elucidating the chemical structure of the compounds. The \(^1\text{H}\) NMR spectrum of PHA showed three characteristic groups signals of PHB: a doublet at 1.29 ppm which is characteristic of methyl group, a doublet of a quadruplet at 2.5 ppm which is attributed to
methylene group and a multiplet at 5.28 ppm characteristic of a methylene group. A triplet at 0.9 ppm and a methylene resonance at 1.59 and methyne resonance at 5.5 indicated the presence of valerate in the polymer.

Several pathways are involved in the synthesis of PHA. Biosynthesis of scl-PHA such as PHB is initiated by the condensation of two acetyl-CoA molecules to acetoacetyl CoA that is catalysed by the enzyme β–ketothiolase. Acetoacetyl CoA is reduced to hydroxybutyryl CoA by the NADPH dependant acetoacetyl CoA reductase. This is polymerized to PHB in the presence of PHA synthase. This pathway has been studied in detail and is known to exist in bacteria such as *Ralstonia eutropha*, *Bacillus spp* etc. The mcl-PHA biosynthesis pathway is closely related to the β-oxidation pathway where fatty acids serve as carbon substrates. Hydroxyacetyl CoA intermediates of β-oxidation pathway function as precursors for the mcl-PHA biosynthesis. Fatty acids are activated by the acyl CoA synthetase leading to the formation of corresponding acyl-CoA thioesters. These are degraded to acetyl CoA through β-oxidation pathway. Acyl CoA is reduced to trans-2, 3- enoyl CoA that is catalysed by acyl CoA dehydrogenase. Further conversion is catalysed by multi enzyme complex. Enzymes such as (R)-specific enoyl CoA hydratase, hydroxyacetyl CoA epimerase, and β-ketoacyl CoA reductase are connected with the β-oxidation pathway for the synthesis of mcl-PHA. Mcl-PHA synthesis is prevalently found in *Pseudomonas spp*.

Several genes that encode enzymes involved in PHA synthesis and degradation have been cloned and characterized from several microorganisms. These studies have shown that several pathways are involved in the synthesis of PHA each of which is optimized for the environment where these microorganisms are found. In addition to diversity in the metabolic pathway for PHA synthesis, there is a divergence in the *pha* gene loci of various bacteria. These *pha* genes are found as clusters in an operon in some bacteria and in others they occur as separate transcriptional units. Even though PHB was
first isolated from *B. megaterium*, biosynthetic mechanism involved in PHA synthesis has not been characterized in *Bacillus spp* in detail.

The pha operon of *Bacillus* consist of five genes such as *phaP, phaQ, phaR phaB* and *phaC*, where the *phaRBC* genes are in one orientation under the control of a single promoter; *phaP* and *phaQ* are in separate orientation. In *Bacillus* the gene coding for β-ketothiolase (*phaA*) is not associated with *pha* operon. In the present study PHA biosynthesis genes were cloned and sequenced. PCR cloning strategy was used to isolate the genes from *Bacillus sp*. Different primers were designed to amplify PHA biosynthesis genes such as *phaA, phaB* and *phaC*. All the genes characterized were of complete ORFs of expected size with a start site and a termination codon. The gene sequences were used to study the gene polymorphism and sequence homology.

The *phaB* gene *Bacillus sp* 256 was 744 bp long. The sequence showed similarity with acetoacetyl CoA reductases of various *Bacillus spp*. Multiple sequence alignment with deduced amino acid sequence of *phaB* gene showed that acetoacetyl CoA reductase of *Bacillus sp* 256 is also similar to that of many other PHA producing organisms in various aspects. This protein is almost similar in most of the PHA producing organisms with an average molecular weight of 27 k Da. The amino acid sequence was conserved towards the C terminal region of the polypeptide chain. *PhaC* gene from *Bacillus sp* was cloned and sequenced completely. The sequence was a complete ORF consisting of 361 amino acids. The PHA synthase of *Bacillus* is reported as a unique one, which requires a *phaR* protein sub-unit for its activity. The gene coding for β-ketothiolase was also cloned from *Bacillus sp* 256 and characterized. The gene was 1173 bp long complete ORF and was similar to that of *B. cereus*. The *Bacillus* ketothiolase was found to be distinct; showed lesser homology with ketothiolas from other PHA producing organisms. Genomic BLAST with *bktB* protein sequence from *R. eutropha* suggested that the ketothiolas present in *Bacillus* genome has an ascending order of similarity with *bktB* protein.
The PHA biosynthesis genes from *Bacillus* and *Pseudomonas* were selected for heterologous expression in *B. subtilis* and *E. coli* for PHA production. Plasmid bearing *phaC1* and *phaJ4* genes from *Pseudomonas aeruginosa* (*pCPC1J4*) were transferred into *B. subtilis* strain by electroporation. The recombinant *B. subtilis* was selected on the basis of kanamycin resistance. The strain was subjected to PHA production in the medium containing nonanoic acid. The recombinant produced only 5% of PHA copolymer.

PHA biosynthesis genes from *Bacillus sp* and *Pseudomonas sp* were cloned for scl-co-mcl PHA production in *E. coli*. *PhaA* and *phaB* genes from *Bacillus sp* 256 were cloned into *E. coli* integration vector *pBRINT*-Cm (*pBRAB* construct). The *E. coli* strain JC7623 was transformed with *pBRBA* vector and the white integrant colonies (*JC7623AB*) were selected on chloramphenicol resistance. The *JC7623AB* strain was subjected to another transformation with *pBSC1J4* plasmid containing *phaC1* and *phaJ4* genes from *Pseudomonas aeruginosa* (Collected from laboratory from Reeta Davis and Chandrashekar A). The expression of all four genes was induced by isopropyl β-D-thiogalactopyranoside. The expression of the cloned genes was monitored on SDS PAGE.

Three recombinant strains such as *E. coli fadBC1J4* (*fadB* mutant LS1298 bearing *phaC1* and *phaJ4* genes from *P. aeruginosa*), *E. coli JC7623C1J4* (*E. coli* strain JC7623 containing *phaC1* and *phaJ4* genes) and *E. coli JC7623 ABC1J4* (*E. coli* strain JC7623 bearing *phaA* and *phaB* gene from *Bacillus sp* 256; *phaC1* and *phaJ4* genes from *P. aeruginosa*) were subjected for PHA production. The strain *JC7623ABC1J4* grew well in PHA production medium and produced highest amount of PHA and hence it was selected for further studies.

Recombinant *E. coli JC7623ABC1J4* possessing PHA synthesis genes of *phaA, phaB* from *Bacillus sp* (involved in scl-PHA synthesis); *phaC1* and *phaJ4* from *P. aeruginosa* (genes involved in mcl-PHA synthesis) was used in the cultivation experiments. The complementation of PHA genes from different hosts in *E. coli* has demonstrated that the system can be used to produce P(HB-co-HV)-co-mcl PHA.
copolymers. Production of mcl-PHA appeared to be dependent on the flux through the β-oxidation pathway and scl-PHA was through the intervention of acetyl CoA. The flexibility in carbon source usage could allow for the production of PHA with different mol% of scl or mcl monomers. The strain was able to produce 2, 2, 1, 3, 1, 4 and 4-mol % of hydroxyvalerate when the cells were grown in butyrate, valerate, hexanoate, heptanoate, octanoate, nonanoate and decanoate, respectively as co carbon substrates in the medium. The PHA yield reached maximum of 51 % when it grew in medium containing butyrate. Analysis of the genome of *Bacillus* indicated the presence of four β–ketothiolase genes. Sequences of these were checked for their similarities with the β-ketothiolase (bktB) of *Ralstonia eutropha*. The similarity of the said genes with bktB in a descending order was 48%, 44%, 43%, 35% and these were designated as PhaA1-PhaA4. PhaA2 from *Bacillus sp* 256, which encodes β–ketothiolase was cloned and sequenced completely. The sequence showed the presence of a complete open reading frame (ORF) of 1173bp in size. The deduced amino acid sequence showed maximum similarity with that of PhaA2 from other *Bacillus spp* such as *B. cereus*, *B. anthrax* and *B. halodurans*. The sequence of PhaA3 and PhA4 from these species differed from that of the *Bacillus sp* 256 sequence. Our data indicate that the PHV production in the recombinant *E.coli* may be possible through the breakdown of fatty acids as evidenced by the increase in the proportion of PHV when the recombinant cells were grown in the presence of higher fatty acids such as heptanoic acid and nonanoic acid. The absence of PHV synthesis in the medium containing only glucose and citric acid suggested the same conclusion.

Quantitative and qualitative analysis of the polymer by GC indicated that the molar percentage of scl:mcl PHA varied, depending on the fatty acid supplemented to the glucose containing medium as a co-substrate, from 91:9 to 24:76. Concentration of mcl-PHA increased under supplementation of heptanoic, octanoic and decanoic acids. \(^1\)H NMR spectra of PHA indicated the synthesis of polyhydroxybutyrate (PHB) and higher alkanoate copolymers in the presence of glucose and fatty acids whereas only PHB was synthesized in the presence of glucose as sole carbon substrate. In PHA synthesis, the
acetyl CoA enters in to biosynthesis pathway with the help of the enzymes coded by \textit{phaA} and \textit{phaB} genes. The presence of mcl-PHA biosynthesis genes such as \textit{phaC1} and \textit{phaJ4} along with \textit{phaA} and \textit{phaB} enabled the recombinant strain to follow a novel PHA biosynthesis pathway.

Response surface methodology (RSM) was used as an efficient method to obtain optimized biomass and PHA production by recombinant bacteria. A complex interaction was observed between factors and their variables, which resulted in quantitative changes in biomass and PHA. From the RSM studies it was possible to conclude that the recombinant could yield high biomass under higher levels of ammonium phosphate. Optimized biomass (11 g/l) and PHA (4 g/l) were obtained in medium containing (g/l): tryptone 2; MgSO$_4$ 1.4; KH$_2$PO$_4$ 18.5; glucose 5; (NH$_4$)$_2$HPO$_4$ 7; citric acid 3 and 10 % inoculum.

The recombinant strain was also cultivated in a fermentor in medium containing glucose as a carbon source. At the end of fermentation period maximum biomass (10 g/l) and PHA (4 g/l) were obtained. Concentration of PHA was 40 % of dry cell biomass.

**Future studies**

\textit{Bacillus sp} 256 is an excellent organism for PHA production. It is able to synthesize PHA from versatile carbon sources such as sugars, fatty acids, oils, molasses and starch hydrolysates. Compared to other \textit{Bacillus sp} that are reported in the literature, \textit{Bacillus sp} 256 produced higher amount of PHA which was composed of P(HB-co-HV) copolymer. This study has indicated that multiple ketothiolases are present in the Bacillus genome, one of which was cloned and characterized in the present assignment. These enzymes can be studied in detail to show the reason for requirement of multiple copies of ketothiolases in the bacterium. In the present study recombinant \textit{E. coli} strain produced moderate amount of PHA (40%), the following points may be considered to improve the strain for enhanced PHA production:- a) Intracellular NADPH level is considered as one of the limiting factors of scl PHA production and intracellular NADPH concentration can be
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Further increased by addition of a gene which reduces NADP molecule such as gdh gene coding for glucose 6-phosphate dehydrogenase in pentose phosphate pathway. b) The recombinant strain was observed to form filaments during fermentation; inhibition of filamentation would lead to enhancement of polymer synthesis. c) *E. coli* JC7623 strain can be subjected to mutation in fatty acid metabolism (*fad* mutation) to diverge the metabolic intermediates in to PHA biosynthesis. d) Enhancement of the ability of the strain to grow on economic substrate such as glycerol, whey etc. e) Extraction of the intracellular PHA can be simplified by cloning an inducible lytic gene in to the recombinant strain.