“Polyhydroxyalkanoates accumulating bacteria from coastal sand-dunes”

A thesis submitted to Goa University

For the Award of the Degree of

DOCTOR OF PHILOSOPHY

In

MICROBIOLOGY

By

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STATEMENT

As required under the Goa University Ordinance, I hereby state that the present thesis for Ph.D. degree entitled "Polyhydroxyalkanoates accumulating bacteria from coastal sand-dunes" is my original contribution and that the thesis and any part of it has not been previously submitted for the award of any degree/diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area.

The literature related to the problem investigated has been cited. Due acknowledgement have been made whenever facilities and suggestions have been availed of.

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CERTIFICATE

This is to certify that the thesis entitled "**Polyhydroxyalkanoates accumulating bacteria from coastal sand-dunes**" submitted by ***Mr. Pramoda Kumar Nayak*** for the award of the degree of ***Doctor of Philosophy*** in **Microbiology** is based on his original studies carried out by him under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any University or Institution.

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Pramoda

Dedicated to my family

LIST OF ABBREVIATIONS

PHAs	Polyhydroxyalkanoates	**NH_4Cl**	Ammonium chloride
PHB	Polyhydroxybutyrate	**DO**	Dissolved oxygen
PhaC	PHA synthase	**°C**	Degree centigrade
PhaP	Phasin	**sp.**	Species
PhaZ	PHA depolymerase	**OD**	Optical density
%	Percentage	**NMR**	Nuclear Magnetic Resonance
w/v	Weight/volume	**HCl**	Hydrochloric acid
v/v	Volume/volume	**NaOH**	Sodium hydroxide
DCW	dry cell weight	**NaOCl**	Sodium hypochlorite
UV	Ultraviolet	**M**	Molar
PCR	Polymerase chain reaction	μ	Specific growth rate of cells
vvm	Volume per volume per minutes	μ_m	Maximum specific growth rate of cells
g/L	Grams per litre	Tm	Melting temperature
L	Litre	^{r}p	Rate of product formation
ml	Millilitre	**v**	Volts
µl	Microlitre	**ANOVA**	Analysis of varience
h	Hours	**N**	Normal
min	Minutes	**PGBP**	PHA-granule bound protein
bp	Base pair	**X**	Active biomass concentration
kb	Kilo base pair	$Y_{x/s}$	Growth yield
kDa	Kilo Dalton	**RT-PCR**	Real time-PCR
MT	Microelement	**MCL-PHA**	Medium-chain-length PHA
nm	Nano meter	**3HHx**	3-hydroxyhexanoate
β	Beta	**DTT**	Dithiothreitol
SEM	Scanning electron microscope	**IAA**	Iodoacetamide
FTIR	Fourier-Transform Infrared	**$AgNO_3$**	Silver nitrate
PBS	Phosphate buffer saline	**HA**	Hydroxyalkanoate
DNA	Deoxyribonucleic acid	**mM**	Millimolar
RNA	Ribonucleic acid	**µM**	Micromolar
rRNA	Ribosomal RNA	**g**	Gram
3HV	3-hydroxyvalerate	**mg**	Milligram
rpm	Revolutions per minute	**Fig.**	Figure
CoA	Coenzyme A	**SCL-PHA**	Short-chain-length PHA
BLAST	Basic Local Alignment Search Tool	**PAGE**	Poly-acrylamide Gel electrophoresis
SDS	Sodium dodecyl sulfate	**DNSA**	Dinitrosalicylic acid
N	Normal	**LPS**	Lipopolysaccharide
dNTP	Deoxyribonucleotide triphosphate	**EDTA**	Ethylenediaminetetraacetic acid
NJ	Neighbor-joining	**FTIR**	Fourier-Transform Infrared

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ABSTRACT

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ABSTRACT

Polyhydroxyalkanoates are natural biopolymers, synthesized and accumulated by many prokaryotic microorganisms under environmental stress. PHAs possess properties similar with synthetic thermoplastics, at the same time are biodegradable, biocompatible and can be produced from renewable resources. All these important properties make this polymer a suitable candidate to replace synthetic plastics in the market. PHAs are produced by various industries and commercially available in the market for various uses. Although PHAs are produced in the industry and has large scope in the market, the wide spread use of this polymer is restricted due to its higher cost of production than synthetic plastics. The present investigation was carried out in order to isolate potential polyhydroxyalkanoate accumulating bacteria from costal sand-dune ecosystem of Miramar beach, Goa. The study dealt with isolation of heterotrophic bacteria from sand-dune ecosystem and screening for their ability to accumulate PHA.

Sand samples of coastal sand dune were used for the isolation of heterotrophic bacteria using nutrient agar and Tryptone glucose yeast extract agar. Eight sand samples were collected, four from the rhizosphere region and four from the non-rhizosphere region of costal sand-dunes. Highest heterotrophic bacterial counts were obtained from rhizosphere samples. Total 171 bacterial isolates were collected, 77 were obtained on Nutrient agar and 94 were on Tryptone glucose yeast extract agar. Maximum numbers of bacterial isolate were also obtained from rhizosphere.

Twenty-two isolates showed PHA accumulation on E2-mineral medium containing glucose as sole source of carbon. All the isolates showed PHA accumulation within 24 h. Among these, bacterial isolates TMR1.3.2, TMR1.26 and TMR1.28 showed maximum PHA accumulation at 48 h. Maximum numbers of PHA accumulating bacterial isolates were obtained from rhizosphere samples. PHA accumulating bacterial isolates was tentatively identified using their phenotypic characteristics and clustering with the standard organisms in the phenogram. Thirteen isolates showed similarity with *B. megaterium*, one with *B. flexus*, one with *Pseudomonas oryzihabitans,* one with *Paracoccus yeei* and one as *Paracoccus* sp.. Five isolates did not showed similarity with any standard species in the tree and as these were clustered near *B. megaterium* were identified as *Bacillus* sp..

Two PCR methods were developed for rapid identification of PHA accumulating members of Bacillales. Method I for rapid identification of PHA accumulating *Bacillus megaterium* and Method II for rapid differentiation of PHA accumulating members of Bacillales. PCR amplification of *phaC* gene from sand-dune bacterial isolates was carried out using Method I. Among 22 bacterial isolates screened, 13 bacterial isolates showed amplification of 900 bp amplicon and were identified as *Bacillus megaterium*. Selected bacterial isolates were used for 16S rRNA gene sequencing. Nucleotide sequences obtained were analyzed and deposited in Genbank. The phylogenetic tree and maximum sequence homology of the isolates identified these isolates as *B. megaterium* (7), *B.flexus* (1), *B. endophyticus* (1), *B. vireti* (2), *Bacillus* sp. (2), *P. oryzihabitans* (1), *Paracoccus yeei* (1) and *Paracoccus* sp. (1). The diversity of PHA accumulating bacteria observed from coastal sand-dune ecosystem includes both Gram positive and negative bacteria. Gram positive bacteria were belonging to the genus *Bacillus* such as, *Bacillus megaterium* (13), *Bacillus flexus* (1), *Bacillus endophyticus* (1), *Bacillus vireti* (2), *Bacillus* sp. (2). Gram negative bacteria were of genus *Pseudomonas* and *Paracoccus* which includes *Pseudomonas oryzihabitans* (1), *Paracoccus yeei* (1) and *Paracoccus* sp. (1).

Production of biomass and PHA was carried out from all the bacterial isolates using glucose as sole source of carbon. Bacterial isolates showed polymer accumulation ranges between 21.8 and 71.2% of their dry cell weight. *Bacillus* sp. NAMR1.8 showed the maximum and *Paracoccus* sp. TMNR1.3 showed the lowest PHA accumulation per gram of biomass among sand-dune bacterial isolates. *Bacillus megaterium* TMR1.3.2 showed maximum over all biomass and PHA accumulation at 48 h. Among the sand-dune isolates, *Pseudomonas oryzihabitans* NAMR1.6, *Bacillus vireti* TMR1.9.1, *Bacillus vireti* TMR1.9.2 and *Bacillus endophyticus* TMR1.22 were being reported for the first time as PHA accumulating bacterial species. Among the other members of Bacillale *Bacillus mojavensis*, *Bacillus niacin*, *Bacillus simplex*, *Marinibacillus marinus* 21AIT and *Paenibacillus dendritiformis* 30A2 were also reported first time for PHA accumulation.

Twenty-two bacterial isolates were screened for PHA accumulation using various organic acids. In the presence of pyruvic acid all the isolates showed PHA accumulation. Seventeen isolates showed accumulation using succinic acid, 9 isolates showed accumulation using propionic acid, 15 isolates showed accumulation using valeric acid and none of the isolates showed growth or PHA accumulation on octanoic acid. Seven bacterial isolates showed PHA accumulation using all the organic acids tested except octanoic acid. All these bacterial

isolates belong to *Bacillus megaterium*. *Bacillus megaterium* TMR1.3.2 was used for polymer production using glucose as sole source of carbon and combinations of glucose along with valeric acid as carbon sources. The polymers extracted were characterized by FTIR, ^{1}H NMR and ^{13}C NMR and identified as polyhydroxybutyrate. The polymers extracted from 22 bacterial isolates of sand-dune ecosystem using glucose as sole source of carbon were characterized by FTIR spectroscopy and identified as polyhydroxybutyrate. All the PHA accumulating bacteria obtained from coastal sand-dunes produces polyhydroxybutyrate only.

Bacillus megaterium TMR1.3.2 and *Bacillus megaterium* Col1/A6 were selected for high cell density PHB production using batch and fed-batch cultivation. The kinetic parameters obtained using batch cultivation of *B. megaterium* TMR1.3.2 with glucose and nitrogen limitations were used for fed-batch cultivation. In fed-batch cultivation of *B. megaterium* TMR1.3.2 was carried out with exponential feeding of substrate concentrations. At 28 h of fermentation the DCW and PHB obtained were 116.88 g/L and 50.61 g/L respectively, with the overall productivity of PHB as 1.823 g/L/h. The same conditions were used for fed-batch cultivation of *B. megaterium* Col1/A6. At 25 h of cultivation the DCW and PHB obtained were 122.68 g/L and 65.76 g/L, respectively. The overall productivity of PHB was 2.63 g/L/h. The biomass and PHB obtained in fed-batch cultivation of *B. megaterium* TMR1.3.2 and *B. megaterium* Col1/A6 were higher than the earlier reports of high cell density PHB production using *B. megaterium.*

Native PHB granules were isolated from *B. megaterium* TMR1.3.2 and *B. megaterium* Col1/A6. Proteins associated with PHB granules were extracted and loaded in SDS-PAGE followed by silver staining. The SDS-PAGE profile of granule associated proteins showed presence of 25-30 visible protein bands. Each protein bands were excised from the gel and processed for in-gel trypsin digestion. The tryptic digest extracts obtained were loaded in LC/MS QToF for protein identification. More than 60 proteins were identified on the proteome analysis of granule associated proteins. Protein involved in PHA metabolism such as PhaC, PhaR, PhaA, PhaB, PhaP, ketol-acid reductoisomerase and 3-hydroxybutyryl-CoA dehydrogenase were identified. Besides proteins of PHA metabolism other proteins such as, pyruvate dehydrogenase complex, acetyl-CoA metabolic process, citric acid cycle, fatty acid β-oxidation, fatty acid biosynthesis, cell wall synthesis, electron transport chain, protein biosynthesis, nucleic acid biosynthesis, amino acid biosynthesis and hypothetical proteins were identified. This is the first report of proteome analysis of whole granule associated proteins from *B. megaterium* by LC-MS Q-ToF.

To understand the type of granule formation, cells of two bacterial strain *B. megaterium* TMR1.3.2 and *B. megaterium* Col1/A6 were analyzed using SEM. PHA granules were observed attached to cell wall in samples treated with lysozyme for 15 and 30 min. In the samples treated with lysozyme for 45 min the PHA granules were not bound with cell wall. The sizes of PHA granules obtained were between 0.28 - 0.95 μm in *B. megaterium* TMR1.3.2 and 0.3 - 1.1 μm in *B. megaterium* Col1/A6. To differentiate between PHA granule and cell wall the EDX of both granule and cell wall was carried out. The EDX of PHA granule showed the presence of C, N, O and P atoms. In the cell wall along with these atoms Al and Cu were also detected.

Publications

Research Paper:

- Nayak, P., Gaonkar, T., Mohanty, A., Kumar, A., Bhosle, S., Garg, S., 2013. Rapid identification of polyhydroxyalkanoate accumulating members of Bacillales using internal primers for *phaC* gene of *Bacillus megaterium*. ISRN Bacteriology. Article ID 562014, pp 1-12. doi:10.1155/2013/562014
- Gaonkar, T., Nayak, P. K., Garg, S., Bhosle, S., 2012. Siderophore producing bacteria from a sand dune ecosystem and the effect of sodium benzoate on siderophore production by a potential isolate. The Scientific World Journal. Article ID 857249, pp 1-8. doi:10.1100/2012/857249

Book Chapter:

- Nayak, P., Gaonkar, T., Mohanty, A., Kumar, A., Bhosle, S., Garg, S., 2013. Isolation and characterization of polyhydroxyalkanoates producing bacteria from coastal sand-dune ecosystem. In Microbial Diversity and its Applications. Barbuddhe *et al.* (Ed). NIPA, India. 7, 75-82.

Poster Presentations:

- Nayak, P., Gaonkar, T., Mohanty, A., Kumar, A., Bhosle, S., Garg, S., (Poster GM-174). "Diversity of polyhydroxyalkanoates accumulating bacteria isolated from coastal sand-dunes" presented at the 50th Annual Conference of AMI December 15-18, 2009 at NCL Pune.
- Nayak, P., Gaonkar, T., Mohanty, A., Kumar, A., Bhosle, S., Garg, S., (Poster -IP.04). Isolation and characterization of polyhydroxyalkanoates producing bacteria from coastal sand-dune ecosystem" presented at the National Symposium on Microbial Diversity and its applications in Health, Agriculture and Industry held on March 4-5, 2011 at ICAR Research Complex Goa.
- Nayak, P., Palanker, N., Bhosle, S., Garg, S., (Poster EM-31). "Studies on polyhydroxyalkanoate accumulating heterotrophic bacteria from coastal sand-dunes of East-Coast of India" presented at the 52th Annual Conference of AMI November 3-6, 2011 at Panjab University, Chandigarh.

Chapter-I

Introduction and Literature survey

1.1 Introduction

Synthetic plastics are inexpensive, and have many useful properties such as, thermostable, durable, lightweight, strength, resistance to degradation, good insulating material and easily mouldable. Due to these properties, synthetic plastics are used as a material in almost all aspects of our daily life as house hold, storage, packaging, electronic products, clothing, footwear, transport and other goods (Andrady and Neal, 2009). These plastics are non-biodegradable and derived from petroleum products. Because of non-biodegradable nature, plastic products accumulate in the environment causing serious problems on wildlife and considerable burden on solid waste management. In 2011, the synthetic plastic production was around 280 million tons and it is expected that by 2050 its production will be close to 810 million tons (Piet, 2010; Bauwens, 2011). It is almost impossible to restrict the wide spread use of plastic products but it can be possible to replace these synthetic plastics with other polymers that have similar properties and at the same time are biodegradable when discarded in the environment. Among various biodegradable polymers, polyhydroxyalkanoates (PHAs) are very well known and commonly studied biopolymer.

Polyhydroxyalkanoates are natural biopolymers, synthesized and accumulated by many prokaryotic microorganisms as insoluble cytoplasmic granules under limitation of essential nutrients such as nitrogen, oxygen or phosphorous in presence of excess of carbon source (Lee, 1996; Sudesh *et al*., 2000; Kessler and Witholt, 2001). It has ecological as well as environmental importance. PHA accumulating bacteria utilize these polymer granules as source of carbon and energy to survive with the changing environmental conditions (Yang *et al*., 2006). PHAs possess properties similar with synthetic thermoplastics, at the same time are biodegradable, biocompatible and can be produced from renewable resources (Patwardhan and Srivastava, 2008). These important properties make this polymer a suitable candidate to replace synthetic plastics in the market. PHAs are produced by various industries, such as Bio-on (Italy), Kaneka (Singapore), Meredian (USA), Metabolix (USA), Mitsubishi Gas Chemicals (Japan), PHB Industrial S/A (Brazil), Tianan Biological Materials (China), Tianjin Green Biosciences (China) and Biomer Inc. (Germany) (Babu *et al*., 2013)

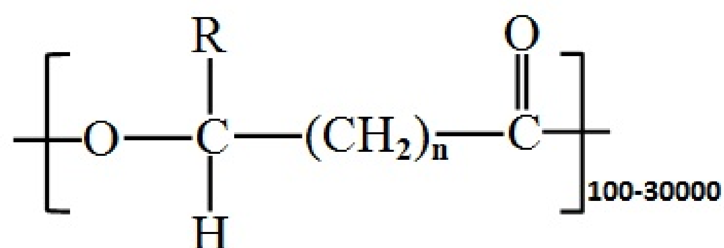
and commercially available in the market for various uses such as packaging, coating, electronic products, agricultural and biomedical applications (Chen, 2010).

1.2 Bacterial polyhydroxyalkanoate

Many prokaryotic microorganisms such as eubacteria and archaea are known to produce PHA granules naturally to store excess of carbon during environmental stress (Anderso and Dawes, 1990). More than 300 species accumulating PHA belongs to 90 genera have been reported from different environments, such as soil (Sabat *et al*., 1998; Wang and Bakken, 1998; Santimano *et al*., 2009; Raj *et al*., 2014), sewage sludge (Sheu *et al*., 2000; Bhuwal *et al*., 2013; Raj *et al*., 2014), organic solid waste (Ibrahim *et al*., 2010), hot springs (Kung *et al*., 2007; Ibrahim *et al*., 2010), ponds (Yellore & Desai, 1998), Antarctic ecosystem (Ayub *et al*., 2004; Lopez *et al*., 2009; Goh and Tan, 2012), Salt lake (Rodriguez-Contreras *et al*., 2013), sea water (Arun *et al*., 2009; Van-Thouc *et al*., 2012), marine sediments (Prabhu *et al*., 2010), mangrove ecosystem (Arun *et al*., 2009), salt pans (Subin*et al*., 2013) and marine microbial mats (Simon-Colin *et al*., 2008; Lopez-Cortes *et al*., 2008). These genera of PHA accumulating bacteria are listed in Table 1.1. Among PHA accumulating bacteria *B. megaterium*, *B. flexus*, *B. circulans*, *B. thuringiensis*, *B. cereus* and *Bacillus* sp. are reported as the most diverse PHA producers. Members of the genus *Bacillus* are known for rapid growth to utilize various carbon sources and produces polymer and co-polymer from single and structurally unrelated carbon sources (Santimano *et al*., 2009).

Polyhydroxyalkanoates are polymers of hydroxyalkanoic acid monomers, which are linked to each other by an ester linkage. The general structure of polyhydroxyalkanoate is shown in Fig. 1.1. Polyhydroxybutyrate (PHB) is the most commonly produced and well studied biopolymer in this group. It was first discovered in *Bacillus megaterium* by Lemoigne (1926). Approximately, 150 different hydroxyalkanoic acids have been characterized as monomeric units of homopolymer or co-polymer of PHA. Co-polymers have more useful industrial and medical (Biocompatible) properties than the homopolymers. PHAs are of two types depending on the number of carbon atoms present in the monomer: short chain length (SCL) PHA and medium chain length (MCL) PHA. The SCL-PHA contains monomers of 3

– 5 carbon atoms. These polymers are stiff and brittle in nature. They possess high degree of crystallinity, lack toughness and show material properties similar to synthetic thermoplastics of petrochemical origin. MCL-PHA has monomers of 6 – 15 carbon atoms. These are flexible and possess low crystallinity, tensile strength and melting point. Due to elastomeric and less crystalline nature these polymer has more industrial application than SCL-PHA (Doi *et al*., 1995; Ojumu *et al*., 2004; Volova, 2004; Suriyamongkol *et al*., 2007). In last two decades, many bacteria reported capable of synthesize PHAs having both SCL and MCL monomeric units. These co-polymers shows improved physical and thermal properties than SCL-PHA and it depends on the mole fraction of SCL to MCL monomers present (Madison and Huisman, 1999; Philip *et al*., 2007; Yu, 2007).



n = 1	R = hydrogen	Poly (3-hydroxypropionate)
	R = methyl	Poly (3-hydroxybutyrate)
	R = ethyl	Poly (3-hydroxyvalerate)
n = 2	R = hydrogen	Poly (4-hydroxybutyrate)
n = 3	R = hydrogen	Poly (5-hydroxyvalerate)

Fig. 1.1: General structure of PHA

Acidovorax	*Erwinia*	*Oscillatoria*
Acinetobacter	*Escherichia* (recombinant)	*Paracoccus*
Actinobacillus	*Ferrobacillus*	*Paucispirillum*
Actinomycetes	*Gamphospheria*	*Pedomicrobium*
Aeromonas	*Gloeocapsa*	*Photobacterium*
Alcaligenes	*Gloeothece*	*Protomonas*
Allochromatium	*Haemophilus*	*Pseudomonas*
Anabaena	*Halobacterium*	*Ralstonia*
Aphanothece	*Haloarcula*	*Rhizobium*
Aquaspirillum	*Haloferax*	*Rhodobacter*
Asticcaulus	*Halomonas*	*Rhodococcus*
Azomonas	*Haloquadratum*	*Rhodopseudomonas*
Azospirillum	*Haloterrigena*	*Rhodospirillum*
Azotobacter	*Hydrogenophaga*	*Rubrivivax*
Bacillus	*Hyphomicrobium*	*Saccharophagus*
Beggiatoa	*Klebsiella* (recombinant)	*Shinorhizobium*
Beijerinckia	*Lamprocystis*	*Sphaerotilus*
Beneckea	*Lampropedia*	*Spirillum*
Brachymonas	*Leptothrix*	*Spirulina*
Bradyrhizobium	*Methanomonas*	*Staphylococcus*
Burkholderia	*Methylobacterium*	*Stella*
Caryophanon	*Methylosinus*	*Streptomyces*
Caulobacter	*Methylocystis*	*Synechococcus*
Chloroflexus	*Methylomonas*	*Syntrophomonas*
Chlorogloea	*Methylovibrio*	*Thiobacillus*
Chromatium	*Micrococcus*	*Thiococcus*
Chromobacterium	*Microcoleus*	*Thiocystis*
Clostridium	*Microcystis*	*Thiodictyon*
Comamonas	*Microlunatus*	*Thiopedia*
Corynebacterium	*Moraxella*	*Thiosphaera*
Cupriavidus	*Mycoplana*	*Variovorax*
Cyanobacterium	*Nitrobacter*	*Vibrio*
Defluviicoccus	*Nitrococcus*	*Wautersia*
Derxia	*Nocardia*	*Xanthobacter*
Delftia	*Nostoc*	*Zoogloea*
Ectothiorhodospira	*Oceanospirillum*	

Table 1.1: Different genera of PHA accumulating bacteria (Koller *et al*., 2010)

1.3 PHA biosynthesis

Many bacterial species are known for PHA accumulation using various carbon sources such as simple carbohydrates, beet/cane molasses, plant oils, fatty acids, and alkanes (Lageveen *et al*., 1988; Hangii, 1990; Page, 1992; Eggink *et al*., 1993; Tan *et al*., 1997; Fukui and Doi, 1998). Biosynthesis of PHA polymer involved in a series of enzymatic reactions. The composition and type of PHA monomer synthesized by bacteria depends on the carbon sources and the metabolic pathways they used (Lu *et al*., 2009). The PHA synthesis is from related carbon sources, when the hydroxyalkanoic (HA) monomers of the PHA polymer are structurally identical with the carbon source used and the PHA synthesis from unrelated carbon sources, if the HA monomers are structurally different from the carbon source provided (Philip *et al*., 2007). Chen (2010) has comprehensively described the pathways involved in PHA synthesis. Depending on the types of monomer incorporated in to PHA by PHA synthase, eight PHA biosynthetic pathways have been explained.

Pathway I: Synthesis of SCL-PHA

Pathway I is represented by *Cupriavidus necator* (Schubert *et al*., 1988; Slater *et al*., 1988; Peoples and Sinskey, 1989). In *C. necator*, the PHA synthesis is a three-step enzyme reaction and acetyl-CoA used as a substrate. The first step involves condensation of two acetyl-CoA molecules to acetoacetyl-CoA by the enzyme β-ketothiolase (encoded by *phaA*). Second step involves reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA by the enzyme NADPH-dependent acetoacetyl-CoA reductase (encoded by *phaB*). Finally, 3-hydroxybutyryl-CoA is polymerized to PHB by PHA synthase (encoded by *phaC*) (Anderson and Dawes, 1990; Singh *et al*., 2009) (Fig. 1.2). Due to the substrate specificity of PHA synthase of *C. necator*, polymers synthesized by this pathway contain monomers of C3-C5 carbon atoms (Steinbuchel and Schlegel, 1991). Utilization of various carbon sources by *C. necator* for PHA production has been reported by many researchers such as lactic acid (Linko and Vaheri, 1993), plant oils (Fukui and Doi, 1998) and carbon dioxide (Ishizaki and Tanaka, 1991). It is also reported that *C. necator* produces P(3HB) homopolymer from *n*-alkanoate having even carbon numbers and co-polymers of 3HB and 3HV from *n*-alkanoates having odd carbon numbers (Akiyama *et al*., 1992).

Including PHB, a number of other PHAs are produced in bacteria (Kim and Lenz, 2001; Luengo *et al*., 2003; Tan *et al*., 2014). For example, production of co-polymer P(3HB-co-3HV) in the presence of glucose as a single carbon source (Reddy *et al*., 2009) and presence of glucose along with propionic acid or valeric acid in the medium (Chen *et al*.,1991; Poirier, 2002). In this co-polymer synthesis, the first step involves condensation of propionyl-CoA with acetyl-CoA molecule by a distinct β-ketothiolase (encoded by *bktB*). Further, 3-ketovaleryl-CoA is reduced to (R)-3-hydroxyvaleryl-CoA by NADPH-dependent acetoacetyl-CoA reductase and finally polymerization of (R)-3-hydroxyvaleryl-CoA to the P(3HB-co-3HV) by PHA synthase.

Pathway II: Synthesis of MCL-PHA from related carbon sources

This pathway is best studied in *Pseudomonas* species belonging to the ribosomal RNA (rRNA)-homology Group I. In this pathway, β-oxidation intermediates of alkanoic or fatty acids serve as hydroxyacyl-CoA (HA-CoA) substrate (C6-C14 carbon length) for synthesis of MCL-PHAs. The MCL-PHA synthase has substrate specificity ranges from C6-C14 (R)-3-hydroxyacyl-CoA, with a liking towards C8, C9 and C10 molecules (Huisman *et al*., 1989). Bacteria of this group synthesize PHA from alkanoic acids or fatty acids and the polymer produced is directly related to the carbon substrates used (Lageveen *et al*., 1988; Rehm, 2007). Brandl *et al*. (1988) reported that when substrates of even carbon atoms are used, only PHA monomers of even carbon are synthesized and for substrates of odd carbon atoms, only PHA monomers of odd carbon are synthesized. In the presence of mixtures of alkanoic acids or fatty acids as carbon sources in the medium results in synthesis of PHAs where the composition reflects the ratio of both the carbon source used. Lageveen *et al*. (1988) reported that when *P. oleovorans* grown with the supply of mixtures of octane and 1-octene in the medium, the ratio of PHA monomers was synthesized containing unsaturated bond of 0 to 50% depending on the fraction of 1-octene in the medium. They suggested that the MCL-PHA synthesis pathway is a direct link of fatty acid β-oxidation pathway.

The β-oxidation pathway intermediates such as trans-2, 3-enoyl-CoA, (S)-3-hydroxyacyl-CoA and 3-ketoacyl-CoA are in (S)-3-hydroxyacyl-CoA form, which

cannot be directly incorporated by MCL-PHA synthase. The enzymes, (R)-specific enoyl-CoA hydratase, hydroxyacyl-CoA epimerase and 3-ketoacyl-CoA reductase that convert intermediates of β-oxidation pathway to (R)-3-hydroxyacyl-CoA have been discovered (Fukui *et al*., 1998; Taguchi *et al*., 1999; Rehm, 2007).

Pathway III: Synthesis of MCL-PHA from unrelated carbon sources

In this pathway, bacteria synthesize MCL-PHA from intermediates of fatty acid biosynthesis. Except *Pseudomonas oleovorans* and *Pseudomonas fragii*, most of the fluorescent pseudomonads belonging to the rRNA-homology group I also synthesize MCL-PHA from unrelated carbon substrates such as carbohydrates (Anderson and Dawes, 1990). *P. aeruginosa* and *P. putida* synthesize MCL-PHA when grown on unrelated carbon substrates such as glucose (Huijberts *et al*., 1992; Steinbuchel and Lutke-Eversloh, 2003). Lee *et al*. (2001) reported that *P. fluorescens* BM07 produces MCL-PHA containing more percentage of unsaturated monomers such as 3-hydroxy-cis-5-dodecenoate or 3-hydroxy-cis-7-tetradecenoate from unrelated substrates. In these bacteria, MCL-PHA is produced from the 3-hydroxyacyl-ACP intermediates of the *de novo* fatty acid biosynthesis pathway. The enzyme 3-hydroxyacyl-CoA-ACP transferase (encoded by *phaG*) is a key enzyme in this pathway, which is responsible for converting the 3-hydroxyacyl-ACP intermediate of the fatty acid biosynthesis pathway to 3-hydroxyacyl-CoA, the substrate for MCL-PHA synthase (Rehm *et al*., 1998). Few *Pseudomonas* sp. also capable of incorporating both SCL and MCL-PHA monomers to the polymer chain, when grown in medium containing unrelated carbon substrates such as 1,3-butanediol and carbohydrates (Steinbuchel and Wiese, 1992; Abe *et al*., 1994; Lee *et al*., 1995; Kato *et al*., 1996).

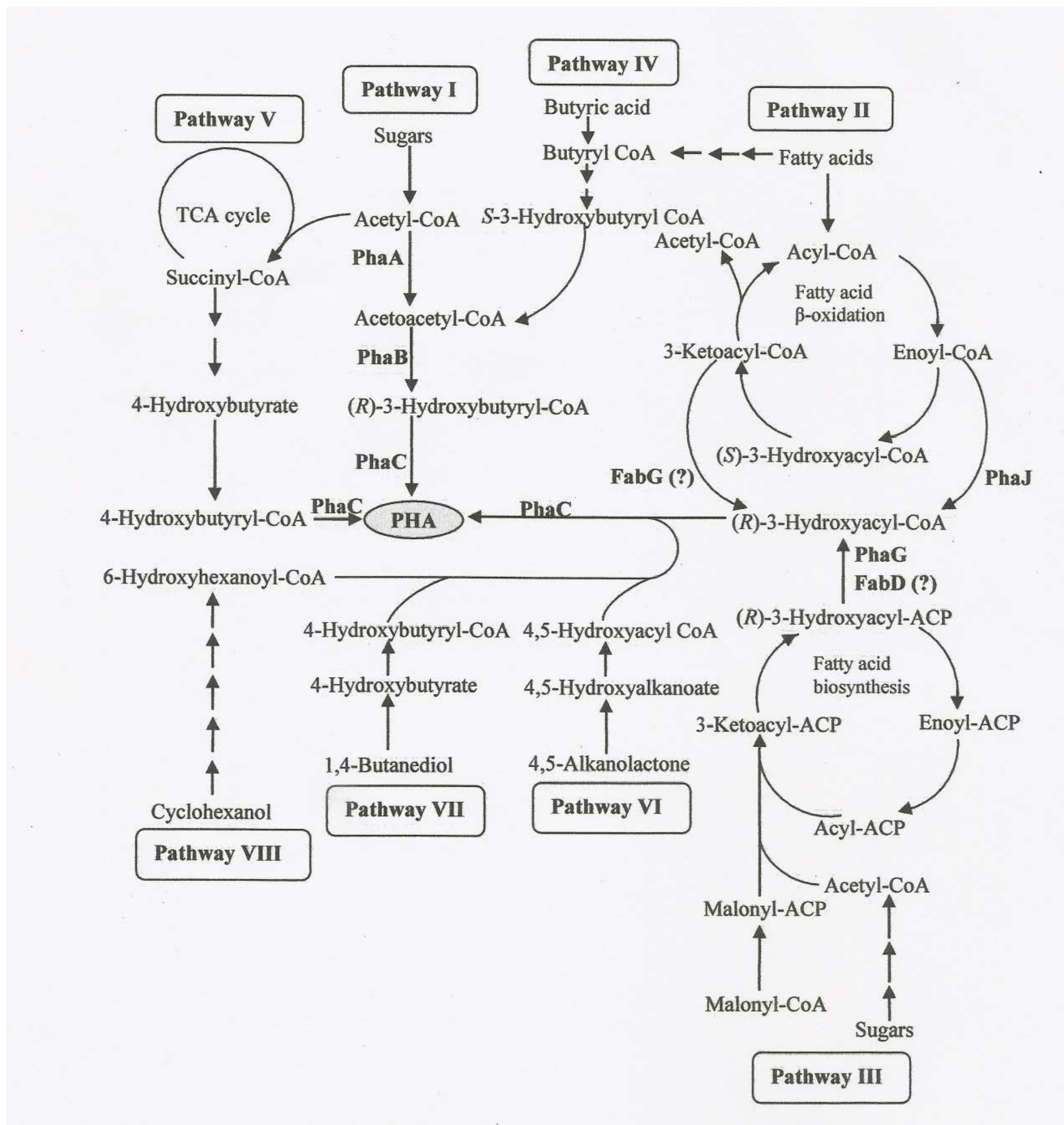


Fig. 1.2: Pathways involved in PHA biosynthesis

Pathway IV: In this pathway *S*-3-hydroxybutyryl-CoA produced from butyric acid and other fatty acids are oxidized by acetoacetyl-CoA reductase to acetoacetyl-CoA (Chohan and Copeland, 1998). Further acetoacetyl-coA is reduced and subsequently polymerized to PHA.

Pathway V: In this pathway 4-hydroxybutyryl-CoA is synthesized from succinyl-CoA by using succinic semialdehyde dehydrogenase, 4-hydroxybutyrate dehydrogenase and 4-hydroxybutyrate-CoA transferase (Valentin and Dennis, 1997).

Pathway VI: This pathway involves production of 4,5-hydroxyacyl-CoA from 4,5-alcanolactone using putative lactonase and hydroxyacyl-CoA synthase (Valentin and Stenbuchel, 1995).

Pathway VII: It involves synthesis of 4-hydroxybutyryl-CoA from 1,4-butanediol using putative alcohol dehydrogenase and hydroxyacyl-CoA synthase (Xie and Chen, 2008).

Pathway VIII: In this pathway 6-hydroxyhexanoyl-CoA is synthesized from cyclohexanol using 9 enzymes (Chen, 2010).

1.4 PHA granule formation in bacteria

The hydroxyalkanoic acid monomers are polymerized by PHA synthase in to high molecular weight polymer ranging from 200 to 3,000 kDa, depending on the bacterial strain (Byrom, 1994). *In vitro* synthesis of PHB and self-assembly of spherical granules was first demonstrated by Gerngross and Martin, (1995) using purified polyester synthase and substrate. They demonstrated that the polyester synthases possess all the necessary features required for self-assembly of the polymer in to spherical granules. This was confirmed by *in vitro* polyester synthesis using purified polyester synthase from different microorganisms, such as *C. necator*, *Allochromatium vinosum*, *Pseudomonas aeruginosa* and *Pseudomonas oleovorans* (Jossek and Steinbuchel, 1998; Jossek *et al*., 1998; Muh *et al*., 1999; Qi *et al*., 2000; Rehm *et al*., 2001). The polymer aggregates inside the bacterial cell to form discrete hydrophobic granules. These granules are of 200 to 500 nm in diameter. Granules contain 97.5% PHA, 2% protein and 0.5% phospholipids (Rehm, 2003). The spherical granules are composed of an amorphous PHA core, surrounded by a 4 nm thick phospholipid monolayer (Barnard and Sanders, 1989; Mayer and Hoppert, 1997; Dennis *et al*., 2003; Grage *et al*., 2009). This monolayer is embedded with granule associated proteins. These proteins form a surface layer on PHA granules and plays an important role in polymer metabolism and granule formation (Potter and Steinbuchel, 2005; Jendrossek, 2009). In the last two decades, many investigations have been carried out on PHA metabolism to increase the understanding of functions of individual proteins associated with PHA granules (Jendrossek and Handrick, 2002;

Rehm, 2003; Stubbe and Tian, 2003; Jendrossek, 2005; Stubbe *et al*., 2005; Potter and Steinbuchel, 2006; Rehm, 2006). However, the initiation of polymer synthesis and granule formation inside the bacterial cell is not yet understood clearly.

1.4.1 Subcellular localization of PHA granules and Models of granule formation

In the past, very little was known about subcellular localization of PHA granules in bacteria and many researchers assumed that PHA granules are randomly localized in the cytoplasm of bacteria. When localization of granules was found in the periphery of bacterial cells, it was assumed to be a consequence of nucleoid occlusion (Peters and Rehm, 2005, 2006). Shekhovtsev and Zharikova, (1978), for the first time, reported the localization of PHB granule formation in *Caryophanon tenue* and *C. latum*. Using light microscopy they observed that early PHB granules were attached to the cytoplasmic membrane but in the later stage of growth granules were found in the whole cell compartment. Further they reinvestigated the PHB granule formation in *C. latum* and confirmed that the newly synthesised granules of PHB were localized close to the cell membrane. This hypothesis got support by reports that claimed cytoplasmic localization of PHB synthase *PhaC* in *Ralstonia eutropha* even in the absence of accumulated PHB (Haywood *et al*., 1989). However, in *C. latum* a direct attachment of PHB granule to the cytoplasmic membrane was never observed (Jendrossek *et al*., 2007). A peripheral localization of PHB granules were observed in several other species, such as *Rhodospirillum rubrum*, *Ralstonia eutropha, Azotobacter vinelandii*, *Hyphomicrobium facile*, *Beijerinckia indica* and *Haloquadrata walsbyi* (Jendrossek,2005; Hermawan and Jendrossek, 2007). Based on theoretical considerations and experimental reports obtained so far, there are three possible models of PHA granule formation in bacterial cell (Jendrossek and Pfeiffer, 2014): a) Micelle, b) Budding and c) Scaffold models.

1.4.1.I Micelle model

In this model the PHB synthase molecules (PhaC1 dimers) are present in the cell as a soluble enzyme, more or less randomly distributed in the cytoplasm. This enzyme starts synthesis of PHB molecule if the substrate (3-hydroxybutyryl-CoA) concentration is sufficiently high (Ellar *et al*., 1968; Griebel *et al*., 1968; Gerngross *et*

al., 1993; Grage *et al*., 2009). Once the polymerization starts, the nascent PHB chain converts the soluble PHB synthase in to an amphipathic molecule. Due to the hydrophobicity and low solubility of PHB molecule in the cell cytoplasm, nascent growing polymer chains aggregate and form micelle like structures with PhaC molecule attached on the polymer surface (Stubbe and Tian, 2003; Stubbe *et al*., 2005; Grage *et al*., 2009). Later, phospholipids, phasins and other PHB granule-associated proteins (PGAPs) gradually attached to the growing PHB granule. A consequence of this model is that the granule formation starts at any localization within the cytoplasm and the granule formed should be randomly localized within the volume of a cell.

1.4.1.II Budding model

This model suggests that the PHB synthase is associated with the inner surface of the cytoplasmic membrane and the newly synthesized PHB chain integrates into the bilayer of the cell membrane. Once the polymer chain is synthesized it interacts hydrophobically with itself and with cytoplasmic membrane to form a PHB core inside the bilayer which results in swelling of the cell membrane. At the later stages, the granules detach from the membrane as buds and granule associated proteins attached to it (Jendrossek, 2009; Jendrossek and Pfeiffer, 2014). The presence of oligo-PHB inside the bacterial cell membrane was reported earlier (Reusch, 1995; Das *et al*., 1997). If this model exists, then PHB granules should be localized inside or near the cell membrane at early stages of granule formation. Most of the recent studies and evidences strongly support the budding model of PHA granule formation.

PHA granule formation was studied by Peters and Rehm (2005) in *Pseudomonas aeruginosa* PAO1 and recombinant *E. coli* by N-terminal fusion of a green fluorescent protein (GFP) with Class I and Class III PHA synthase, respectively. In both the organisms nascent PHA granules were observed near the cell poles. Jendrossek (2005) investigated early stages of PHB accumulation *in vivo* in *Rhodospirillum rubrum*, *C. necator* and recombinant *E. coli* using confocal laser scanning fluorescence microscopy (CLSM) by Nile red staining of PHB granules and fusion of enhanced yellow fluorescent protein (EYFP) to a phasin. In the early stages of PHB accumulation, he observed that most of the time PHB granules localized near

the cell poles or near the cell wall. Hermawan and Jendrossek (2007) studied the PHB granule formation in *Azotobacter vinelandii* by fluorescence microscopy of cells stained with Nilered and Backlight®. They observed the fluorescent PHB granules near the cell periphery at the early stages of granule formation and later these granules become detached from the cell periphery. The authors suggested that the granule formation starts at the inner site of the cytoplasmic membrane. Jendrossek *et al*. (2007) analyzed *Caryophanon latum* by CLSM of cell stained with Nile red, TEM in combination with immunogold labeling and SEM analysis of PHB granules after cell lysis. They found that at the early stages of granule formation PHB granules were predominately localized close to the cytoplasmic membrane. PHA synthase fused with GFP was investigated in recombinant *E. coli* expressing PHA synthase of *R. eutropha* and *P. aeruginosa*, where both the PHA synthase were localized at the cell poles (Peters and Rehm, 2005; Peters *et al*., 2007). Boatman (1964) demonstrated TEM analysis of PHA accumulating *Rhodospirillum rubrum* cells and showed that the PHB granules was covered by a ~4 nm thick surface layer. The author also suggested that the surface layer of PHB granules might be a phospholipid monolayer.

1.4.1.III Scaffold model

Scaffold model assumes that PHB synthase of nascent polymer chain is attached to an unknown scaffold molecule referred to as mediation element within the cell. These mediation elements probably serve as scaffolds for the initiation of PHB granule formation. In this model subcellular localization of PHB granules mainly depends on the nature and the position of scaffold molecule of the PHB accumulating cell (Jendrossek and Pfeiffer, 2014). Tian *et al*. (2005) studied the PHB granule biogenesis in *C. necator* by TEM analysis. They found that early PHB granules are attached to a darkly stained structure referred as mediation elements in the centre of the cell. In the same study of older PHB accumulating cells, they could not detect the darkly stained structure, which might be degraded or totally covered by the granule. Recently, Beeby *et al*. (2012) studied the PHB granule formation in *R. eutropha* by Electron cryotomography. They observed PHB granules localized in the cell centre. In this study they could not detect PHB granules attached to the cytoplasmic membrane but PHB synthase of a nascent granule was never detected attached to scaffold molecule.

1.5 Proteins associated with PHA granules

PHA granule associated proteins plays important role in biosynthesis and degradation of PHA and in the formation of granule (Potter and Steinbuchel, 2005). These proteins are PHA synthase (PhaC), PHA depolymerase (PhaZ), regulatory protein (PhaR), phasins (PhaP) and many other proteins such as membrane proteins, soluble proteins of tricarboxylic acid cycle (Jendrossek and Pfeiffer, 2014).

1.5.1 PHA synthase (PhaC)

PHA synthase has been identified as a crucial enzyme of PHA biosynthesis, which catalyses the selective conversion of (R)-3-hydroxyacyl-CoA thioester substrates to PHA with the concomitant release of free CoA molecules (Rehm, 2003; Grage *et al.* 2009). All PHA synthases possess a conserved amino acid (cysteine) at its catalytic active site to which the growing polymer chain is covalently bound (Jendrossek, 2009; Prabhu *et al*., 2010). The first PHA synthase gene (*PhaC*) of *R. eutrophus* H16 was cloned in 1988 (Schubert *et al*., 1988; Slater *et al*., 1988). Currently, the nucleotide sequences of more than 88 PHA synthases have been characterized, it includes two potential PHA synthase gene recently characterized from halobacterial species (Han *et al*., 2007; Lu *et al*., 2008). Based on primary structures, subunit composition and substrate specificity, PHA synthases have been classified in to four major classes (Table 1.2) (Rehm, 2003; Jendrossek, 2009).

Class I PHA synthases

These synthases are represented by *Cupriavidus necator* and consists of only one type of subunit (PhaC) with molecular weight ranging between 61 and 73 kDa. These synthases preferentially utilize (R)-3-hydroxy fatty acid substrates comprising of 3-5 carbon atoms (SCL-HA) and produce PHAs composed of short chain length monomers (SCL-PHA). Although these enzymes prefer SCL-HA, they can also incorporate small amount of 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO) and 3-hydroxydodecanoate (3HDD) to synthesize co-polymers (Dennis *et al*., 1998; Antonio *et al*., 2000).

Class	Microorganism	Subunits	Substrate specificity
I	*Ralstonia eutropha*	PhaC ~60-73 kDa	$3HA_{SCL}$-CoA (~C3-C5) $4HA_{SCL}$-CoA, $5HA_{SCL}$-CoA, $3MA_{SCL}$-CoA
II	*Pseudomonas aeruginosa*	PhaC ~60-73 kDa	$3HA_{MCL}$-CoA (~C6-C14)
III	*Allochromatium vinosum*	PhaC ~40 kDa PhaE ~40 kDa	$3HA_{SCL}$-CoA $3HA_{MCL}$-CoA (~C6-C8), $4HA_{SCL}$-CoA, $5HA_{SCL}$-CoA
IV	*Bacillus megaterium*	PhaC ~40 kDa PhaR ~22 kDa	$3HA_{SCL}$-CoA

Table 1.2: Different classes of PHA synthases

Class II PHA synthases

These synthases are represented by *Pseudomonas aeruginosa* and consists of one type of subunit (PhaC) with molecular weight between 61 to 73 kDa. These enzymes efficiently incorporate CoA thioester of (R)-3-Hydroxy fatty acids containing 6-14 carbon atoms from the intermediates of fatty acid β-oxidation and fatty acid biosynthesis pathway and they produce polymers composed of medium chain length monomers (MCL-PHA) (Sudesh *et al*., 2000).

Class III PHA synthases

This type of synthases is represented by *Allochromatium vinosum* and consists of two different types of subunits such as PhaC and PhaE of 40 kDa each. In this class both the subunits are required for the functional activity of this enzyme during polymer synthesis. The PhaC subunit shows 21-28% amino acid sequence similarity to Class I and Class II PHA synthases, whereas PhaE shows no sequence similarity with any of the synthases. These synthases prefer CoA thioester of (R)-3HAs comprising 3-5 carbon atoms and they produce PHAs composed of short chain length monomers (SCL-PHA) (Liebergessell *et al*., 1992; Liebergessell and Steinbuchel, 1992).

Class IV PHA synthases

This type of synthases is represents by *Bacillus megaterium* and consists of two subunits similar to class III PHA synthases. However, in this case, the second subunit PhaE is replaced by PhaR having a molecular weight of 20 kDa (McCool and Cannon, 2001). Class IV PHA synthases prefer CoA thioesters of (R)-3HAs comprising 3-5 carbon atoms and produces PHAs composed of short chain length monomers (SCL-PHA).

1.5.2 Unclassified PHA synthases

There are few exceptions to the current classification of PHA synthases. PHA synthase of *Thiocapsa pfennigii* consist of two subunits with strong similarity to class III PHA synthases but shows broad substrate specificity comprises CoA thioesters of SCL and MCL 3-hydroxy fatty acids (Liebergessell *et al.*, 1993). *Aeromonas punctata* PHA synthase consist of one subunit with strong similarity to class I PHA synthases but catalyses the synthesis of a co-polymer of 3-hydroxybutyrate-co-3-hydroxyhexanoate (3HB-co-HHx) (Fukui and Doi, 1997). *Pseudomonas* sp. 61-3 possesses two PHA synthases (*phaC1* and *phaC2*), which shows strong similarity to class II PHA synthases. These synthases catalyzes polymerization of co-polymer consisting of 3-hydroxybutyrate and MCL-3HAs (Matsusaki *et al.*, 1998).

1.5.3 PHA depolymerases (PhaZ)

PHA depolymerases are enzymes that degrade PHA. These are of two types, namely extracellular depolymerase and intracellular depolymerase. Extracellular depolymerases are enzymes that specifically degrade denatured PHA (dPHA). In the last two decades, more than 20 extracellular PHA depolymerases have been characterized and their functions are known (Jendrossek and Handrick, 2002). In 2006, the crystal structure of first extracellular PHA depolymerase has been reported (Hisano *et al.*, 2006). However, in comparison to extracellular depolymerases, the structure and function of intracellular depolymerases are less studied.

Intracellular depolymerases are enzymes that catalyse the depolymerisation of native PHA (nPHA) within PHA accumulating bacteria. The intracellular PHA depolymerases are associated with PHA granules by non-covalent interactions (Saegusa *et al.*, 2002). The first nucleotide sequence of an intracellular PHA depolymerase (PhaZ1) was obtained from *C. necator* (Saegusa *et al.*, 2001). Saegusa

et al. (2001) reported the intracellular depolymerase activity of PhaZ of *C. necator* by using amorphous PHA granules as substrate. Potter and Steinbuchel (2005) demonstrated the presence of intracellular PhaZ1 on the surface of native PHA granules by Western blot analysis using anti-PhaZ1 antibodies. In addition to PhaZ1, four more intracellular PHA depolymerases were identified in *C. necator*. These are known as PhaZ2, PhaZ3, PhaZ4 and PhaZ5 (York *et al*., 2003; Schwartz *et al*., 2003; Potter *et al*., 2004). Gebauer and Jendrossek (2006) demonstrated the intracellular depolymerase activity of PhaZ5 of *R. eutropha* H16 by using native PHB granules as substrate. An intracellular PHB depolymerase PhaZ of *Paracoccus denitrificans* was demonstrated for *in vitro* degradation of protease-treated native PHB granules, where the main breakdown products were 3HB dimmers and oligomers (Gao *et al*., 2001). Tseng *et al*. (2006) have identified a novel intracellular PHB depolymerase Phaz from *B. thuringiensis*, which shows no significant amino acid sequence similarity with any PHA depolymerases. This PHB depolymerase has strong hydrolyzing activity towards native PHB and can also hydrolyze the protease-treated native PHB. Chen *et al*. (2009) have been identified an intracellular PHB depolymerase PhaZ in *B. megaterium* ATCC 11561, which shows rapid degradation activity towards native PHB granules *in vitro.*

1.5.4 Phasins (PhaP)

Phasins are the most abundant proteins present on the surface of PHA granules. These proteins were named phasins due to its analogy with oleosins, proteins found on the surface of triacylglycerol inclusions present in seeds and pollen of plants (Huang, 1992; Murphy, 1993). These are non-catalytic proteins, having molecular weight between 11 and 25 kDa (Wieczorek *et al*., 1995). These proteins are bound to the PHA core of the granule through hydrophobic interactions. Phasins helps in stabilizing PHA granules in the cell cytoplasm and prevents its coalescence with other granules (Steinbuchel *et al*., 1995; Potter and Steinbuchel, 2005). Atomic force microscopy of PHB granules isolated from *R. eutropha* reveals that surface of PHB granules is covered by a network or a skeleton like structure (Dennis *et al*., 2003). Later, it was suggested that PhaP1 is required for the formation of skeleton like structure on the surface of PHA granules (Kuchta *et al*., 2007) and possibly this protein is part of it (Dennis *et al*., 2008).

There are four homologous phasin proteins such as, PhaP1, PhaP2, PhaP3 and PhaP4 were reported in *R. eutropha*, where PhaP1 is confirmed as the major phasin protein among them (Wieczorek *et al*., 1995; Potter *et al*., 2004). Phasins are only expressed under conditions favourable for PHA accumulation and their concentrations are parallel to the number of PHA granules present in the bacteria (Qi *et al*., 2000; York *et al*., 2001; Almeida *et al*., 2007). Wieczorek *et al*. (1995) reported that phasins are not required for PHA accumulation, but in the absence of phasin protein bacteria could accumulate a single large PHA granule. On the other hand, the overproduction of phasin protein resulted in accumulation of many small PHA granules in the bacterial cell (Potter *et al*., 2002). York *et al*. (2002) demonstrated that deletion of *PhaP* gene, the amount of PHB production was reduced by 50% in comparison to wild-type *R. eutropha*.

1.5.5 Regulatory proteins

Regulatory proteins are non-covalently bound to the PHA granule surface and play a major role in regulating the granule formation and phasin production. Various regulatory proteins have been identified in PHA accumulating bacteria such as, PhaM in *R. eutropha* H16 (Pfeiffer and Jendrossek, 2011, 2013, 2014; Pfeiffer *et al*., 2011; Cho *et al*., 2012; Wahl *et al*., 2012), PhaR in *R. eutropha* (Potter et al., 2002; Pfeiffer and Jendrossek, 2011), PhaF in *P. putida*and *P. oleovorans* (Prieto *et al*., 1999; Moldes *et al*., 2004; Galan *et al*., 2011), PhaD in *P. Putida* (Klinke *et al*., 2000; de Eugenio *et al*., 2010b), PhaQ in *B. megaterium* (Lee *et al*., 2004) and PhaR in *Paracoccus denitrificans* (Maehara *et al*., 1999, 2002; Yamada *et al*., 2007).

PhaR has the capability to bind to the promoter region of PhaP1, the promoter region of PhaR and the surface of PHB granules. During PHB accumulation PhaR binds to growing PHB granules and allows the expression of PhaP and PhaR. Once all the binding sites for PhaR on surface of PHB granules are occupied by this protein, excess PhaR binds to the promoter region of PhaP and PhaR, which results in the repression of these proteins. The regulations of phasin expression and PHB accumulation are same in *Paracoccus denitrificans* (Maehara *et al*., 1999; 2001). PhaD is a transcriptional regulator, which controls the expression of phaI and PhaF proteins in *Pseudomonas putida* (Sandoval *et al*., 2007). Klinke *et al*. (2000) reported that in *PhaD* mutant the PHA production was reduced and in wild type the number of

PHA granules increased with decrease in size. Lee *et al*. (2004) reported a new class of transcriptional regulator PhaQ in *B. megaterium*, which directly interacts with PHB granules and regulates PhaP expression. PhaF plays a crucial role on granule localization in the cells of PHA accumulating *Pseudomonas putida* (Galan *et al*., 2011). Like PhaF, in *R. eutropha* depending on the concentrations of PhaM and phasin PhaP5 the subcellular localization of PHB granules are controlled and PhaM helps in binding of PHB granules to nucleoid (Wahl *et al*., 2012).

1.5.6 Other PHA granule associated proteins

SDS-PAGE of purified native PHA granule associated proteins from *R. eutropha* H16 reveals the presence of large number of proteins (Jendrossek and Pfeiffer, 2014). Except few proteins, such as PHA synthase, PHA depolymerase, phasins and transcriptional regulators the subcellular localization and function of other granule associated proteins are not yet understood. Several granule associated proteins such as GroEL, EF-Tu, PhaA, PhaB, PhaP1, PhaP3, PhaP4, heat-shock proteins and few unknown proteins were reported on the proteome analysis of granule associated proteins in recombinant *E. coli* (Mee-Jung *et al*., 2006). Seven enzymatic activities were identified in isolated PHA granules from *P. putida*: other than PHA synthase and PHA depolymerase, acyl-CoA synthetase, acyl-CoA dehydrogenase, enoyl-CoA hydratase, hydroxyacyl-CoA reductase and ketoacyl-CoA reductase (Fuller *et al*., 1992; Stuart *et al*., 1996; de Eugenio *et al*., 2007). Proteins isolated from native PHB granules of *R. eutropha* were subjected to proteome analysis by trypsin digestion followed by LC-MS/MS (Jendrossek and Pfeiffer, 2014). More than 400 different polypeptides were identified. Besides well-known PHB granule associated proteins (PhaC, PhaZ1, PhaPs and transcriptional regulators), they also found several outer membrane proteins, soluble proteins and proteins of the tricarboxylic acid cycle.

1.6 Industrial PHA production

Polyhydroxyalkanotes (PHAs) are a group of biopolyesters accumulated by many prokaryotic microorganisms as a carbon and energy storage material (Madison and Huisman, 1999). Beijerinck (1888) first observed granule like structure inside *Rhizobium* cells as extremely refractile globules. Lemoigne (1923) noticed the production of an unknown acid substance in cells *Bacillus subtilis*. Later this acid was

identified as monomer of poly-β-hydroxybutyric acid (Lemoigne, 1926). Lemoigne *et al*. (1950) noticed different amount of accumulation of PHB in *Bacillus megaterium* by changing the type of growth medium. The first functional P(3HB) biosynthetic pathway was proposed by Macrae and Wilkinson (1958). They observed that *Bacillus megaterium* accumulates the polymer especially when the ratio of glucose to nitrogen in the growth medium was high and subsequently degrades the accumulated polymer with the depletion of carbon source in the medium.

The potential usefulness of PHAs was recognised since the first half of the 1960s, when the patents related to P(3HB) production process (Baptist, 1963), polymer extraction from biomass (Baptist, 1962a), plasticization with additives (Baptist, 1962b) and use of pure polymer for absorbable prosthetic devices were started (Grace and Co 1963). In between 1970 and 1990, many hydroxyalkanotes (HAs) monomers have been identified. Wallen and Rohwedder (1974) reported the presence of 3HB, 3-hydroxyvalerate (3HV), 3-hydroxyhexanoate (3HHx), and 3-hydroxyheptanoate (3HHp) monomers among the polymer extracted from activated sewage sludge. Findlay and White (1983) identified 3HB, 3HV and 3HHp in polymer extracted from *B. megaterium*. Huisman *et al*. (1989) reported the production of 3-hydroxyoctanoate (3HO) and 3HHx monomers when *Pseudomonas oleovorans* was fed with *n*-octane. The commercial scale production of PHA was begun in the year 1980 by the UK chemical group, Imperial Chemical Industry (ICI); this unit is currently known as Zeneca Bioproducts. The company produced copolymer P(3HB-co-3HV) with the trade name Biopol® by fed-batch cultivation of a mutant strain of *R. eutropha* H16 using glucose and propionic acid as sources of carbon (Lee and Chang, 1995). A number of companies globally engaged in PHA research and development as well as production are listed in the Table 1.3. Few companies have stopped their PHA production in 1990s but many of them have plans to increase their production rate in the future. Chemie Linz, (Austria), produced PHB 1,000 kg per week in a 15,000 L fermentor using *Alcaligenes latus* DSM 1124 grown in a mineral medium containing sucrose as carbon source (Hrabak, 1992). In 1995, Copersucar, (Brazil) build a pilot-scale plant to produce PHB enough to supply the market for tests and trials. This company managed to produce 120–150 g/L DCW containing 65–70% PHB with a PHB productivity of 1.44 g/L/h and a PHB yield of 3.1 g sucrose per 1 g of PHB. In 1996, Zeneca sold its Biopol® business to Monsanto Co. USA but continued PHA

production by using genetically modified crops. Monsanto produced Biopol® commercially with 20% HV contents by bacterial fermentation. However, at the end of 1999 Monsanto ceased commercial PHA production and sold its Biopol® assets to Metabolix Inc. USA (Braunegg, 2003). The Institute of Microbiology, Chinese Academy of Sciences and Tianjin Northern Food, China as a joint program started producing PHB. *R. eutropha* was grown in a 1,000 L fermenter containing glucose mineral medium. After 48 h of fermentation they obtained 160 g/L DCW and 128 g/L PHB (Chen, 2010). At the same time, Lantian Group (Jiangsu, China) used recombinant *E. coli* and produced PHB amounts to 168 g/L DCW in a 10,000 L fermentor (Yu *et al*., 2003). Akiyama *et al*. (2003) demonstrated large-scale poly-3-hydroxybutyrate-co-3-hydroxyhexanoate P(3HB-co-3HHx) production by a recombinant *Wautersia eutropha* from soybean oil. The annual production of 5,000 tons of P(3HB-*co*-5 mol% 3HHx) is estimated to cost from US $3.5 to 4.5 kg^{-1}, depending on the presumed production performances. Similar-scale production of PHB from glucose is estimated to cost US $3.8–4.2 kg^{-1}.

In 2006, Metabolix a joint venture with Archer Daniels Midland (ADM) Chicago, (USA) started producing PHA by fermentation using commercial grade corn sugar in a 50,000L fermenter under the trade name Mirel™ (Gilliland, 2006). ADM alone began to build its first plant in Clinton, Lowa (USA) in 2009, with a production capacity of 50,000 tons per year. Meanwhile, Metabolix started investigation on PHA production using genetically modified crops. In 2009, the company announced having completed a field trial of tobacco, genetically engineered to produce PHA. This company has also announced that, in greenhouse trials, switchgrass plants engineered to produce PHA. Meredian, Inc. is the world's largest PHA producer, with a production capacity of 300,000 tons of PHA per year (www.meredianinc.com).

At present the PHA produced by various industries with their technological advancement and production capacity, could not match economically with the production cost of synthetic plastics which is < 1 US$ per kg. PHA finds applications in the market such as, packaging, food services, biomedical and agricultural industries. Biomedical application offers the highest growth of industrial PHAs produced today. In 2003, the market price of PHA was roughly between 10 and 20 € per kg (Jacquel *et al*., 2008). In 2010, this price was reduced to 1.5 – 5 € per kg

(Chanprateep, 2010). According to the report by MarketsandMarkets (www.marketsandmarkets.com) the global PHA market consumption will grow from an estimated 10,000 MT in 2013 to 34,000 MT by 2018, with a compound annual growth rate (CAGR) of 27.7%. The higher market price of PHAs is mainly due to cost of raw material, processing cost and the small production quantities.

There is a scope for improvement of the current technology for the whole process of PHA production from the start to the final step. It includes isolation of new bacterial strain accumulating higher amount of PHA with less incubation time, utilization of cheaper carbon substrates for higher production, optimization of fermentation strategies for high cell density PHA production and strain improvement by characterizing the proteins associated with PHA granules (Amache *et al.*, 2013).

Company	Country	Brand	Type of PHA	Production capacity (metric tons/year)
Biomer[1]	Germany	Biomer	PHB, PHBV	Pilot scale
Bio-on[2]	Italy	Minerv-PHA	PHB, PHBV	1,000
Kaneka[1]	Japan	Kaneka	PHBHx	Pilot/Industrial scale
ADM with Metabolix[3]	USA	Mirel	Unknown	5,000
Meredian, Inc.[4]	USA	-	Several PHA	1,500
Metabolix[2]	USA	Mirel	Several PHA	5,000
Mitsubishi Gas Chemicals[2]	Japan	Biogreen	PHB	5
PHB Industrial S/A[2]	Brazil	Biocycle	PHB, PHBV	5
Shenzen O'Bioer[2]	China	-	Several PHA	Unknown
TEPHA[2]	USA	ThephaFLEX/ ThephELAST	Several PHA	Unknown
Tianan Biological Materials[2]	China	Enmat	PHBV	200
Tianjin Green Biosciences[2]	China	Green Bio	P3HB4HB	1,000
Yikeman Shandong[3]	China	-	PHA (unclear)	300
Zhejiang Tian An[3]	China	-	PHBV	200
Biocycle[3]	Brazil	Biocycle	PHB	10
Shandong Lukang[3]	China	-	Several PHA	Pilot scale
Jiangsu Nantian[3]	China	-	PHB	0.5

Table 1.3: Globally companies engaged in PHA production and their production capacity.

1: Averous and Pollet, (2012); 2: Babu *et al.*, (2013); 3: Chen, (2009) and 4: Meredian 2014.

1.7 Strategies for improved PHA production

1.7.1 Bacterial strains

For a cost effective PHA production, the first step is the utilization of highly robust production strains. The importance of bacterial species for the industrial production of PHA varies depending on factors that includes utilization of an inexpensive carbon

source (agricultural wastes and industrial by-products), the cost of the fermentation broth, the growth rate, the rate of PHA accumulation, the polymer types and the cost of downstream processes (Lee and Choi, 2001). Although there are more than 300 bacterial species were known for accumulating PHA (Hazer and Steinbuchel, 2007; Chanprateep, 2010), only a few bacterial strains, such as *Cupriavidusnecator* (*Ralstonia eutropha*), *Alcaligenes latus*, *Azotobacter vinelandii*, *Aeromonas hydrophilia*, *Rhodopseudomonas palustris*, *Burkholderia sacchari*, *Pseudomonas oleovorans*, *Pseudomonas fluorescens*, *Paracoccus denitrificans*, *Protomonas extorquens*, *Halomonas boliviensis*, *Bacillus megaterium, Zobellella denitrificans* and recombinant *E. coli*, are studied for large-scale PHA production (Verlinden *et al*., 2007; Jiang *et al*., 2008; Ibrahim and Steinbuchel, 2009; Kanjanachumpol *et al*., 2013).

Cupriavidus necator is the most extensively studied and first bacterial strain used in the industry for PHA production by the UK chemical group, Imperial Chemical Industries (ICI) (Byrom, 1990). Metabolix, Inc. developed recombinant *E. coli* for production of PHB (Lee *et al*., 2004). So far PHB produced in the industry is mainly by Gram-negative bacteria such as *Cupriavidus necator*, *Alcaligenes latus*, *Pseudomonas oleovorans* and recombinant strains of *E. coli* (Lopez *et al*., 2012; Faccin *et al*., 2013).

1.7.2 Carbon substrates

The carbon source contributes as much as 40-50 % of the overall PHA production cost. Many authors reported the importance of the selection of suitable carbon sources for maximum PHA accumulation, on which the production cost could be reduced for commercialization of the polymer (Lee *et al*., 1990; Lee and Chang, 1995; Tsuge, 2002; Lenz and Merchessault, 2005). Plenty of renewable carbon sources are available around the globe, which can be used for PHA production. However, the selection of carbon sources should not focus only on low cost but also on its easy availability near the production area is very important. Many wastes of agricultural and industrial by-products such as molasses, starch based waste, whey, fats and oils and glycerol have been considered as potential carbon substrates for industrial PHA production (Santimano *et al*., 2009; Du *et al*., 2012). Use of these waste carbon

substrates for PHA production will reduce the raw material cost and simultaneously save energy as well as its disposal cost.

1.7.2.I Molasses

Molasses is a sugar-rich by-product generated during sugar manufacturing, it has been widely used as a carbon substrate in commercial fermentation due to its low cost, abundance and it contains different vitamins and trace elements, which promotes microbial growth (Zhang *et al*., 1994; Bengtsson *et al*., 2010). There are three types of molasses that have been tested for PHA production. Page (1992) first time tested sugar beet molasses as a carbon substrate for PHB production by *Azotobacter vinelandii* UWD. He achieved PHB concentration of 19 to 22 g/L with productivity of 0.50 to 0.55 g/L/h. Chen and Page (1997) improved the process for PHB production with a two-stage fermentation strategy using the above mentioned strain. In which, they reported PHB concentration 32 g/L at a productivity of 1 g/L/h. Omar *et al*. (2001) reported PHB production in *Bacillus megaterium* using sugar beet molasses. After 48 h of cultivation 3.7 g/L of DCW was obtained containing 50% w/w PHB. Mercan and Beyatli (2005) demonstrated PHB production in *Rhizobium meliloti* using sugar beet molasses as carbon source and reported 56% w/w of the dry cell weight as PHB. Yilmaz and Beyatli (2005) reported PHB production in *Bacillus cereus* M5 using sugar beet molasses. They obtained 74% w/w of the dry cell weight as PHB. Wu *et al*. (2001) used sugar cane molasses for PHB production by *Bacillus* sp. JMa5. After fed-batch cultivation 70 g/L DCW was obtained containing 25 to 35% w/w PHB. Chaijamrus and Udpuay (2008) studied PHB production using sugar cane molasses by *Bacillus megaterium* ATCC 6748. A maximum dry cell weight (DCW) of 5 g/L containing 43% w/w of PHB was obtained. Liu *et al*. (1998), demonstrated PHB production using sugar cane molasses by recombinant *E. coli* and reported production of DCW 39.5 g/L containing 80% PHB with productivity of 1 g/L/h. Jiang *et al*. (2008) in a fed-batch cultivation of *Pseudomonas fluorescens* A2a5 used sugarcane molasses for PHB production and reported DCW 32 g/L with PHB 22 g/L. Santimano *et al*. (2009) studied PHB production in *Bacillus* sp. Col1/A6 using sugar cane molasses. They obtained 3.3 g/L of DCW containing 55% w/w PHB. In fed-batch cultivation, *Bacillus megaterium* BA-019 was tested for PHB production using sugar cane molasses as carbon source (Kulpreecha *et al*., 2009). After 24 h of

cultivation 72.6 g/L of DCW and 30.52 g/L PHB was obtained with an overall PHB productivity of 1.27 g/L/h. The above mentioned strain was used for high cell density fed-batch cultivation using sugar cane molasses (Kanjanachumpol *et al*., 2013). In which 90.7 g/L of PHB and 41.6 g/L of DCW was obtained after 24 h of cultivation with a final PHB productivity of 1.73 g/L/h. Besides beet molasses and cane molasses, PHA production was reported from soy molasses (Solaiman *et al*., 2006a). In which 1.5-3.6 g/L DCW containing 5-17% of mcl-PHA was produced by using *Pseudomonas corrugate*.

1.7.2.II Starch based waste

Starch based wastes are present in wastewaters from industries such as paper, beverages, food processing and fermentation. These wastes are easily utilized by various bacterial species for accumulation of PHA. *Alcaligenes eutrophus* DSM 545 showed utilization of potato waste hydrolyzed by barley-malt and produced 76.9% of DCW as PHA (Rusendi and Sheppard, 1995). Kimand Chang (1998) investigated fed-batch cultivation of *Azotobacter chroococcum* for PHB production using starch. Fed-batch cultivation with oxygen limitation, 54 g/L DCW containing 46% w/w PHB was obtained and without oxygen limitation, 71 g/L DCW containing 20% w/w PHB was obtained. Huang *et al*. (2006) demonstrated PHA production by *Haloferax mediterranei* using extruded corn starch as the major carbon source, 62.6 g/L of DCW and 24.2 g/L of PHA were obtained. Halami (2008) used *Bacillus cereus* for PHA production by utilizing starch. He found that the isolate could secrete the enzyme amylase for hydrolysis of starch and simultaneously produce PHB with productivity of 0.48 g/L/h. *Ralstonia eutropha* NCIMB 11599 utilizes saccharified potato starch waste for PHB production (Haas *et al*., 2008). The DCW and PHB produced were 179 g/L and 94 g/L, respectively.

1.7.2.III Whey

Whey is the main by-product of dairy industry. It is produced during the conversion of milk to cheese and casein. It is rich in lactose, proteins, lipids and lactic acids (Yang *et al*., 1994). The global whey production in 2012 is more than 24 million tons (http://www.dairyco.org.uk/market-information/processing-trade/dairy-product-

production/world-dairy-product-production, Dated: 11/26/2014). The annual whey formation is more than 40 million tons in the European Union (Koller *et al*., 2008). Young *et al*. (1994) reported, for the first time, the production of PHB using whey lactose in the *Burkholderia cepacia*. Lee *et al*. (1997) demonstrated PHB production using whey with recombinant *E. coli* GCSC4401 and GCSC6576 strains. These strains express the PHB biosynthesis genes of *R. eutropha*. Recombinant *E. coli* GCSC6576 showed 87 g/L DCW with 69 g/L PHB accumulation in fed-batch cultivation after 47 h incubation (Wong and Lee, 1998). Ahn *et al*. (2001) in a fed-batch cultivation of recombinant *E. coli* GCSC4401 used whey solution for PHB production and reported 119.5 g/L DCW with 96.2 g/L PHB in 37.5 h. In the same year Ahn *et al*. (2001) tried fed-batch cultivation with cell recycling to improve the PHB yield. They achieved 194 g/L DCW with 168 g/L PHB in 36.5 h. Park *et al*. (2002) used the same system for scale-up in a 30 L and a 300 L fermentor, in which they achieved 51 g/L DCW containing 70% PHB and 30 g/L DCW containing 67% PHB respectively.

Besides recombinant *E. coli*, other potential PHA accumulating bacteria recombinant as well as wild strains were exploited for PHA production from whey. These are *Ralstonia eutropha* DSM545 (Marangoni *et al*., 2002), *Pseudomonas hydrogenovora* (Koller *et al*., 2008), *Thermus thermophilus* HB8 (PantaZaki *et al*., 2009), *Methylobacterium sp*. ZP24 (Yellore and Desai, 1998; Nath *et al*., 2008) and *Hydrogenophaga pseudoflava* DSM1034 (Koller *et al*., 2011). However, PHA accumulations were less than 10 g/L, which was significantly lower than the PHA accumulation by recombinant *E. coli*.

1.7.2.IV Fats and oils

In 1990s, triacylglycerydise (TAG) and its derived fatty acids attracted industrial PHA production greatly due to its renewability and low cost. For MCL-PHA production TAGs could be a valuable alternative carbon source, as the constituents of PHA under these conditions would be directly derived from fatty acids of TAGs (Ashby and Foglia, 1998; Solaiman *et al*., 2006). Shimamura *et al*.(1994) were the first to demonstrate PHA production using *Aeromonas caviae* directly from TAG. *Pseudomonas resinovorans* showed PHA accumulation up to 15% of its DCW from

tallow (Cromwick *et al*., 1996). Further, Ashby and Foglia (1998) investigated MCL-PHA production using *Pseudomonas resinovorans* from a whole range of TAGs, such as lard, butter oil, olive oil, coconut oil and soybean oil. They reported accumulation of 1.2 to 1.9 g/L PHA having monomers of 4 to 14 carbon atoms. Also, they observed the kind of monomer incorporated in PHB had a strong relationship with the type of substrate used. When coconut oil containing high levels of saturated fat was used, saturated monomers were incorporated in PHA. In contrast when soybean oil containing high levels of unsaturated fat was used, unsaturated monomers were produced and incorporated in PHA. In the last decade, many researchers have carried out PHA production using wild and genetically modified strains with different TAGs such as, palm oil (Wu et al., 2009), olive oil (Ntaikou *et al*., 2009), corn oil (Chaudhry *et al*., 2011), coconut oil (Thakor *et al*., 2005), soy bean oil (He *et al*., 1998; Kahar *et al*., 2004), other vegetable oils and animal fats (Du *et al*., 2012). In most of these studies, PHA accumulation was reported less than 10 g/L. However, Kahar *et al*. (2004) reported 138 g/L DCW containing 71-74% (w/w) P(3HB-co-3HHx) using soybean oil as the carbon substrate.

1.7.2.V Glycerol

Glycerol is the main by-product of biodiesel industry. It is produced with about 10% (v/v) of the volume of biodiesel. In 2009, the biodiesel production in Europe was increased, which resulted in huge increase in production of glycerol. This resulted in drop of price of glycerol in the world market. The availability and low market price makes glycerol a promising carbon substrate for industrial microbiology (da Silva *et al*., 2009). Borman and Roth (1999) produced PHB by *Methylobacterium rhodesianum* using glycerol and casein peptone as carbon and nitrogen sources, respectively and reported accumulation of 10.5 g/L. Borman and Roth (1999) used the same experimental setup for *R. eutropha* DSM 11348 and achieved 15 to 17.6 g/L PHB. Ashby *et al*. (2004) used crude glycerol for PHA production by *P. oleovorans* NRRL B-14682 and *P. corrugate* 388. In case of *P. oleovorans* the molecular weight of PHA decreased with increasing concentration of crude glycerol in the medium but similar effect was not observed with the *P. corrugate* strain. Ashby *et al*. (2005) attempted PHA productions by *Pseudomonas oleovorans* NRRL B-14682 and *Pseudomonas corrugate* 388 with increasing concentration of glycerol in the

fermentation broth but they have failed to achieve the increased in PHA accumulation. Sujatha and Shenbagarathai (2006) used recombinant *E. coli* harbouring *PhaC1* gene of *Pseudomonas* sp. LDC-5 and reported 3.4 g/L PHA with glycerol and fish peptone derived medium. de Almeida *et al*. (2007) studied the effect of PhaP in recombinant *E. coli*, on cell growth and PHB accumulation using glycerol and noted maximum 7.9 g/L PHB in 48 h. Cavalheiro *et al*. (2009) reported 68.8 g/L DCW containing 38% PHB with a productivity of 0.84 g/L/h by cultivating *C. necator* DSM 545 on waste glycerol. Further, earlier nitrogen limitation in the fermentation broth enhanced PHB accumulation, which resulted increased of productivity to 1.1 g/L/h with 50% of DCW as PHB in *C. necator*.

1.8 Fermentation strategies for high-cell-density PHA production

The fermentation strategy for bacterial PHA production depends on various factors such as, carbon source, type of bacterial strain, fermentation parameters, mode of fermentation (batch, fed-batch and continuous), PHA synthesis phase (growth associated or non-growth associated) and rate of PHA accumulation (Byrom, 1992). Batch fermentation is a popular method for PHA production due to low operation cost and easy to handle. However, this process is associated with low PHA productivity. After utilization of available carbon source in the growth medium, bacteria depolymerises the accumulated PHA for carbon and energy source resulted in decrease of PHA concentration (Zinn *et al*., 2001). The main goal behind the development of fermentation strategies is to maximize the product concentration in the growth medium within short incubation time. Since PHA accumulated intracellular high cell densities are a requirement for high productivities. Continuous and fed-batch cultivation methods are the main operation modes for high cell density cultivation (HCDC) of bacterial cells. Bacteria accumulate PHA under limitation of an essential nutrient (nitrogen, phosphorus, oxygen, etc.) with excess of carbon source. Accumulated PHA is directly related to the residual biomass (Xr) present in the production medium. Hence, processes with high cell densities (higher Xr concentration) are desired because they favour PHA production, especially in terms of reduction of the culture volume, reduction of residual liquids, lower production costs, and lower investment in equipment compared to the low-cell-density processes (Ienczak *et al*., 2011; Cavalheiro *et al*., 2012). Currently, fed-batch and continuous

cultivations are the main cultivation methods being tried to achieve high cell density PHA production.

1.8.1 Fed-batch cultivation

Fed-batch cultivations have been widely attempted for high cell density PHB production. In fed-batch, bacterial cells are grown in batch mode for some time and then the fermentor is fed with concentrated substrate solutions without removal of the culture broth, until the desired volume is achieved in the fermentor. Depending on the bacterial strain two fed-batch cultivation processes are tested for PHA production. The most commonly attempted method is a two-phase fermentation process consisting of a growth phase, in which a high cell density is achieved under favourable growth conditions, followed by PHA accumulation phase, where an essential nutrient is limited in the growth medium to enhance PHA accumulation (Ryu *et al*., 1997; Grousseau *et al*., 2013). During two phase fermentation, both growth phase and PHA accumulation phase need to be balanced in order to obtain higher productivity. The second cultivation mode consists of a single-phase during which cell growth and PHA accumulation occur simultaneously (Yamane *et al*., 1996). Fed-batch cultivation methods investigated for high cell density PHA production by different bacterial strains have been summarized in Table 1.4.

Kim *et al*. (1994) suggested that in production phase to keep the glucose concentrations between 10 and 25 g/L is crucial for obtaining high productivity. Ryu *et al*. (1997) reported the importance of glucose feeding in fed-batch cultivation of *C. necator* to obtain high cell density and high PHB productivity. The glucose feeding was monitored on-line to maintain the carbon concentration between 0 and 20 g/L in the fermentor. The best results for total biomass, residual biomass, PHB content and productivity were 221g/L, 42 g/L, 81% and 3.75 g/L/h, respectively. Shang *et al*. (2003) maintained residual glucose concentrations of 2.5, 9, 16 and 40 g/L in separate cultivations to obtain high cell density PHB production with *C. necator*. The residual glucose concentration 9 g/L was showed the best results, where 208 g/L of DCW and 139 g/L of PHB was obtained with a PHB productivity of 3.1 g/L/h. Ienczak *et al*. (2011) shown that the importance of maintaining carbon source concentration (around 10 g/L) in the production phase to obtain high productivity in *C. necator*. Cavalheiro

et al. (2012) demonstrated HCDC for P(3HB-co-4HB) and P(3HB-4HB-3HV) production by *C. necator* using fed-batch cultivation. Along with glycerol the fermentation broth was supplemented with γ-butyrolactone (GBL) and propionic acid (PA). The best results obtained were PHA content 36.9%, PHA concentration 16.7 g/L, 4HB content of 43.6% and 3HV content 6.0%. Total biomass concentration reached to 45 g/L at 64.3 h.

Yamane *et al*. (1996) with a fed-batch cultivation using *Alcaligenes latus* showed high-cell-density PHB production from sucrose. Biomass of 143 g/L, residual biomass of 71.6 g/L and PHB productivity of 3.97 g/L/h were obtained by feeding the fermentor with nutrient solutions to maintain a constant nutrient concentration throughout the fermentation. Lee *et al*. (2000) reported high-cell-density MCL-PHA production by *Pseudomonas putida* from oleic acid under phosphorus limitation. A total biomass of 141 g/L, residual biomass of 69.1 g/L, and productivity of 1.9 g/L/h were achieved. *Burkholderia sacchari* shows growth associated as well as non-growth associated PHA accumulation. Rocha *et al*. (2008) carried out P(3HB-co-3HV) production by fed-batch cultivation of *B. sacchari* IPT 189 using different ratio of sucrose to propionic acid (PA). At 250 g/L of sucrose and in high sucrose/PA ratio, total biomass of 221 g/L containing 45% of PHA with productivity of 1.04 g/L/h were achieved. Pradella *et al*. (2010) performed high-cell-density PHB production from *B. sacchari* IPT 189 under nitrogen limiting conditions. They achieved total biomass of 150 g/L with PHB production of 63 g/L.

Kulpreecha *et al*. (2009) studied high-cell-density PHB production using *Bacillus megaterium*. In fed-batch cultivation the fermentor was fed with sugar cane molasses and urea in a ratio of 10:1 throughout fermentation. At the end of the fermentation total biomass of 72.6 g/L, residual biomass of 42.1 g/L and PHB productivity of 1.27 g/L/h were achieved. Kanjanachumpol *et al*. (2013) reported high-cell-density PHB production using *B. megaterium*. In this study concentrations of sugar cane molasses and urea were maintained in a ratio of 12.5:1 throughout fermentation that resulted in improved biomass and PHB production. The final results obtained were biomass of 90.71 g/L, residual biomass of 49.1 g/L and PHB productivity of 1.73 g/L/h.

Bacteria	Carbon source	PHAs	Xt (g/L)	PHA (%)	PHA Productivity (g/L/h)	References
Alcaligenes latus	Sucrose	P(3HB)	143	50	3.97	Yamane *et al.*, 1996
Alcaligenes eutrophus	Tapioca	P(3HB)	106	58	1.04	Kim *et al.*, 1995
Aeromonas hydrophila	Glucose/lauric acid	P(3HB-co-3HV)	50	50	0.54	Chen *et al.*, 2001
Bacillus sacchari	Sucrose/PA	P(3HB-co-3HV)	221	45	1.04	Rocha *et al.*, 2008
Bacillus megaterium	Sugar cane molasses	P(3HB)	72.6	42.1	1.27	Kulpreecha *et al.*, 2009
Bacillus megaterium	Sugar cane molasses	P(3HB)	73	43	1.3	Kanjanachumpol *et al.*, 2013
Bacillus megaterium	Sugar cane molasses	P(3HB)	90.7	46	1.73	Kanjanachumpol *et al.*, 2013
Cupriavidus necator	Waste glycerol/GBL	P(3HB-co-4HB)	30	36.1	0.17	Cavalheiro *et al.*, 2012
Cupriavidus necator	Waste glycerol/GBL/PA	P(3HB-4HB-3HV)	45	36.9	0.25	Cavalheiro *et al.*, 2012
Cupriavidus necator	Glucose	P(3HB)	221	81	3.75	Ryu *et al.*, 1997
Cupriavidus necator	Glucose/fructose	P(3HB)	40	68	0.45	Ienczak *et al.*, 2011
Cupriavidus necator	Glucose	P(3HB)	208	67	3.1	Shang *et al.*, 2003
Pseudomonas putida	Glucose/nonanoic acid	MCL-PHA	56	66.9	1.44	Sun *et al.*, 2007
Pseudomonas putida	Oleic acid	MCL-PHA	141	51	1.91	Lee *et al.*, 2000
Pseudomonas putida	Glucose	MCL-PHA	61.8	67.1	0.83	Poblete-Castro *et al.*, 2014
Recombinant *E. coli*	Beet molasses	P(3HB)	39.5	80	1.00	Liu *et al.*, 1998
Zobellella denitrificans	Glycerol	P(3HB)	81.2	67	1.09	Ibrahim and Steinbuchel, 2009

Table 1.4: Fed-batch cultivation methods investigated for high cell density PHA production by different bacterial strains

1.8.2 Continuous cultivation

A continuous cultivation method is of great commercial importance due to its high productivities, more importantly for strains with high maximum specific growth rates. The continuous process is characterized by continuously feeding and removing the fermentation broth at a given constant flow to maintain the working volume.

Ramsay *et al*. (1990) showed high cell density PHB and P(3HB-co-3HV) production in single stage continuous cultivation by *C. necator* and two-stage continuous cultivation by *A. latus*, respectively. In the single stage continuous cultivation, *C. necator* accumulated 33% of the DCW as PHB when fed with nitrogen limiting mineral medium containing glucose as carbon source. Similarly in two-stage continuous cultures, *A. latus* accumulated 58% of DCW as P(3HB-co-3HV) when fed with sucrose and propionic or valeric acid. Du *et al*. (2001) demonstrated PHB production in a two-stage continuous culture by *C. necator* using fructose as carbon source. In this process total biomass of 50 g/L containing 73% PHB and residual biomass of 13.5 g/L with productivity of 1.25 g/L/h were obtained. Khanna and Srivastava (2008) tested a two-stage continuous culture of *C. necator* using fructose as carbon source. They achieved biomass of 20 g/L and PHB of 1.5 g/L. Atlic *et al*. (2011) in a five-stage continuous process produced PHA by *C. necator* using glucose as carbon source. The results obtained were residual biomass of 18.6 g/L with PHB

productivity of 1.85 g/L/h. Tan *et al*. (2011) with an unsterile two-stage continuous process produced PHB from glucose using *Halomonas* TD01, a halophilic strain. The total biomass of 20 g/L containing 52% of PHA was obtained.

1.9 *Bacillus megaterium* and PHA production

Bacillus megaterium is a Gram-positive, aerobic, spore forming bacterium present in diverse habitats from terrestrial to marine sediments. This "big beast" has been identified as an experimental organism for studies on various cell structures and functions (Hrafnsdottir *et al*., 1997; McCool and Cannon, 2001). Polyhydroxybutyrate is the first PHA discovered in *B. megaterium* by Lemoigne (1926). Since this discovery many prokaryotic microorganisms are reported for PHA accumulation and these polymers gained the interest for industrial production due to its biodegradability and similar properties with synthetic plastic. So far PHA produced in the industry is mainly by Gram-negative bacteria such as *Cupriavidus necator*, *Alcaligenes latus*, *Pseudomonas oleovorans* and recombinant strains of *Escherichia coli* (Lopez *et al*., 2012; Faccin *et al*., 2013). These bacteria contain pyrogenic lipopolysaccharide (LPS) endotoxin and co-purify with PHB during extraction. The presence of LPS limits the biomedical application of PHB produced by these species (Santimano *et al*., 2009).

Bacillus megaterium do not contain LPS. In addition to this, strains of this bacterium are known for rapid growth, use diverse cheaper carbon substrates, shows resistance to high osmotic pressure and are capable of accumulating homopolymer and co-polymer in the presence of a single carbon source or cheaper carbon sources (Otari and Ghosh, 2009; Reddy *et al*., 2009; Santimano *et al*., 2009). This make *Bacillus megaterium* an ideal candidate for industrial PHB production. The list of different strains of *B. megaterium* reported for PHA accumulation using various carbon sources are summarized in the Table.1.5. This bacterium could accumulate PHA in between 20 and 70% of their dry cell weight depending on the culture condition and carbon source they used (Rodriguez-Contreras *et al*., 2013).

Lemoigne *et al*. (1950) for the first time produced PHB with varying concentrations by growing *B. megaterium* on different media and showed that accumulated PHB could act as sole source of carbon and energy for growth of the organism in the

absence of carbon source in the growth medium. Macrae and Wilkinson (1958) showed that when the glucose concentration of the growth medium is increased more polymers are produced and in the later stages of growth the depletion of the polymer was observed. *B. megaterium* has been reported for PHA production using different carbon substrates such as glucose, fructose, sucrose, lactose, sodium succinate, sodium acetate, starch, glycerol, beet molasses, date syrup, corn steep liquor, sugarcane molasses, wafer residue, citrus pulp and dairy waste (Santimano *et al.*, 2009). Due to low productivity, the industrial PHB production using this bacterium is still limiting.

As batch and fed-batch cultivation has been the most popular and effective cultivation methods to achieve high cell density PHA production, in the recent past many attempts are made on optimization of these methods using *B. megaterium*. Omar *et al.* (2001) using date syrup showed PHB production in *B. megaterium.* The best results obtained were 3.4 g/L of total biomass containing 25% of PHB. Gouda *et al.* (2001) used sugarcane molasses as carbon source for PHB production and reported 3.6 g/L of total biomass and 2.2 g/L of PHB. Sabra and Abou-Zeid (2008) have reported 0.19 g/g/h specific PHB productivity in fed-batch compare to 0.09 g/g/h in batch and demonstrated that cell could accumulate 65% PHB production under optimized fed-batch cultivation. Kulpreecha *et al.* (2009) tested batch and fed-batch cultivation using sugarcane molasses. Twelve hours of cultivation gave 8.78 g/L DCW and 5.41 g/l PHB in batch while at 24 h of cultivation gave 72.6 g/L biomass with 30.5 g/L PHB in fed-batch. Pandian *et al.* (2010) using fed batch cultivation with dairy waste showed 11.32 g/L PHB production at 36 h of incubation period. Naranjo *et al.* (2013) using batch cultivation with glycerol showed 7.7 g/L DCW and 4.8 g/L PHB production at 48 h of incubation. Rodriguez-Contreras *et al.* (2013) in a fed-batch cultivation showed PHB production using glucose as carbon source, where they obtained 70% of the DCW as PHB with a productivity of 0.25 g/L/h. The authors concluded that PHB accumulation occurred under nitrogen limiting conditions and reached to higher polymer concentration without spore formation. Kanjanachumpol *et al.* (2013) have demonstrated a fed batch cultivation method for high cell density and PHB production using sugarcane molasses. They achieved 90.7 g/l DCW and 41.6 g/l PHB at 24 h of cultivation with a productivity of 1.73 g/L/h, which is the highest cell density and PHB production, reported so far using *B. megaterium*.

Bacterial strain	Substrate	Culture strategy	PHA (%)	References
B. megaterium BA-019	Molasses	Fed-batch	42.1	Kulpreecha *et al.* 2009
B. megaterium BA-019	Molasses	Fed-batch	43	Kanjanachumpol *et al.* 2013
B. megaterium BA-019	Molasses	Fed-batch	46	Kanjanachumpol *et al.* 2013
B. megaterium BA-019	Molasses	Batch	61.62	Kulpreecha *et al.* 2009
B. megaterium BA-019	Molasses	Batch	27	Kanjanachumpol *et al.* 2013
B. megaterium DSM 32	Sucrose	Batch	62	Faccin *et al.*, 2013
B. megaterium NQ-11/A2	Glucose	Shake flask	61	Prabhu *et al.*, 2010
B. megaterium Col1/A6	Glucose	Shake flask	65.25	Santimano *et al.*, 2009
B. megaterium Col1/A6	Wafer residue	Shake flask	62.41	Santimano *et al.*, 2009
B. megaterium Col1/A6	Molasses	Shake flask	54.68	Santimano *et al.*, 2009
B. megaterium Col1/A6	Citrus pulp	Shake flask	47.5	Santimano *et al.*, 2009
B. megaterium	Glucose	Batch	59.1	Naranjo *et al.*, 2013
B. megaterium	Glycerol	Batch	62.4	Naranjo *et al.*, 2013
B. megaterium NCIM 2475	Sucrose	Shake flask	-	Otari and Ghosh, 2009
B. megaterium SW1-2	Lactose	Shake flask	15	Berekaa and Thawadi, 2012
B. megaterium SW1-2	Glucose	Shake flask	36	Berekaa and Thawadi, 2012
B. megaterium SW1-2	Sodium acetate	Shake flask	28	Berekaa and Thawadi, 2012
B. megaterium	Glucose	Shake flask	41.95	Krueger *et al.*, 2012
B. megaterium	Starch	Shake flask	30.45	Krueger *et al.*, 2012
B. megaterium	Molasses	Batch	46.2	Gouda *et al.*, 2001
B. megaterium	Glucose/fructose	Shake flask	49	Bora, 2013

Table 1.5: Strains of *Bacillus megaterium* reported for PHA accumulation using various carbon sources.

Bacterial strain	Substrate	Culture strategy	PHA (%)	References
B. megaterium DSMZ 90	Sucrose	Fed-batch	65	Sabra and Abou-Zeid, 2008
B. megaterium BBST4	Glucose	Batch	59	Lopez *et al.*, 2012
B. megaterium BBST4	Glucose	Fed-batch	29	Lopez *et al.*, 2012
B. megaterium BBST4	Glycerol	Batch	60	Lopez *et al.*, 2012
B. megaterium SRKP-3	Dairy waste	Shake flask	-	Pandian *et al.*, 2010
B. megaterium OU303A	Glucose	Shake flask	62.43	Reddy *et al.*, 2009
B. megaterium OU303A	Glycerol	Shake flask	58.63	Reddy *et al.*, 2009
B. megaterium Ou303A	Sodium acetate	Shake flask	50.75	Reddy *et al.*, 2009
B. megaterium uyuni S29	Glucose	Shake flask	-	Rodriguez-Contreras *et al.*, 2013
B. megaterium uyuni S29	Glucose	Fed-batch	31	Rodriguez-Contreras *et al.*, 2013
B. megaterium KM	Glucose	Shake flask	40	Macrae and Wilkinson, 1958
B. megaterium P7	Yeast extract peptone	Shake flask	14.04	Yilmaz *et al.*, 2005
B. megaterium	Date syrup	Shake flask	52	Omar *et al.*, 2001
B. megaterium	Molasses	Shake flask	50	Omar *et al.*, 2001
B. megaterium	Date syrup	Batch	25	Omar *et al.*, 2001
B. megaterium ATCC 6748	Molasses and Corn steep liquor	Shake flask	43	Chaijamrus and Udpuay, 2008

Table 1.5 contd: Strains of *Bacillus megaterium* reported for PHA accumulation using various carbon sources.

1.10 Applications of polyhydroxyalkanoates

The problems associated with the use of synthetic plastics are produced from non-renewable petroleum products that are, not biodegradable, extremely persistent and accumulate in the ecosystem, resulting in a significant burden in solid waste management. Due to these problems researchers are forced to find out an alternative source for replacement of synthetic plastics in the market. Among the various types of biodegradable plastics, polyhydroxyalkanoates have shown great potential as a replacement for synthetic plastics. In April 1990, the first commercial PHA came into market in the form of shampoo bottles made by ICI, UK (Weiner, 1997). Considering the current advances, PHAs have found a wide range of applications in industries, bio-medics and agriculture (Lee, 1996; Chen, 2010).

1.10.1 Industrial applications

Packaging and food services are main consumers in global PHA market (www.marketsandmarkets.com). Proctor and Gamble, Biomers, Metabolix and several other companies were engaged in developing PHA materials as packaging films mainly for use as shopping bags, containers, paper coatings, disposable items such as razors, utensils, diapers, feminine hygiene products, shampoo bottles and cups as well as medical surgical garments, upholstery, carpet, packaging, compostable bags and lids or tobs for thermoformed articles, foils, films etc. (Weiner, 1997; Clarinval and Halleux, 2005; Mikova and Chodak, 2006; Philip *et al.*, 2007). Due to piezoelectric nature, PHAs are used to make articles such as pressure sensors for keyboards, stretch and acceleration measuring instruments, shock wave sensors, gas lighters, microphones, loudspeakers, ultrasonic therapy, atomization of liquids etc. (Babel *et al.*, 1990). The poly(hydroxybutyrate-co-hydroxyvalerate) has gas barrier properties, which makes this polymer useful for food packaging and making beverage bottles. It also can be used for coating paper and films (Hocking and Marchessault, 1994). PHAs are also being processed in to toners for printing and adhesives for coating applications (Madison and Huisman, 1999).

The polyhydroxybutyrate-co-polyhydroxyhexanoate (P(HB-co-HHx)) produced by Metabolix, a US-based company, has been approved by the Food and Drug Administration (FDA) for production of food additives (Clarinval and Halleux, 2005).

Composites of PHAs are used to develop electronic products, like mobile phones (NEC Corporation and UNITIKA Ltd. 2006). Zhang *et al*. (2009) for the first time showed that biofuel can be produced from PHA by methyl esterification of 3-hydroxyalkanoates. Since it has low viscosity, can be melted and used for injection molding of thin wall objects (Chen, 2005). High tensile strength PHB fibres were prepared by stretching the fibres after isothermal crystallization near the glass-transition temperature (Tanaka *et al*., 2007). Vogel *et al*. (2007) tried to improve the crystallization of PHB in a melt spinning process by using reactive extrusion with peroxide as a comfortable pathway. They improved the crystallization and finally strong fibres are produced with promising applications. This PHB is used in the industry to make articles such as combs, pens and bullets. PHA latexes have been used for surface coating of paper and as sizing agents in the paper industry (Ariffin *et al*., 2011).

1.10.2 Biomedical applications

PHAs have shown attracted biomedical applications due to its biocompatible and biodegradable nature. Only few PHA, such as PHB, PHBV, P4HB, PHBHHx, and PHO have been used as materials for implants in biomedical, tissue engineering and specific drug delivery (Hrabak, 1992; Chen *et al*., 2001). Since 1990, PHA have been used to manufacture devices such as sutures, sutures fasteners, meniscus repair devices, guided tissue repair or regeneration devices, articular cartilage repair devices, atrial septal defect repair devices, tendon repair devices, repair patches, cardiovascular patches, pericardial patches, rivets, tacks, staples, screws, surgical mesh, orthopaedic pins, stents, slings, bone plates and bone plating systems, bulking and filling agents, vein valves, bone marrow scaffolds, ligament and tendon grafts, spinal fusion cages, ocular cell implants, skin substitutes, dural substitutes, bone graft substitutes, bone dowels and wound dressings (Abe *et al*., 1995; Chen and Wu, 2005; Wang *et al*., 2008a; b; Bian *et al*., 2009; Dai *et al*., 2009). Xiao *et al*. (2007) reported that 3HB and its derivatives have effects on cell apoptosis and the cytosolic Ca2+ concentration of mouse glial cells. TephaFLEX® suture fabricated from P4HB is the first US FDA approved and most well-known product of Tepha, Inc. which also produces surgical meshes and films fabricated from PHA (Brigham and Sinskey, 2012).

PHAs have been gaining interest as drug delivery systems for tissue specific release of therapeutics over a period. Eldridge *et al*. (1990) reported the use of microsphere of PHB for the targeted delivery of formalinized vaccine, to the gut-associated lymphoid tissues. Shishatskaya *et al*. (2008) demonstrated the release of anti-tumor drug rubomycin by using PHB microspheres, which inhibited proliferative activity of Ehrlich's carcinoma in mice. Yao *et al*. (2008) in a receptor-mediated drug delivery system used rhodamine B isothiocyanate (RBITC) model drug by incorporating with P(HB-co-HHx) and associating with a recombinant PhaP phasin protein to target cancer cells or macrophages. PhaP phasins were fused to ligands of human $\alpha 1$ acid glycoprotein (hAGP) or human epidermal growth factor (hEGF) for targeting cancer cells or macrophages, respectively. In vitro testing of PHB microsphere has been carried out for releasing the antibiotics tetracycline and gentamycin (Francis, 2011). Francis (2011) also has been discussed the multifunctional PHB/45S5Bioglass composite system as a drug delivery agent for certain bone tissue engineering applications. Kilicay *et al*. (2011) demonstrated the delivery of antineoplastic agents to cancer cells by a matrix of poly-3-hydroxybutyrate-co-3-hydroxyhexanoate (PHBHHX) nanoparticles. Lee *et al*. (2011) examined the PHB nanoparticles functionalized with a tumor-specific ligand for specifically targeting certain breast cancer cells.

Wang *et al*. (2010) has been reported the use of poly-3-hydroxybutyrateb-3-hydroxyvalerate-b-3-hydroxyhexanoate (PHBVHHx) as scaffold to promote differentiation of human bone marrow mesenchymal stem cell in to nerve cells. Scaffolds prepared from poly-3-hydroxybutyrate-co-3-hydroxyhexanoate have been tested for eyelid reconstruction in experimental animals (Zhou *et al*., 2010). The result was satisfactory but scaffold produced inflammation which took about 2 weeks to clear. Yan *et al*. (2011) reported poly-3-hydroxybutyrate-co-3-hydroxyhexanoate induces cartilage development from mouse mesenchymal stem cells and preserve the chondrocytic phenotype of the cells.

1.10.3 Agricultural applications

PHAs are biodegradable in soil under aerobic and anaerobic conditions, due to which it finds promising applications in agricultural field. The main agricultural application of co-polymer P(3HB-co-3HV) is the control release of insecticides (Holmes, 1985; Philip *et al*., 2007). These insecticides could be integrated in to co-polymer pellets and sown along with the crop. The P(3HB-co-3HV) also can be used as seedling containers, plastic sheaths for protecting saplings, biodegradable matrix for drug release and tubing for crop irrigation (Jendrossek, 2001). Bacterial inoculants are used in agricultural field to enhance nitrogen fixation in plants. These inoculants are prepared from PHA accumulating bacteria, which could withstand environmental stress during storing for longer period of time. Studies using *Azospirillum brasilense* inoculants, it was found that the promotion of plant growth was constant with *A. brasilense* inoculants having higher intracellular PHA even though carriers were different (Fallik and Okon, 1996). Further to confirm this, field experiments were carried out with maize and wheat in Mexico. Consistency in increasing of crop yield was obtained with peat inoculants prepared from PHA-rich *Azospirillum* cells. The authors concluded that, intracellular PHA significantly improves the self-life, efficiency and reliability of commercial bacterial inoculants (Reddy *et al*., 2003; Philip *et al*., 2007). PHAs have been used as mulch films for crop production because it controls weeds, conserve soil moisture, increases soil temperature; improve crop yield and quality (Arun *et al*., 2009). Bioplastics manufacturer Ecomann Biotechnology Co. (China) manufactures mulch films, which finds huge demands in China and Europe (Plastics News Correspondent, June 3, 2014). Nodax is a co-polymer consist of mainly 3(HB) and small amount of MCL-monomer can be used to manufacture agricultural film, for coating urea fertilizers or for herbicides and insecticides to be used in rice field (Hocking and Marchessault, 1994; Philip *et al*., 2007).

Aims and objectives of the research work

Synthetic plastic products are not biodegradable and are often discarded to the environment after use. Due to non-biodegradable nature of synthetic plastic products, they persist in the ecosystem for many years causing a significant burden on solid waste management. It is almost impossible to restrict the use of plastic products but it can be possible to replace synthetic plastics with alternative materials, which are similar to plastics and biodegradable in nature. Polyhydroxyalkanoates possess such properties, and are synthesized by bacteria under limitations of essential nutrients such as nitrogen, phosphorus or oxygen with excess of available carbon source. PHAs are commercially available in the market for various uses such as packaging, biomedical and agricultural applications (Chen, 2010).

Polyhydroxybutyrate is the most commonly produced and widely studied biopolymer in this group. Including 3-hydroxybutrate, more than 150 different hydroxyalkanoate monomers have been characterized as constituents of homopolymer and copolymer of PHA (Potter and Steinbuchel, 2005). The composition and percentage of polymer accumulation depends on the bacterial strain and the type of carbon source they utilize. PHA synthases are the key enzymes involved in PHA biosynthesis. PHA synthesis results in formation of water insoluble PHA granules inside the cell cytoplasm. The granule associated proteins play a crucial role in synthesis, depolymerisation, granule formation and granule stabilization. Very few reports are available on characterization of granule associated proteins. So it is necessary to characterize the proteins associated with PHA granules in order to improve its production and quality.

PHA produced in the industry is mainly by using Gram-negative bacteria such as *Cupriavidus necator*, *Alcaligenes latus*, *Pseudomonas putida* and recombinant *Escherichia coli*. These bacteria contain pyrogenic lipopolysaccharide (LPS) endotoxin, which get co-purified with PHA during extraction. The separation of LPS from PHA requires additional purification cost, results in increase of overall production costs (Santimano *et al*., 2009). Gram-positive bacteria such as *Bacillus* sp. do not contain LPS; grow rapidly using various cheaper carbon substrates and shows resistance to high osmotic pressure. These important characteristics of *Bacillus* sp.

can be exploited for industrial scale PHA production. In the last decade, *B. megaterium* has been studied for PHA production using cheaper carbon substrates (Santimano *et al*., 2009). Due to low productivity, industrial scale PHA production using this bacterium is still limiting. Recently, a few researchers have shown interest in developing high cell density cultivation for PHA production using *B. megaterium* (Kulpreecha *et al*., 2009; Kanjanachumpol *et al*., 2013; Rodriguez-Contreras *et al*., 2013).

Although PHAs are produced in the industry and has large scope in the market, the wide spread use of this polymer is restricted due to its higher cost of production compared to synthetic plastics. To reduce the production cost of this polymer, there is scope for improvement on current research area for the whole process from starting to final step. It includes isolation of new bacterial strain accumulating higher amount of PHA with less incubation time, utilization of cheaper carbon substrates for higher production, optimization of fermentation strategies for high cell density PHA production and strain improvement by characterizing the proteins associated with PHA granules.

In view of the above problems, the following objectives were proposed for this study:

1. **Isolation of heterotrophic bacterial cultures from marine coastal sand-dunes and screening of PHA accumulating bacterial isolates.**
2. **Development of high cell-density cultivation process for the selected bacterial isolates for production of PHB and/or co-polymer of PHA.**
3. **Characterization of PHA synthesis enzyme/proteins associated with granules present in selected bacterial isolates.**

Chapter-II

Isolation and characterization of PHA producing bacteria from sand-dune ecosystem

2.1 Introduction

Polyhydroxyalkanoate was first discovered in *Bacillus megaterium* by Lemoigne (1926). PHAs are biodegradable, biocompatible and show properties similar to synthetic thermoplastics. Due to these important properties it is recognised as a strong candidate for the replacement of synthetic plastic in the market. PHAs are produced in the industry using bacterial strains such as *Cupriavidus necator*, *Alcaligenes latus*, *Pseudomonas putida* and recombinant *Escherichia coli* (Faccin *et al*., 2013). However, higher production cost of this polymer than synthetic plastic limits its wide spread usage in the market. Bacterial strain used for PHA production is one of the factors that determine its production cost. In addition, the composition and quality of the polymer are also determined by the PHA accumulating bacteria (Chein *et al*., 2007; Nath *et al*., 2008). Therefore, it is necessary to isolate new bacterial strains accumulating higher amount of PHA with less incubation time, which will reduce the final production cost of this polymer.

Since its discovery, more than 300 bacterial species accumulating PHA have been reported from various environments (Koller *et al*., 2010; Prabhu *et al*., 2010). In the last decade, many attempts have been made on screening of PHA accumulating bacteria from various environments, such as soil (Santimano *et al*., 2009; Chaudhry *et al*., 2011; Michael *et al*., 2012; Preethi *et al*., 2012; Raj *et al*., 2014), sewage sludge (Borah *et al*., 2002; Reddy *et al*., 2009; Bhuwal *et al*., 2013), marine and mangrove ecosystems (Chien *et al*., 2007; Arun *et al*., 2009; Prabhu *et al*., 2010; Van-Thouc *et al*., 2012) and marine microbial mats (Simon-Colin *et al*., 2008; Lopez-Cortes *et al*., 2008). Coastal sand-dune is a unique ecosystem present along the coastal areas of sea, all over the world. This ecosystem faces drastic fluctuations in physico-chemical status within a span of small time. Sand dunes have low nutrient content, especially lack in nitrogen (N) and phosphorus (P), have low moisture content and high salinity (Arun *et al*., 1999; Kurtboke *et al*., 2007). The nutrient limitation could provide selective pressure on microorganisms present in such environments, to accumulate PHA as a source of carbon and energy for future use. Rhizosphere region of coastal sand-dune harbour diverse microbial community, as part of a plant-microbe symbiosis (Park *et al*., 2005; Lee *et al*., 2006). Very few reports are available on isolation and characterization of bacteria from such ecosystems. Park *et al*. (2005) have characterized bacteria associated with two plant species from a sand-dune ecosystem of Korea. These bacterial isolates were checked for their plant growth promoting activity. Plant growth promoting mesophilic actinomycetes were

isolated from sand-dune vegetations of Fraser Island, Australia (Kurtboke *et al*., 2007). Godinho *et al*. (2010) isolated bacteria from coastal sand-dunes of Goa, promoting plant growth in Eggplant. Gaonkar *et al*. (2012) have isolated and characterized siderophore producing bacteria from coastal sand-dunes of Miramar beach, Goa.

Diversity of microbes has a major contribution to the functioning and restoration of the ecosystem. Microorganisms present in such ecosystems could have different survival strategies including synthesis of PHA, spore formation, cyst development etc. Sand-dune ecosystems have low nutrient content. Bacteria here can therefore produce and accumulate PHA in presence of excess carbon. The present study is concentrating on isolation of potential PHA accumulating bacteria from coastal sand-dune ecosystem. Heterotrophic bacteria were isolated from rhizosphere and non-rhizosphere regions of the sand-dune ecosystem and screened for PHA accumulation by alcoholic Nile blue method. Bacterial isolates showing PHA accumulations were identified by phenotypic and genotypic characterization.

2.2 Materials and methods

2.2.1 Isolation of PHA accumulating bacteria from coastal sand-dunes

2.2.1.I Sample collection

Total eight sand samples were collected, four from the rhizosphere region of the *Ipomoea pes-caprae* and four from the non-rhizosphere region of coastal sand dunes of Miramar beach, Goa (Fig. 2.1). The sampling site was 100 meters away from the high-tide line. Samples were collected in sterile plastic bags, brought to the laboratory and stored at 4 °C before processing for bacteriological analysis.

2.2.1.II Isolation of heterotrophic bacteria and determination of total viable counts

One gram of sand sample was suspended in sterile physiological saline to 10 ml suspension. Suspension was diluted serially up to 10^{-4}. Samples of 0.1ml from dilutions 10^{-2}, 10^{-3} and 10^{-4} were spread plated on to Nutrient Agar (NA) and Tryptone Glucose Yeast extract Agar (TGYA) (Appendix A). Plates were incubated at 28 °C for 48 h. Total Viable Counts on plates were recorded. Morphologically distinct colonies were streaked on to respective medium, purified, maintained and stored at 4 °C.

Rhizosphere region

Non-rhizosphere region

Fig. 2.1: Sampling sites of coastal sand-dune ecosystem of Miramar beach, Goa.

2.2.2 Screening of PHA accumulating bacteria

All the bacterial isolates obtained were tested for PHA accumulation using E2-mineral medium (Lageveen *et al*., 1988) containing glucose as sole source of carbon (Appendix A). Bacterial isolates were spot inoculated on E2-mineral agar plates and incubated at 28 °C. After each 24, 48 and 72 h of incubation the colonies on the plates were flooded with 0.05% (w/v) Nile blue A in ethanol (Appendix B) and incubated in the dark for 20 min (Kitamura and Doi, 1994). Extra stain was decanted and stained colonies were visualized under UV-transilluminator for the presence of orange fluorescence. Depending on the intensity of orange fluorescence the degree of PHA accumulation was recorded.

2.2.3 Identification of bacterial isolates accumulating PHA

2.2.3.1 Phenotypic characterization

Bacterial isolates were phenotypically characterized using the methods described in Bergey's Manual of Systematic Bacteriology (Sneath *et al*., 1986; Vos *et al*., 2009) (Appendix A, B). The data was analyzed numerically, using the simple matching coefficient (S_{SM}). Clustering was achieved by unweighted pair group average linkage (UPGMA). The computations were performed using the Probiosys Software.

2.2.3.2 Genotypic characterization

2.2.3.2.1 Specific PCR amplification of *PhaC* gene of *Bacillus megaterium*

2.2.3.2.1.I Bacterial strains and growth medium

Bacterial cultures used in present investigation and their sources are listed in Table 2.1. All the bacterial isolates were maintained and grown on the respective medium (Table 2.1). All the bacterial isolates were screened for PHA accumulation on E2- mineral medium containing 2% w/v glucose and visualized with Nile blue A staining method (as described in section 2.2.2). Except those isolates did not grow on E2-mineral medium were checked for PHA accumulation on Nutrient Agar plates.

Serial No.	Bacterial species	Medium	Source	16S rRNA sequence Accession No.	PHA accumulation (% w/w)
1	*Aneurinibacillus migulanus* 81A1^T	NA	NRS 1137T*	ND	-
2	*B. amyloliquefaciens*10A1	NA	BGSC 10A1*	ND	+ (18.246)
3	*B. aquimaris*	NA	MTCC6722	AF483625$	-
4	*B. coagulans* 61A1^T	NA	ATCC 7050*	DQ297928$	+ (15.020)
5	*B. cereus* 6A5	NA	ATCC 14579*	AE016877$	-
6	*B. circulans*16A1^T	NA	ATCC 4513*	FJ560956$	+ (18.000)
7	*B. endophyticus* TMR1.22	TYGA	Coastal sand-dune (R)	HQ897169#	+ (39.409)
8	*B. firmus* 29A1^T	NA	NRS 613T*	ND	+ (20.078)
9	*B. flexus* NAMR4.1	NA	Coastal sand-dune (R)	HM026605#	+ (47.476)
10	*B. licheniformis* 5A1	NA	ATCC 8480*	ND	-
11	*B. megaterium*	NA	MTCC428	ND	+ (32.630)
12	*B. megaterium*7A16	NA	QM B1551*	CP001983$	+ (32.814)
13	*B. megaterium* NQ-11/A2	NA	Arabian sea- continental shelf sediment sample-NCIM5334	FJ392860#	+ (61.000)
14	*B. megaterium* COL1/A6	NA	Humus sample	EU702754#	+ (65.510)
15	*B. megaterium* BLQ-2/A7	NA	sediment sample	EU924811#	+ (59.870)
16	*B. megaterium* TMR1.3.2	TYGA	Coastal sand-dune (R)	GU984576#	+ (39.356)
17	*B. megaterium* TMR1.4	TYGA	Coastal sand-dune (R)	GU951918#	+ (40.801)
18	*B. megaterium* NAMNR3.7	NA	Coastal sand-dune (NR)	GU951917#	+ (41.776)
19	*B. mojavensis*	NA	MTCC8604	AF440779$	+ (12.345)
20	*B. mycoides* 6A19	NA	ATCC 31101*	EF210306$	-
21	*B. niacin*	NA	MTCC8323	ND	+ (11.702)

Table 2.1.a: Bacterial cultures their source and growth medium

NA, Nutrient Agar; TYGA, Tryptone Yeast extract Glucose Agar; ATCC, American Type Culture Collection, USA; MTCC, Microbial Type Culture Collection, India; NCIM, National Collection of Industrial Micro-organisms, India; RCPFBS, Russian commercial powder formulations Bacticide and Sphericide; BGSC, Bacillus Genetic Stock Center, Columbus; NRRL, Northern Regional Research Laboratory, USA; NCIB, National Collection of Industrial, Marine and Food Bacteria, Scotland; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany; NRS, Northern Research Station, USA; *, Cultures obtained from BGSC; #, Sequences obtained in current study; $, Sequences obtained from NCBI; R, Rhizosphere; NR, Non rhizosphere; PHA, Polyhydroxyalkanoate; ND, Not done; + and -, Nile Blue A staining method; +, accumulating PHA; -, negative for PHA accumulation; PHA accumulation (% w/w), gravimetric method.

Serial No.	Bacterial species	Medium	Source	16S rRNA sequence Accession No.	PHA accumulation (% w/w)
22	*B. pumilus* 8A3	NA	ATCC 7061*	EU138517$	+ (23.428)
23	*B. simplex*	NA	MTCC7284	ND	+ (26.972)
24	*B. spizizenii*	NA	ATCC 6633	AB018486$	-
25	*B. subtilis* subsp. *subtilis* 3A1	NA	NCIB 3610*	ND	+ (18.729)
26	*Bacillus* sp. TMR1.10.1	TYGA	Sand-dune (R)	HM035484#	+ (51.860)
27	*Bacillus* sp. NAMNR4.4	NA	Coastal sand-dune (NR)	JX194167#	-
28	*Bacillus* sp. NAMNR3.5	NA	Coastal sand-dune (NR)	JX194166#	+ (23.624)
29	*Bacillus* sp. TMNR4.1.1	TYGA	Coastal sand-dune (NR)	JX194168#	+ (27.369)
30	*Bacillus* sp. MS4.SE3	TYGA	Sediment sample	ND	-
31	*B. thurigiensis* 164 H-14	NA	RCPFBS	ND	+ (37.812)
32	*B. weihenstephanensis* 6A24	NA	BGSC 6A24*	ND	-
33	*Geobacillus stearothermophilus* 9A20	NA	ATCC 12980*	AY608928$	+ (13.394)
34	*Lysinibacillus fusiformis* 19A1^T	NA	ATCC 7055T*	AF169537$	+ (21.794)
35	*Lysinibacillus sphaericus* 13A10	NA	ATCC 12123*	ND	+ (26.785)
36	*Lysinibacillus* sp.KSD-4	NA	Stagnant water - MTCC3672	FJ473365#	+ (25.233)
37	*Marinibacillus marinus* 21A1^T	NA	DSMZ 1297*	AJ237708$	+ (21.198)
38	*Paenibacillus dendritiformis* 30A2	NA	C168*	AB045092$	+ (18.859)
39	*Paracoccus yeii* TMR3.1	TYGA	Coastal sand-dune (R)	GU906275#	+ (28.205)
40	*Pseudomonas aeruginosa*	NA	ATCC 9027	ND	-
41	*Pseudomonas aeruginosa* TMR2.13	TYGA	Coastal sand-dune (R)	HM030825#	-

Table 2.1.b: Bacterial cultures their source and growth medium

NA, Nutrient Agar; TYGA, Tryptone Yeast extract Glucose Agar; ATCC, American Type Culture Collection, USA; MTCC, Microbial Type Culture Collection, India; NCIM, National Collection of Industrial Micro-organisms, India; RCPFBS, Russian commercial powder formulations Bacticide and Sphericide; BGSC, Bacillus Genetic Stock Center, Columbus; NRRL, Northern Regional Research Laboratory, USA; NCIB, National Collection of Industrial, Marine and Food Bacteria, Scotland; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany; NRS, Northern Research Station, USA; *, Cultures obtained from BGSC; #, Sequences obtained in current study; $, Sequences obtained from NCBI; R, Rhizosphere; NR, Non rhizosphere; PHA, Polyhydroxyalkanoate; ND, Not done; + and -, Nile Blue A staining method; +, accumulating PHA; -, negative for PHA accumulation; PHA accumulation (% w/w), gravimetric method.

2.2.3.2.1.II Quantitative analysis of PHA

The isolates which showed orange fluorescence on Nile blue A staining method were selected for PHA extraction. These isolates were grown in 250-ml Erlenmeyer flask containing100 ml E2 mineral medium supplemented with glucose (20 g/L) as sole carbon source. The flask was incubated on an Orbitek environmental shaker (170 rpm) for 48 h at 30 °C. The bacterial isolates which could not grow in E2 broth were grown in Nutrient broth. Twenty-ml of culture broth was transferred to 50ml centrifuge tube and centrifuged at 10,000 rpm for 10 minutes. Cell pellet was oven dried for biomass determination. Another cell pellet was suspended in 10 ml of sodium hypochlorite solution (4% available chlorine). The suspended pellet was shaken at 170 rpm on orbital shaker at room temperature for 20 minutes. Twenty-ml of distilled water was added to the suspension and centrifuged at 12,000 rpm for 20 minutes. The supernatant was discarded and the pellet was resuspended in distilled water and centrifuged. Pellet obtained was suspended in 10ml of chilled ethanol (95%) and centrifuged at 12,000 rpm for 20 minutes. The supernatant was discarded and the pellet was oven dried till constant weight was obtained (Santimano *et al.*, 2009). Biomass and PHA weight was measured by gravimetrically.

2.2.3.2.1.III Characterization of PHA using Fourier Transform Infrared spectroscopy (FTIR)

PHA samples were dissolved in chloroform and made to thin film. The FTIR spectrum of the film of polymer was recorded at 400-4000 cm^{-1} in FTIR (Divyashree *et al.*, 2009).

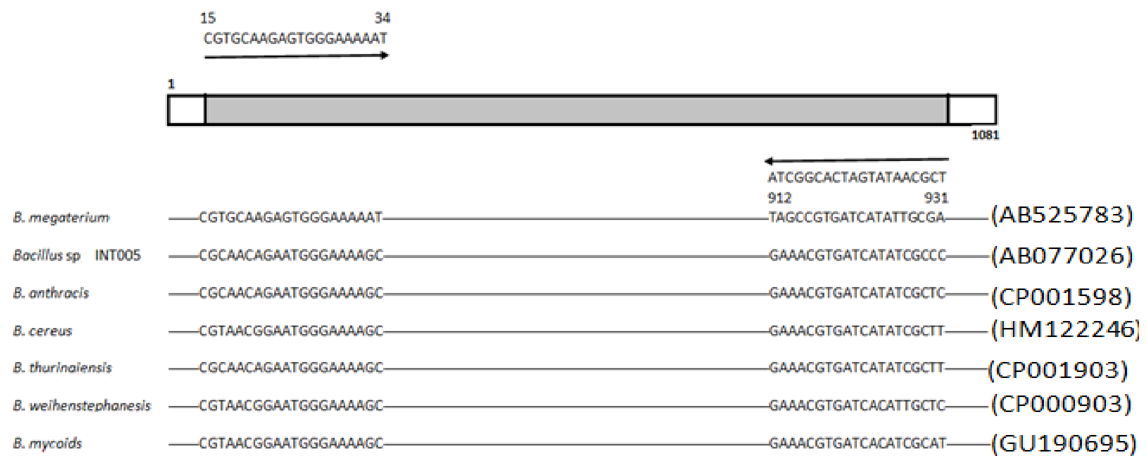
2.2.3.2.1.IV Physiological characterization

All the strains of *Bacillus* used in this study were tested for several physiological characteristics using the methods described in Bergey's Manual of Systematic Bacteriology (Sneath *et al.*, 1986; Vos *et al.*, 2009). Tests used in the present study are listed in Table 2.5.

2.2.3.2.1.V Designing specific primers

Complete sequences of *phaC* genes of *B. megaterium* (AB525783), *B. anthracis* (CP001598), *B. cereus* (HM122246), *B. mycoids* (GU190695), *B. thuringiensis* (CP001903), *B. weihenstephanesis* (CP000903) and *Bacillus* sp. INT005 (AB077026) were obtained from the

NCBI nucleotide database and used for designing specific primers. The sequences were aligned using Clustal-X in order to search for internal region of *phaC* gene specific for *B. megaterium* (Fig. 2.2). A pair of oligonucleotides, forward and reverse was selected. The primers were BmphaC015F (CGTGCAAGAGTGGGAAAAAT) as forward and BmphaC931R (TCGCAATATGATCACGGCTA) as reverse with t_m value 63.9 °C. These were synthesized by Bangalore Genei Pvt. Ltd., Bangalore, India. The sequences and positions of oligonucleotides are presented in Fig. 2.2.



BmphaC015F (CGTGCAAGAGTGGGAAAAAT)

BmphaC931R (TCGCAATATGATCACGGCTA)

Fig. 2.2: Location of forward and reverse primers on *phaC* gene of *B. megaterium*. The numbers indicate the positions of the primers on the *phaC* gene. Arrow indicates the direction of forward and reverse primers on the gene. Accession numbers of the sequences used to draw the diagram are given on the right and name of the species on the left.

2.2.3.2.1.VI Genomic DNA extraction

Bacterial isolates were grown for 24 h in respective media broth at 30 °C. DNA was extracted using the protocol described in Sambrook *et al*. (1989). The culture broth was centrifuged at 3000 rpm for 5 min at 4 °C. The cell pellet was washed in de-ionized water and resuspended in 465 µl of 1X TE (Tris EDTA) buffer, pH 8.0. Then 5 µl of lysozyme (10 mg/100 µl) was added in to the suspension and incubated at 37 °C for 45 min. Further, 30 µl of 10% sodium dodecyl sulphate (SDS) was added and incubated at 60 °C for 15 min. An equal volume of phenol-chloroform solution (1:1) was added, mixed gently and centrifuged at 12000 rpm for 10 min. This step was repeated for two times. The aqueous layer was collected and to it an

equal volume of chloroform-isoamyl alcohol solution (24:1) was added, mixed gently and centrifuged at 12000 rpm for 10 min. The aqueous layer was collected and to it 3 M sodium acetate ($1/10^{th}$ of the aqueous layer) and 0.6 volume of chilled isopropanol were added and centrifuged at 12000 rpm for 10 min. The supernatant was discarded. 1 ml of chilled ethanol (70%) was added to the pellet and centrifuged at 12000 rpm for 10 min. The ethanol was discarded and the tube was allowed to dry completely. The isolated DNA was stored at -20 °C for further use.

2.2.3.2.1.VII PCR optimization of *phaC*

PCR using the internal primers (Fig. 2.2) for *phaC* was optimized. Genomic DNA of six bacterial species *Bacillus megaterium*, *Bacillus flexus*, *Bacillus endophyticus*, *Bacillus* sp., *Paracoccus yeii* and *Pseudomonas aeruginosa* were used for this experiment. The specificity was optimized by adjusting annealing temperature from 51 to 64 °C and primer concentration from 1 to 10 µM.

PCR reactions were performed in a total volume of 50 µl. The reaction mixture contains 2 µl of genomic DNA as template, 10 X PCR Buffer [100 mM Tris-HCl (pH 9), 500 mM KCl and 0.1% gelatin], 1.5 mM $MgCl_2$, 10 mM *d*NTP Mix, 1 µM – 10 µM of each primer depending on the requirement and 5 units/µl of *Taq* polymerase (Bangalore Genei, India). Reactions were carried out in a thermocycler (BIOER XP Cycler, China) consisting of initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 51 to 64 °C (as required) for 1 min and elongation at 72 °C for 1 min. Final extension was carried out at 72°C for 5 min and reaction mix tube was stored at 4 °C.

PCR master mix (50 µl)

Template (DNA sample)	2 µl
PCR buffer	5 µl
*d*NTPs	1 µl
Forward primer	1 µl
Reverse primer	1 µl
Taq polymerase	0.75 µl
$MgCl_2$	3 µl
Nuclease free de-ionized water	36.25 µl

2.2.3.2.1.VIII Detecting PCR products

Electrophoresis on 1% (w/v) agarose gel was used for detecting PCR amplification products (Sambrook *et al.*, 1989). 100 or 500 bp ladders were used as DNA size marker. Run conditions were 100 volts for 2 hours. The gel was stained with ethidium bromide solution (0.5 µg/ml). Amplified DNA fragments were visualized under UV light and recorded using a Gel-Doc Alpha Imager (Alpha Innotech, USA).

2.2.3.2.1.IX Validating PCR

Amplified products from strains *B. megaterium* TMR1.3.2 and *B. megaterium* TMR1.4 under optimum conditions having expected size were purified using purification kit (Qiagen, India) as per the instructions of the manufacturer. Purified amplicons were sequenced using forward primer (BmphaC015F) at Bangalore Genei, India. Sequences obtained were compared with sequences in the NCBI nucleotide database using Blastn (Altschul *et al.*, 1990). Sequences were deposited in GenBank with accession numbers. Amplified nucleotide sequences were analyzed and aligned with the reference sequences of *phaC* of various bacterial species using ClustalX (Larkin *et al.*, 2007) and Neighbor-joining (NJ) tree was obtained with 1000 seeds and 10000 bootstraps. The final tree obtained was rooted and drawn using MEGA 4.0 (Tamura *et al.*, 2007).

Optimized PCR conditions with 1 µM primer concentration and 64 °C annealing temperature were used for the amplification of 0.9 kb from *phaC* of *B. megaterium* by SYBR Green based Real-Time PCR (Queipo-Ortuno *et al.*, 2005). The melting curve of the amplicon was performed from 55 to 94 °C for detecting t_m of amplicon.

All the bacterial isolates mentioned in Table 1 were used for PCR amplification of *phaC* at optimum conditions (Method-I) of 1 µM primer concentration and 64 °C annealing temperature and sub-optimal conditions (Method-II) of 10 µM and 51 °C. Presence of genomic DNA was confirmed using amplification of 16S rRNA gene using universal primers as a positive control (Prabhu *et al.*, 2010). Entire experimental sets were repeated thrice to determine reproducibility of Method I and Method II.

A blind folded test was carried out using a few bacterial isolates. *B. megaterium* BLQ-2/A7 and *B. simplex* MTCC7284, known for PHA accumulation were taken as positive controls. Other six bacteria were randomly selected from genus *Bacillus*. These were not reported for

PHA accumulation earlier. Three of these strains were *B. aquimaris* MTCC6722, *B. mojavensis* MTCC8604 and *B. niacin* MTCC8323 and three were unidentified *Bacillus* sp. NAMNR3.5, *Bacillus* sp. TMNR4.1.1 and *Bacillus* sp. MS4.SE3 (Table 1). These bacterial isolates were tested for biochemical characteristics, PHA accumulation and PCR amplification using Method I and Method II. All the experiments were repeated three times.

2.2.3.2.2 PCR amplification of *PhaC* gene from sand-dune bacterial isolates

Using PCR Method I, all the PHA accumulating bacterial isolates obtained from sand-dune ecosystem were screened for amplification of *PhaC* gene for the rapid identification of PHA accumulating *B. megaterium*.

2.2.3.2.3 PCR amplification of 16S rRNA gene

The 16S rRNA gene was amplified using universal primers such as, S-D-Bact-0011-a-S-17 5`-TTGATCCTGGCTCAG-3` as forward and S-*-Univ-1392-b-A-15 5`-ACGGGCGGTGTGTTC-3` as reverse primer (Alm *et al*., 1996). PCR reactions were performed as described by Prabhu *et al*. (2010). The PCR product obtained were gel purified using purification kit (Qiagen, India) and were sent for sequencing at Bangalore Genei, India. Sequences obtained were compared with sequences in the NCBI nucleotide database using BLASTn (Altschul *et al*., 1990) and deposited in the GenBank with accession numbers (Table 2.7). Further, sequences were aligned with the reference sequences of 16S rRNA gene of closely related bacterial species using ClustalX (Larkin *et al*., 2007) and neighbour-joining tree was constructed with 1000 seeds and 10000 bootstraps. The final tree obtained was rooted and drawn using MEGA 4.0 (Tamura *et al*., 2007).

2.3 Results and discussion

2.3.1 Isolation and determination of total viable counts

Heterotrophic bacterial activities were observed in both rhizosphere and non-rhizosphere sand samples of coastal dune (Table 2.2). Four sand samples of each zone were analysed for heterotrophic bacterial count. Sand-samples of rhizosphere showed highest average

heterotrophic bacterial count of 1.95±1.06 x 10^6 cfu/g on NA. Lowest average counts of 1.32±0.19 x 10^4 cfu/g of heterotrophic bacteria were obtained on TGYA from non-rhizosphere sample. Nutrient agar supported more heterotrophic bacterial counts during isolation irrespective of types of sand-sample used. Chi square Test of Independence gave value of chi-square as 16.67084 which is greater than the 10.83 for $\alpha = 0.001$, indicates $p<0.001$. This clearly states that the viable counts for rhizosphere and nude sand dunes have no relationship. Further, the media also play an important role in variable viable counts obtained from different zones of sand-dunes. In Rhizosphere, plants root exudates continuously provides energy-rich carbon and other metabolites, which enhance the microbial diversity and activity in this region compare to bulk soil (Faure *et al*., 2009). De Ridder-Duine *et al*. (2005) reported the average bacterial count of rhizosphere was 8.92 x 10^8 cfu/g on Tryptic Soy Agar (TSA), which was 20 times higher than the bulk soil. A maximum viable count of 21 x 10^8 cfu/g of rhizospheric bacteria associated with *Ipomoea pes-caprae* was reported from Goa coast on NA (Godinho, 2007). The author also noted that depending on the seasonal variation from pre-monsoon, monsoon and post-monsoon the rhizospheric bacterial counts differs, where the highest total viable count was observed in the post-monsoon sample. In a similar study, Muthezhilan *et al*. (2012) reported the total viable count between 4.4 x 10^6 and 7.5 x 10^7 cfu/g on King's B medium of rhizospheric bacteria associated with *Ipomoea* sp. in Chennai Coast. In the present study the higher bacterial counts obtained in the rhizosphere samples compared to nude sand-dune samples may be due to the secretion of root exudates from the plants.

Zone of sand-dune	**Viable counts (cfu/g)**	
	Media used for isolation	
	NA	**TGYA**
Rhizosphere	1.95±1.06 x 10^6	3.22±2.26 x 10^5
Non-rhizosphere	8.33±2.94 x 10^4	1.32±0.19 x 10^4

Table 2.2: Viable counts (cfu/g) of heterotrophic bacteria from various zones of sand-dunes on different media. Where, viable counts of heterotrophic bacteria are average of four samples with standard error.

Morphologically distinct bacterial colonies were purified and obtained on NA and TGYA medium. Total 171 bacterial isolates were obtained, 77 were obtained on NA and 94 on TGYA. All the bacterial isolates were tested for their ability to accumulate PHA by Nile blue A staining. Colonies showing orange fluorescence (Fig. 2.4) under UV light were considered

as PHA positive. In total, 22 bacterial isolates showed PHA accumulation (Table 2.3a and b), out of which 18 isolates were obtained from rhizosphere samples and 4 from non-rhizosphere samples. Interestingly, 77% of PHA producers are bacterial isolates obtained on TGYA and 23% are on NA. The maximum numbers of PHA accumulating bacteria were obtained from rhizosphere. The numbers of isolates showed PHA accumulation were few from non-rhizosphere region of sand-dune ecosystem. This is probably due to lack of essential nutrients such as nitrogen or phosphorous in sand-dune and at the same time root exudates secreted by rhizospheric plants provides carbon source (Kurtboke *et al*., 2007; Hirsch *et al*., 2013). This is the first report on isolation of PHA accumulating bacteria from such a unique coastal ecosystem. The intensity of orange fluorescence of bacterial isolates was observed along the prolonged incubation time (Table 2.4). All the isolates showed PHA accumulation within 24 h. Further incubation resulted in the increase in PHA accumulation. Among these, bacterial isolates TMR1.3.2, TMR1.26 and TMR1.28 showed maximum PHA accumulation at 48 h. At 72 h of incubation bacterial isolate TMR1.7, TMR1.10.1, TMR1.22, TMR1.4 and NAMR1.8 showed maximum PHA accumulation.

2.3.2 Phenotypic characterization of PHA accumulating bacteria

Heterotrophic bacterial isolates accumulating PHA were obtained from sand samples of rhizosphere and non-rhizosphere present in coastal dune ecosystem of Goa. These isolates were characterized morphologically and biochemically. Biochemical characteristics with similarity analysis and UPGMA clustering placed these isolates along with respective standard organisms in the dendogram (Fig. 2.5). All the PHA accumulating bacterial isolates were tentatively identified as per their phenotypic characteristics and clustering with the standard organisms in the phenogram. Maximum numbers of isolates (13) showed similarity with *B. megaterium*, one with *B. flexus*, one with *Pseudomonas oryzihabitans,* one with *Paracoccus yeei* and one as *Paracoccus* sp.. Five isolates did not showed similarity with any standard species in the tree and as they were clustered near *B.megaterium* were identified as *Bacillus* sp..

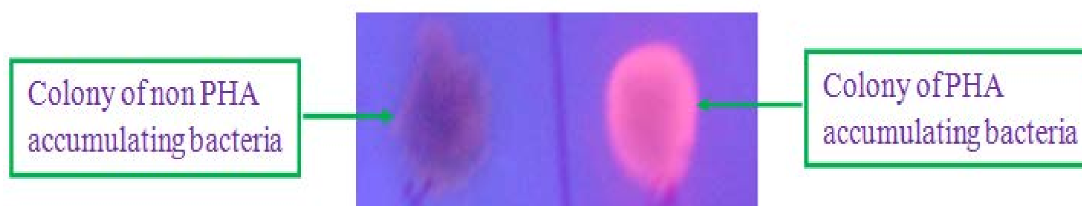


Fig. 2.3: Colony of PHA accumulating bacteria showing orange fluorescence under UV light

Source	Number of isolates	
	Total	PHA positive
R1	14	3
R2	4	0
R3	19	0
R4	12	1
NR1	8	0
NR2	2	0
NR3	9	1
NR4	9	0
Total	**77**	**5**

Table 2.3a: Number of isolates obtained on nutrient agar from rhizosphere (R) and non-rhizosphere (NR) region along with the isolates showing PHA accumulation on E2-mineral medium

Source	Number of isolates	
	Total	PHA positive
R1	34	11
R2	12	1
R3	11	1
R4	10	1
NR1	8	2
NR2	9	1
NR3	3	0
NR4	7	0
Total	**94**	**17**

Table 2.3b: Number of isolates obtained on tryptone glucose yeast extract agar from rhizosphere and non-rhizosphere region along with the isolates showing PHA accumulation on E2-mineral medium

Isolates	Incubation time		
	24 hours	48 hours	72 hours
NAMR1.6	+	+	+
NAMR1.8	+	++	+++
NAMR1.12	++	+++	+++
NAMR4.1	++	+++	+++
NAMNR3.7	++	+++	+++
TMR1.4	+++	+++	++++
TMR1.9.1	++	+++	+++
TMR1.9.2	++	+++	+++
TMR1.22	++	+++	++++
TMR1.26	+++	++++	++++
TMR1.28	+++	++++	++++
TMR1.10.1	+	++	+++
TMR2.4	++	+++	+++
TMR1.3.1a	++	+++	+++
TMR1.3.1b	++	+++	+++
TMNR1.3	++	++	++
TMNR2.4	++	+++	+++
TMNR1.5	++	+++	+++
TMR4.3	+++	+++	+++
TMR1.7	++	+++	++++
TMR1.3.2	+++	++++	++++
TMR3.1	+	++	++

Table 2.4: Extent of PHA accumulation by the isolates with prolong incubation on E2-mineral medium with glucose as sole carbon source

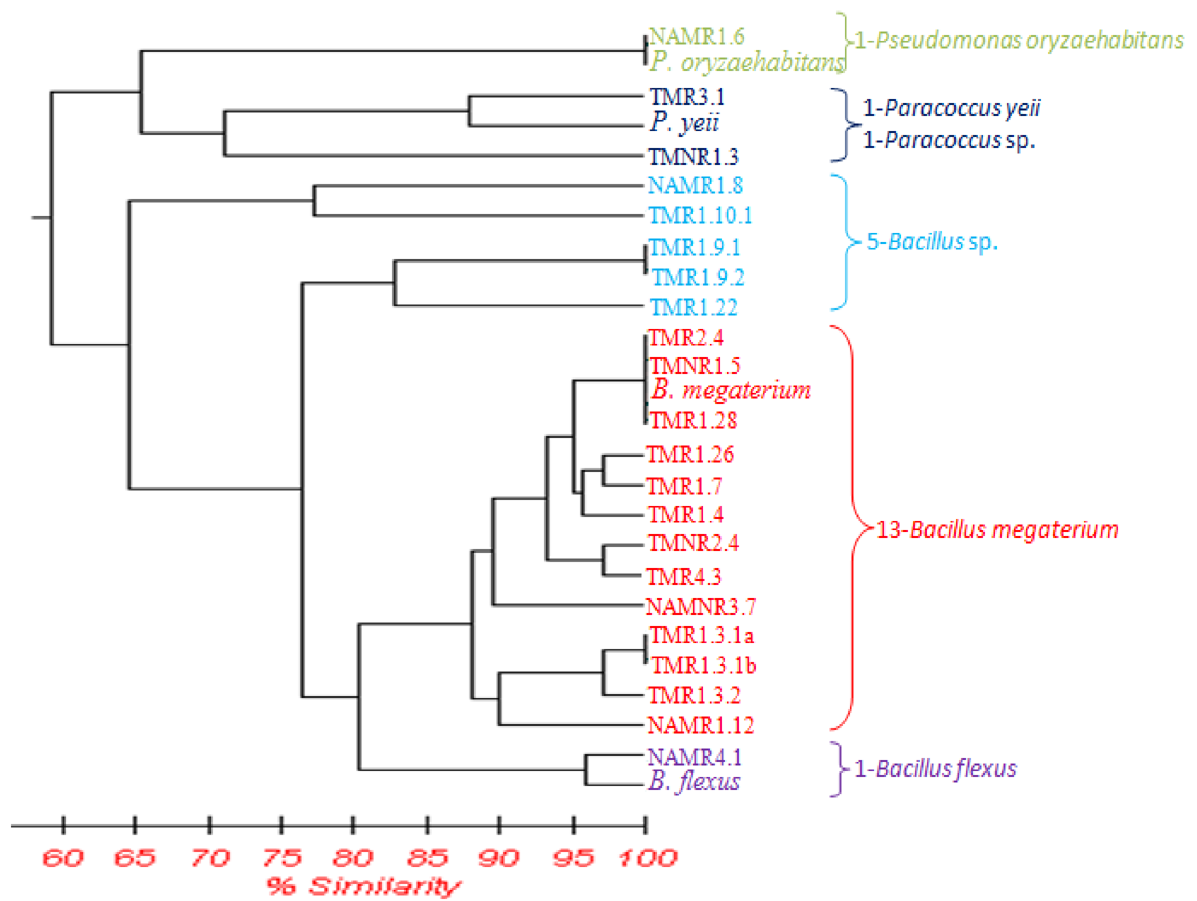


Fig. 2.4: Phenogram of PHA accumulating bacterial isolates along with its tentative identification

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Pigmented colony	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	+	-	-
Gram character	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Swell sporangia	-	-	-	-	-	-	+	+	+	+	-	+	+	-	-	+	+	+	-	+	+	-	+	+	-	-	-	+	-	-	-	-	-	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Oxidase	-	-	-	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	-	+	-	-	-	+	+	-	+	+	-	+	+	-	-
Aerobic growth	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anaerobic growth	+	-	+	+	-	+	-	-	-	-	-	+	-	+	+	+	-	+	-	-	+	+	+	+	+	-	+	-	-	+	-	-	+	+
VP	-	-	-	-	-	+	-	-	-	-	nd	+	nd	+	+	-	-	-	+	-	+	+	-	-	+	+	+	+	+	-	+	+	+	-
Nitrate reduction	-	+	-	-	-	+	-	-	-	-	-	-	+	-	+	-	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+
Acid from																																		
D-glucose	+	+	+	+	+	+	+	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	-	+	+	+	-	-	-	-	-	+	-	-	-	-	+	-	+	+	-	+	-	+	-	+	-	-	+	+	-	-	-	-	-
Glycerol	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	+	+	+	+	-	-	+	+	+	+	-	+
Trehalose	+	+	+	+	+	+	nd	-	-	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
D-Xylose	+	+	+	+	-	-	-	-	-	-	+	-	-	+	+	+	-	+	-	-	+	-	+	+	+	+	-	+	+	-	+	+	-	-
Hydrolysis of																																		
Casein	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+
Gelatin	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch	+	+	-	+	+	+	+	-	-	-	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Aesculin	+	+	+	+	-	+	+	-	-	-	+	nd	-	nd	nd	-	-	nd	+	-	+	nd	+	+	+	nd	+	nd	+	+	nd	nd	+	nd
Urea	-	+	+	+	+	+	-	+	+	+	-	+	-	-	-	-	+	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-
Citrate utilization	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	-	+	-	-	+	+	+	+	+	-	+	+	+	-
Growth in NaCl																																		
2%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
5%	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+
7%	+	+	+	+	+	+	-	+	-	+	+	-	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	-	+	-	+	+	+	+
10%	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	-	+	+	+	-	-	+	+	-	+	-	-	+	-	-	-	+	+
PHA accumulation	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	-	+	+	-	+	-	+	+	+	-	+	+	-
Growth at temp.																																		
10°C	+	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	+	+	+	-	+	+	-	-
20°C	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
40°C	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50°C	+	+	-	+	+	-	+	-	+	-	-	-	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	+	-	+	+	-	+
60°C	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2.5: Physiological characteristics of bacterial cultures belong to Bacillales

All the organisms were positive for endospore formation, catalase and growth at 30°C; +, Positive; -, Negative; nd, Not determined; **1**, *B. megaterium* MTCC428 and *B. megaterium* QMB1551; **2**, *B. megaterium* TMR1.3.2; **3**, *B. megaterium* TMR1.4; **4**, *B. megaterium* NAMNR3.7, *B. megaterium* NQ-11/A2, *B. megaterium* Col1/A6 and *B. megaterium* BLQ-2/A7; **5**, *B. flexus* NAMR4.1; **6**, *B. weihenstephanensis*; **7**, *Geobacillus stearothermophilus*; **8**, *Lysinibacillus fusiformis*; **9**, *Lysinibacillus sphaericus*; **10**, *Lysinibacillus* sp.KSD-4; **11**, *Marinibacillus marinus*, **12**, *Paenibacillus dendritiformis*; **13**, *Aneurinibacillus migulanus*; **14**, *Bacillus* sp. NAMNR3.5; **15**, *Bacillus* sp. TMNR4.1.1; **16**, *B. endophyticus* TMR1.22; **17**, *Bacillus* sp. TMR1.10.1; **18**, *Bacillus* sp. NAMNR4.4; **19**, *B. amyloliquefaciens*; **20**, *B. aquimaris*; **21**, *B. coagulans*; **22**, *B. cereus*; **23**, *B. circulans*; **24**, *B. firmus*; **25**, *B. licheniformis*; **26**, *B. mojavensis*; **27**, *B. mycoides*; **28**, *B. niacini*; **29**, *B. pumilus*; **30**, *B. simplex*; **31**, *B. spizizenni*; **32**, *B. subtilis*; **33**, *B. thuringiensis*; **34**, *Bacillus* sp. MS4.SE3

2.3.3 Genotypic characterization

2.3.3.I Specific PCR amplification of *PhaC* gene of *Bacillus megaterium*

All the bacterial cultures used in this experiment were tested for their ability to accumulate PHA on solidified E2-mineral medium with 2% w/v glucose (Table 2.5). *B. aquimaris* and *B. niacin* did not show any growth on E2-mineral medium. These strains were therefore grown on NA and screened for PHA accumulation. All the strains of *B. megaterium* showed PHA accumulation. *Aneurinibacillus migulanus, B. aquimaris, B. cereus, B. licheniformis*, *B. mycoids, B. spizizenii, Bacillus* sp. NAMNR4.4, *Bacillus* sp. MS4.SE3, *B. weihenstephanensis* and *Pseudomonas aeruginosa* did not show PHA accumulation. The remaining strains showed PHA accumulation. The PHA content of all the bacterial isolates were found between 10 to 66% w/w (Table 2.1). PHA content varied among the isolates of *B. megaterium*. FTIR spectroscopy of PHA extracted from all the bacterial isolates showed intense absorptions typical to PHA at 1724-1740 cm^{-1} and at 1280 cm^{-1} corresponding to C=O and C-O stretching groups in ester, respectively.

The physiological characteristics of the *Bacillus* strains are listed in Table 2.5. All the strains of *Bacillus* are endospore producers, catalase positive and showed growth at 30 °C. Different strains of *B. megaterium* including the type strain *B. megaterium* QMB1551 showed variations in the physiological tests and accordingly are separated in four groups as indicated in Table 2.5.

Primers BmphaC015F and BmphaC931R were used for amplifying *phaC* gene (Fig. 2.2). Different annealing temperatures such as 51, 55, 60 and 64 °C and different primer concentrations such as 1, 2.5, 5 and 10 µM were used for optimization (Table 2.6). *B. megaterium* at different annealing temperatures with primer concentrations of 1 and 2.5 µM gave amplification of a single band of 0.9 kb. With primer concentrations of 5 and 10 µM along with 0.9 kb an additional faint band of 1.9 kb was present. In *B. flexus* and *B. endophyticus* at annealing temperatures 51, 55, 60 and 64 °C with primer concentrations 1 and 2.5 µM there was no amplification, and with primer concentrations of 5 and 10 µM, multiple bands were seen in all the temperatures except at 64°C. PHA accumulating *P. yeii* and PHA negative *P. aeruginosa* and *Bacillus* species gave no amplification with any combination tested. It was found that the 0.9 kb fragment was amplified only in *B. megaterium* and not amplified in any other bacterial isolate used in this study. When the primer concentration was

increased above 5 µM with annealing temperature ≤60 °C, multiple bands were detected in PHA accumulating *Bacillus* species but no amplification was detected in Gram-negative PHA accumulating *P. yeei,* PHA negative *Bacillus* species and *P. aeruginosa*. The optimum conditions chosen for PCR amplification of *phaC* of *B. megaterium* were as 64 °C annealing temperature and 1 µM for primer concentration.

Primer (µM)	Annealing temperature (°C)	Bacterial species											
		***B. megaterium* (MTCC428)**		***B. flexus* (NAMR4.1)**		***B. endophyticus* (TMR1.22)**		***Bacillus sp.* (NAMNR4.4)**		***P. yeei* (TMR3.1)**		***P. aeruginosa* (TMR2.13)**	
		PHA +		**PHA +**		**PHA +**		**PHA -**		**PHA +**		**PHA -**	
		0.9 Kb	**MB**	**0.9 Kb**	**MB**	**0.9 Kb**	**MB**	**0.9 Kb**	**MB**	**0.9 Kb**	**MB**	**0.9 Kb**	**MB**
1	**51**	+	-	-	-	-	-	-	-	-	-	-	-
	55	+	-	-	-	-	-	-	-	-	-	-	-
	60	+	-	-	-	-	-	-	-	-	-	-	-
	64	+	-	-	-	-	-	-	-	-	-	-	-
2.5	**51**	+	-	-	-	-	-	-	-	-	-	-	-
	55	+	-	-	-	-	-	-	-	-	-	-	-
	60	+	-	-	-	-	-	-	-	-	-	-	-
	64	+	-	-	-	-	-	-	-	-	-	-	-
5	**51**	+	*	-	+	-	+	-	-	-	-	-	-
	55	+	*	-	+	-	+	-	-	-	-	-	-
	60	+	*	-	+	-	+	-	-	-	-	-	-
	64	+	*	-	-	-	-	-	-	-	-	-	-
10	**51**	+	*	-	+	-	+	-	-	-	-	-	-
	55	+	*	-	+	-	+	-	-	-	-	-	-
	60	+	*	-	+	-	+	-	-	-	-	-	-
	64	+	*	-	-	-	-	-	-	-	-	-	-

Table 2.6: Optimization of PCR condition for amplification of internal region of *phaC* gene using primer set described in Figure 2.2

PHA +, Polyhydroxyalkanoate accumulating bacteria; PHA -, Bacteria not accumulating polyhydroxyalkanoate; *, Double band; MB, Multiple band; +, Positive; -, Absent.

2.3.3.II Validation of method

Observations of PCR amplifications using primer set performed with all the bacterial cultures at optimal conditions (Method-I) are seen in Fig. 2.5 and Fig. 2.7a and under sub-optimal conditions (Method-II) in Fig. 2.6 and Fig. 2.7b. PCR amplification of *phaC* in all the strains of *B. megaterium* was of single band of 0.9 kb fragment in Method-I (1 µM primer concentration and 64 °C annealing temperature). No amplification was detected in bacterial species other than *B. megaterium*. 16S rRNA gene was amplified in all the bacterial isolates.

PCR amplification in Method-II (10 µM primer concentration and 51 °C annealing temperature) showed presence of two bands in all the strains of *B. megaterium* with sizes 0.9 kb and 1.9 kb (Fig. 2.6 and 2.7b). Interestingly, members of Order Bacillales accumulating

PHA showed multiple bands of non-specific amplicons unique to their respective species. Strains of *B. licheniformis*, *B. cereus,B. mycoids* and *B. weihenstephanensis* did not show any amplification in Method-II. Other than *B. megaterium,* none of the isolates showed presence of the 0.9 kb band of amplification. All other bacterial species tested in present study did not show any band in PCR amplification. The banding pattern showed complete reproducibility during repeated extractions and amplifications using Method I and Method II. Two per cent deviation was noted in molecular weights of bands.

Results of PCR amplification of Method I and Method II using eight additional bacterial isolates as part of the blind folded experiment were seen in Fig. 2.8. In Method I only strain of *B. megaterium* BLQ-2/A7 showed amplification of 0.9 kb fragment, whereas no amplification was observed with other bacterial strains. Method II showed amplification of two bands (0.9 kb and 1.9 kb) in *B. megaterium* and non-specific bands were observed in PHA accumulating *B. mojavensis* MTCC8604, *B. niacin* MTCC8323, *B. simplex* MTCC7284, *Bacillus* sp. NAMNR3.5 and *Bacillus* sp. TMNR4.1.1. The strains of *B. aquimaris* MTCC6722 and *Bacillus* sp. MS4.SE3 showed no PHA accumulation and did not give amplification with Method II.

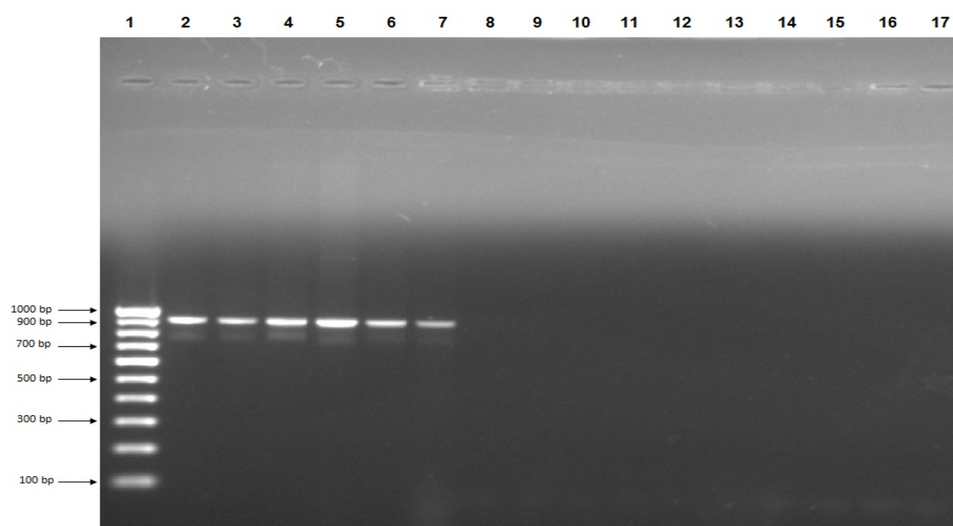


Fig. 2.5: PCR amplification of 0.9 kb internal region of *phaC* gene under optimum condition i.e. 1 µM of each primer concentration and 64 ^{o}C annealing temperature.
Where Lane **1** – molecular weight marker; **2** – *Bacillus megaterium* MTCC428; **3** – *Bacillus megaterium* TMR1.3.2; **4** – *Bacillus megaterium* TMR1.4; **5** – *Bacillus megaterium* NAMNR3.7; **6** – *Bacillus megaterium* NQ-11/A2; **7** – *Bacillus megaterium* COL1/A6; **8** – *Bacillus flexus* NAMR4.1; **9** – *Bacillus endophyticus* TMR1.22; **10** – *Bacillus thuringiensis* 164(H-14); **11** – *Bacillus sphaericus* KSD-4; **12** – *Bacillus* sp. TMR1.10.1; **13** – *Paracoccus yeii* TMR3.1; **14** – *Bacillus spizizenii* ATCC 6633; **15** – *Pseudomonas aeruginosa* ATCC 9027; **16** – *Bacillus* sp. NAMNR4.4; **17** – *Pseudomonas aeruginosa* TMR2.13

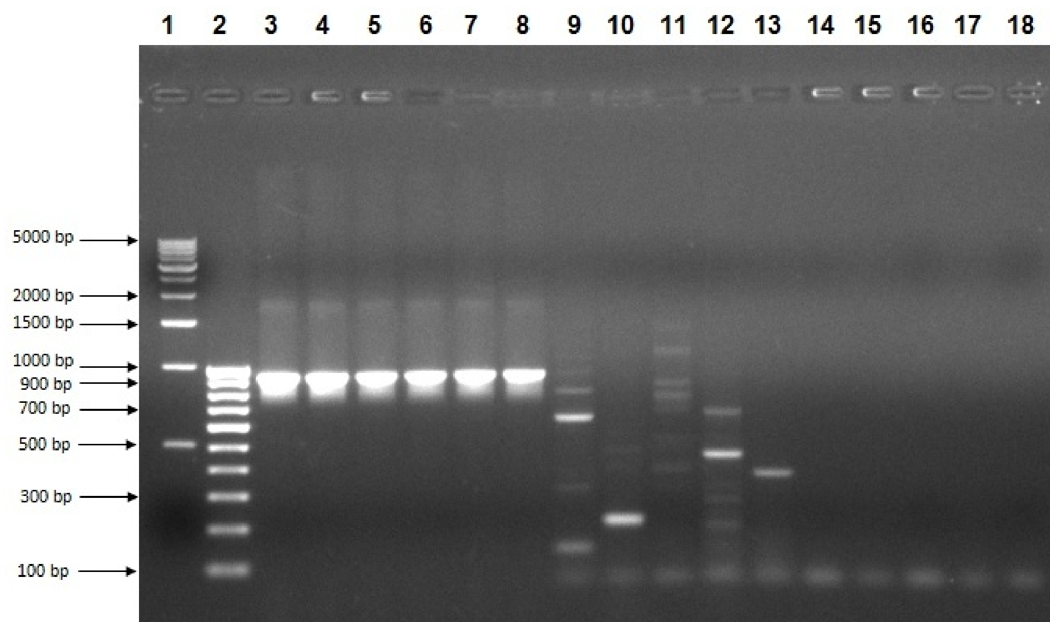


Fig. 2.6: PCR amplification of 0.9 kb internal region of *phaC* gene under sub-optimum condition i.e. 10 µM of each primer concentration and 51 °C annealing temperature.
Where Lane **1**– molecular weight marker (500 bp); **2**– molecular weight marker (100 bp); **3**– *Bacillus megaterium* MTCC 428; **4**– *Bacillus megaterium* TMR1.3.2; **5**– *Bacillus megaterium* TMR1.4; **6**– *Bacillus megaterium* NAMNR3.7; **7**– *Bacillus megaterium* NQ-11/A2; **8**– *Bacillus megaterium* COL1/A6; **9**– *Bacillus flexus* NAMR4.1; **10**– *Bacillus endophyticus* TMR1.22; **11**– *Bacillus thuringiensis* 164(H-14); **12**–*Bacillus sphaericus* KSD-4; **13** – *Bacillus* sp. TMR1.10.1; **14**– *Paracoccus yeii* TMR3.1; **15**– *Bacillus spizizenii* ATCC 6633; **16**– *Pseudomonas aeruginosa* ATCC 9027; **17**– *Bacillus* sp. NAMNR4.4; **18**– *Pseudomonas aeruginosa* TMR2.13

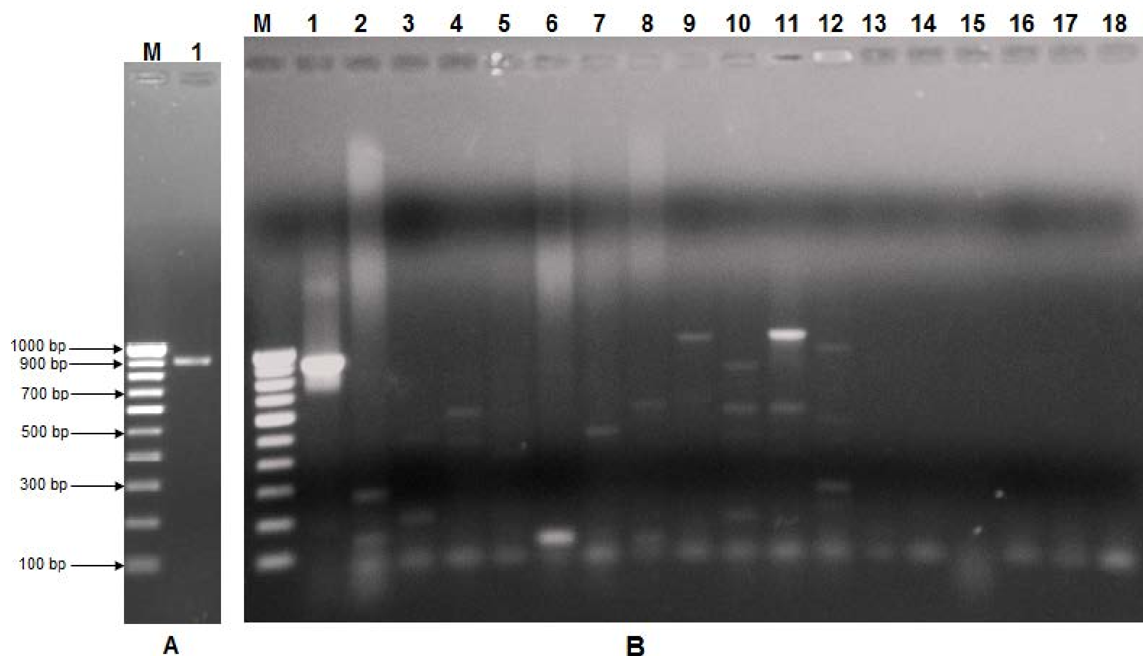


Fig. 2.7A: PCR amplification of 0.9 kb internal region of *phaC* gene under optimum condition. Where Lane **M**– molecular weight marker (100 bp); **1**– *Bacillus megaterium* QM B 1551

Fig. 2.7B: PCR amplification of 0.9 kb internal region of *phaC* gene under sub-optimum condition. Where Lane **M**– molecular weight marker (100 bp); **1**– *Bacillus megaterium* QM B 1551; **2**– *Bacillus pumilus* ATCC 7061; **3**– *Bacillus amyloliquefaciens* BGSC 10A1; **4**– *Bacillus Coagulans* ATCC 7050; **5**– *Bacillus firmus* NRS 613T; **6**– Geo*bacillus stearothermophilus* ATCC 12980; **7**– *Bacillus subtilis subsp. subtilis* NCIB 3610; **8**– *Paenibacillus dendritiformis* C168; **9**– *Marinibacillus marinus* DSMZ 1297; **10**– *Lysinibacillus sphaericus* ATCC 12123; **11**–*Lysinibacillus fusiformis* ATCC 7055T; **12** – *Bacillus circulans* ATCC 4513; **13**– *Bacillus mycoides* ATCC 31101; **14**– *Bacillus weihenstephanensis* BGSC 6A24; **15**– *Bacillus licheniformis* ATCC 8480; **16**– *Aneurinibacillus migulanus* NRS 1137T; **17**– *Bacillus cereus* ATCC 14579; **18**– negative control

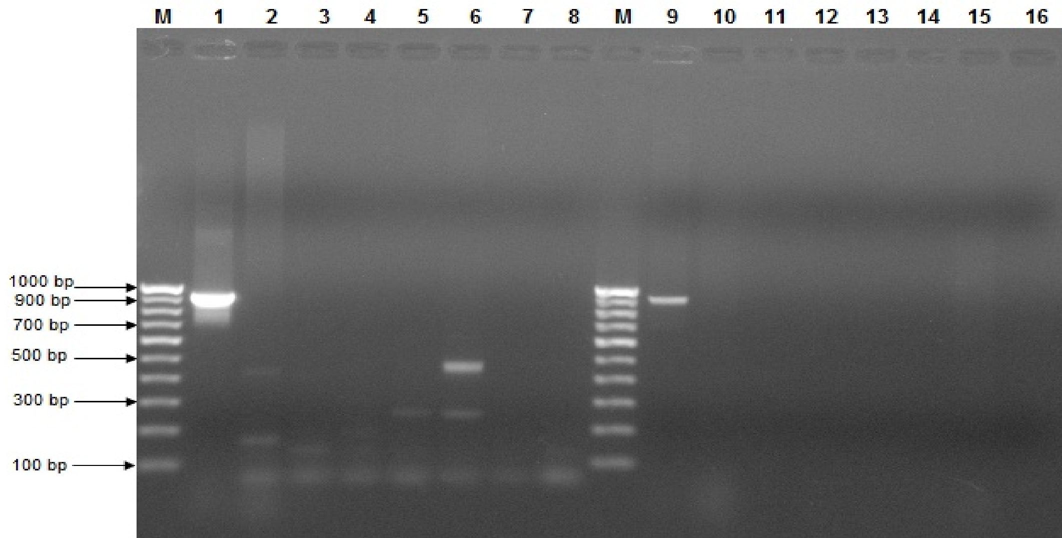


Fig. 2.8: PCR amplification of 0.9 kb internal region of phaC under sub-optimum condition (Lane 1-8) and optimum condition (Lane 9-16). Where Lane **M** – molecular weight marker (100 bp); **1**– *Bacillus megaterium*BLQ-2/A7; **2**– *Bacillus niacini* MTCC 8323; **3**– *Bacillus simplex* MTCC 7284; **4**– *Bacillus mojavensis* MTCC 8604; **5**– *Bacillus* sp. NAMNR3.5; **6**– *Bacillus* sp. TMNR4.1.1; **7**– *Bacillus aquimaris* MTCC 6722; **8**– *Bacillus* sp. MS4.SE3; **M** – molecular weight marker (100 bp); **9**– *Bacillus megaterium*BLQ-2/A7; **10**– *Bacillus niacini* MTCC 8323; **11**– *Bacillus simplex* MTCC 7284; **12**– *Bacillus mojavensis* MTCC 8604; **13**– *Bacillus* sp. NAMNR3.5; **14**– *Bacillus* sp. TMNR4.1.1; **15**– *Bacillus aquimaris* MTCC 6722; **16**– *Bacillus* sp. MS4.SE3

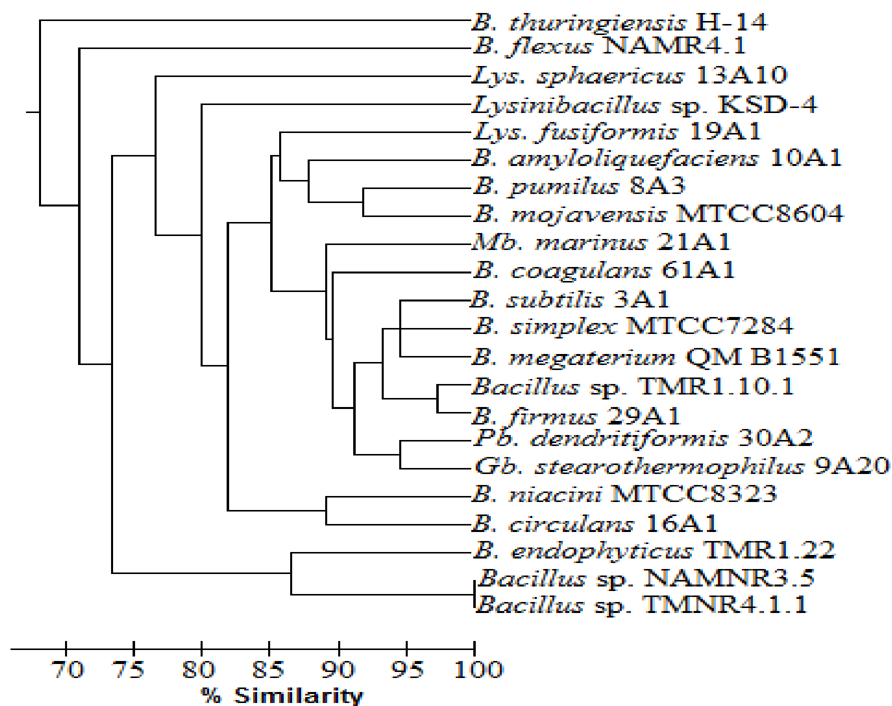


Fig. 2.9: Dendogram of multiple banding patterns obtained in different PHA accumulating *Bacillus* specieses. Clustering was achieved by un-weighted pair group average linkage (UPGMA). The computations were performed using the Probiosys Software

The dendogram constructed using multiple banding patterns of amplicon showed all the strains of Bacillales clustered differently (Fig. 2.9). Each pattern of band showed very little similarity to each other. Further, Method-II gave multiple banding patterns for PHA accumulating *Bacillus subtilis* subsp. *subtilis* but did not give any amplification for non-PHA accumulator *Bacillus subtilis* subsp. *spizizenii*. The clusters formed due to multiple bands among members of Bacillales were different from the clustering obtained with 16S rRNA gene of these species. Method-II therefore was able to discriminate PHA accumulating Bacillales up to species level. Although results were reproducible, it is recommended that it should be used cautiously in combination with other methods.

The melting curve of amplicon from *B. megaterium* TMR1.4 showed the presence of a single peak at 85 °C. This confirms the specificity of the primers for the amplification of 0.9 kb of *phaC* in *B. megaterium.*

PCR products of 0.9 kb obtained with *Bacillus megaterium* TMR1.3.2 and *Bacillus megaterium* TMR1.4 were sequenced and deposited in GenBank with accession numbers JF423932 and JF423933, respectively. Nucleotide blast of these sequences showed 100% similarity with *phaC* gene of *B. megaterium*. Pair-wise alignment of sequences obtained from *B. megaterium* TMR1.4 with *phaC* of other *Bacillus* sp. showed 73-75% similarity with *B. anthracis*, *B. cereus*, *B. mycoids*, *B. thuringiensis*, and *B. weihenstephanesis*. Phylogenetic tree constructed using these sequences with sequences of *phaC* of various species showed alignment of the sequences with *phaC* of *B. megaterium* (Fig. 2.10).

2.3.2.III PCR amplification of *PhaC* gene from sand-dune bacterial isolates

Genomic DNA of bacterial isolates obtained from sand-dune was subjected to PCR amplification of *PhaC* gene using Method I (Fig. 2.11). Out of 22 bacterial isolates screened, 13 bacterial isolates showed amplification of 900 bp amplicon and 9 bacterial isolates showed no amplification. As per Method I, those 13 bacterial isolates showed amplification of 900 bp amplicon were identified as *B. megaterium*.

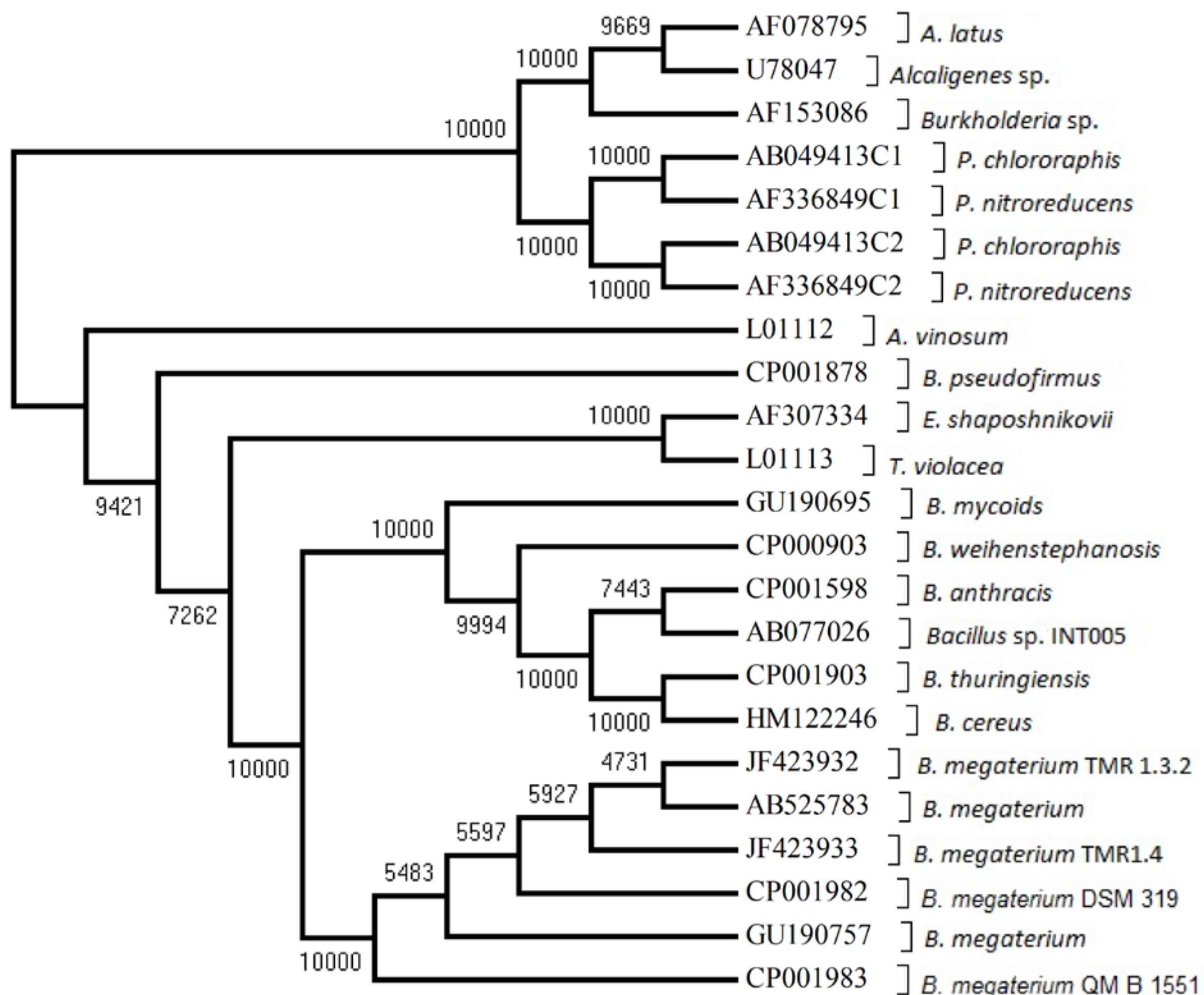


Fig. 2.10: Phylogenetic tree of *phaC* genes of various bacterial species. Tree was constructed using 1000 seeds and 10000 bootstraps. Except for the strains *B. megaterium* TMR1.3.2 and *B. megaterium* TMR1.4 all the sequences used were complete. Node present the value of bootstrap obtained out of 10000

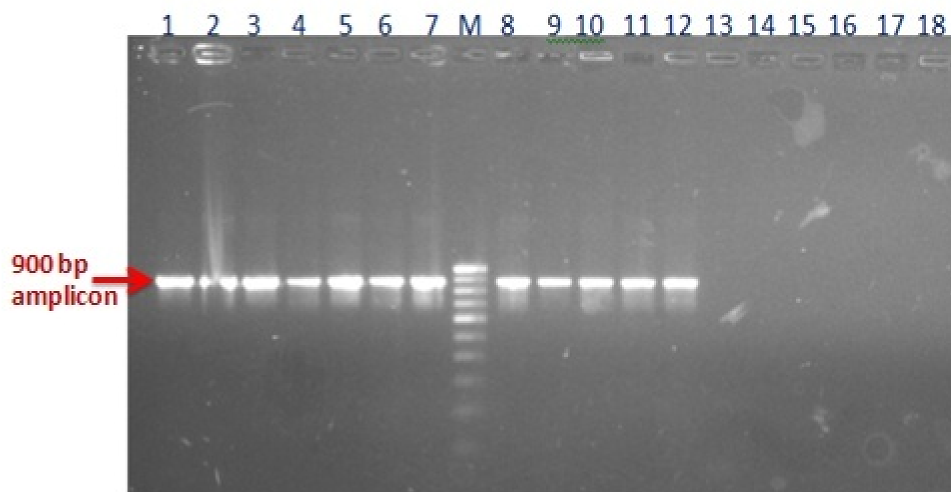


Fig. 2.11: PCR amplification of 0.9 kb internal region of *phaC* gene from PHA accumulating sand-dune bacterial isolates. Where Lane **1**– *Bacillus megaterium* QM B 1551; **2**– TMR4.3; **3**– NAMR1.12; **4**– TMR1.7; **5**–TMNR1.5; **6**–TMR1.28; **7**–TMR1.3.1a; **M**– molecular weight marker (100 bp); **8**– TMR1.3.1b; **9**–TMR1.26; **10**–TMR2.4; **11**–TMNR2.4; **12** –NAMNR3.7; **13**–NAMR1.8; **14**–NAMR1.6; **15**–TMR1.9.1; **16**–TMR1.9.2; **17**–TMR1.10.1; **18**– TMNR1.3

Nucleotide sequences of *phaC* gene from *B. megaterium* showed 73-75% homology with *phaC* gene of other polyhydroxyalkanoate accumulating *Bacillus* sp. This indicates the uniqueness of the sequences of *phaC* of *B. megaterium*. The differences in the gene sequences serving as recognition of unique regions present at 15-34 and 912-931 on *phaC* were utilised to design the primers for identification of *B. megaterium*. Earlier Shamala *et al*. (2003) designed a set of primer using *phaC* gene sequences of *B. megaterium* for detection of PHA producing *Bacillus* sp., where the amplification product was 590 bp in different PHA accumulating *Bacillus* sp. including *B. megaterium*. In the present study the internal primers designed are unique and binds in the *phaC* gene at 15-34 (Forward primer) and 912-931 (Reverse primer). PCR amplification using this primer set results in single amplicon of 0.9 kb seen only in *B. megaterium* at specified optimum conditions. Specificity of the primers were validated by sequencing of 0.9 kb PCR amplification product from *B. megaterium,* that gave 100% similarity with *phaC* of *B. megaterium*, and Real-Time PCR that gave T_m of the amplicon as 85 °C. Recently genomes of two strains of *Bacillus megaterium* namely QM B1551 and DSM 319 were sequenced completely (Eppinger *et al*., 2011). One strain namely *Bacillus megaterium* QM B1551 was incorporated for cross verification of specificity of the primers and method thereof. The method showed amplification of 0.9 kb fragment with this strain. This confirms the specificity of the primers. The ubiquity of *B. megaterium* in the natural environment and its emerging industrial importance could help if identification of new potential strains of this bacterium becomes rapid and easier. In comparison to routinely used molecular methods for identification of *B. megaterium*, the present method is rapid and specific. Further, this method does not require sequencing of amplicon or any additional test to confirm its identification. However, use of additional method is prerogative of researcher.

PCR based randomly amplified polymorphic DNA (RAPD) technique has been used for molecular typing and identification among closely related species of the Genus *Bacillus* (Qingming and Zongping 1997; Matarante *et al*., 2004). The pattern of amplified DNA fragments produce during RAPD-PCR provides information on genetic variability between organisms of different species. Interestingly, in Method-II *B. megaterium* and other PHA producing Bacillales gave multiple banding patterns of non-specific amplicon unique to respective species. Members of Bacillales such as *Lysinibacillus, Marinibacillus, Geobacillus, Aneurinibacillus, Paenibacillus* and all the species of *Bacillus* reported for accumulation of PHA were included in present study. Although, *B. licheniformis*, *B. cereus,*

B. mycoids and *B. weihenstephanensis* were reported as PHA accumulators, the isolates of these species used in this study neither showed PHA accumulation nor any amplification in Method-II.

Different methods have been used for the identification of *B. megaterium* which are both laborious and time consuming. During the last decade a number of new species of the genus *Bacillus* have been described showing very close similarity to *B. megaterium*. This has resulted in difficulty in accurate nomenclature of the isolates. Even though several methods are used, identification is limited to genus level only (Law *et al*., 2001; Santimano *et al*., 2009). It is known that among the members of Bacillales, *B. flexus* and *B. simplex* show very close similarity with *B. megaterium* in physiological characteristics and 16S rRNA sequencing but in this study PCR amplification of the 0.9 kb region of *phaC* gene under optimum condition resulted in no amplification in *B. flexus* and *B. simplex*. Pair-wise alignment of 16S rRNA sequences of *B. megaterium* and *B. flexus* showed 11 nucleotide differences in the hyper variable region in between 150-200 bp region of their 16S rRNA gene. Inconsistency in biochemical test results and errors in sequencing of 16S rRNA gene may lead to wrong identification. Hence, this method can be ideally used to differentiate *B. megaterium* from *B. flexus* and *B. simplex*. With the increasing importance of *B. megaterium* in the field of biotechnology, the ambiguity observed with its identification by conventional biochemical and molecular methods would limit its application. The present simple yet rapid method counteracts these problems and is thus a suitable alternative for the accurate identification of the organism.

Like *B. megaterium* other members of Bacillales are attractive industrial organisms with known capabilities to produce enzymes, recombinant proteins, antibiotics, purine nucleotides, insecticidal proteins, vitamins, sugars biopolymer and biofertilizers (Haki and Rakshit 2003; Schallmey *et al*., 2004; Tsai *et al*., 2007; Valappil *et al*., 2007; Raza *et al*., 2008; Maki *et al*., 2009; Park *et al*., 2010). These strains are gaining interest for economic production of these compounds for a variety of reasons including high growth rates, short fermentation cycle times, ability to tolerate wide pH and temperature ranges, easy to maintain in spore forms, capacity to secrete proteins into the extracellular medium, amenable to genetic engineering and the GRAS (generally regarded as safe) status (Schallmey *et al*., 2004). Further, the sub-optimal conditions described as Method-II was suitable for rapid differentiation for identification of polyhydroxyalkanoate accumulating members of Bacillales.

2.3.3.IV PCR amplification of 16S rRNA gene

Those bacterial isolates did not showed any amplification and few bacterial isolates which are positive for amplification of *PhaC* gene, were processed for 16S rRNA gene sequencing for their identification. Nucleotide sequences obtained were analyzed and deposited in Genbank with their accession numbers. Nucleotide blast results of these sequences showed 99-100% similarity with 16S rRNA gene of their respective species from NCBI data base. Phylogenetic tree was constructed using these sequences along with 16S rRNA gene reference sequences of various species. The clustering of these sequences with the reference sequences of same species in the phylogenetic tree (Fig. 2.12) and maximum sequence homology of the isolates showed that seven isolates were matching with *B. megaterium*, one with *B. flexus*, one with *B. endophyticus*, two with *B. vireti*, two with *Bacillus* sp., one with *P. oryzihabitans,* one with *Paracoccus yeei* and one with *Paracoccus sp.*.

In combination with phenotypic and genotypic characterization all the PHA accumulating bacterial isolates were identified (Table 2.7). Most of the bacterial isolates were identified up to their species level but three isolates could be identified only to their genus level. Diversity of PHA accumulating bacteria was observed from coastal sand-dune ecosystem, which includes both Gram positive and negative bacteria. Gram-positive bacteria belonged to the genus *Bacillus* and included, *Bacillus megaterium* (13), *Bacillus flexus* (1), *Bacillus endophyticus* (1), *Bacillus vireti* (2), *Bacillus* sp. (2). Gram-negative bacteria belonged to the genera *Pseudomonas* and *Paracoccus* and included *Pseudomonas oryzihabitans* (1), *Paracoccus yeei* (1) and *Paracoccus* sp. (1). Among PHA producing bacteria, member of *Bacillus* contributes up to 86.4% of the total PHA producing isolates obtained from sand-dune ecosystem in this study. The predominance of *Bacillus* sp. could be due to their inherent properties like PHA accumulation and endospore production to survive in such extreme ecosystem in terms of nutrient availability and continuously changing environmental conditions (Zhao *et al*., 2007; Bibi *et al*., 2011). In one of our studies on screening of PHA accumulating bacteria from coastal sand-dunes of East Coast of India, it is noted that PHA accumulating Gram-positive heterotrophic bacteria accounted for 66% of the bacterial population in the Rhizosphere region (Palanker, 2011). Siderophore producing bacteria such as *Bacillus* sp., *Brochothrix* sp., *Corynebacterium* sp., *Renibacterium* sp., *Kurthia* sp., *Azotobacter* sp., *Pseudomonas* sp. and *Streptomyces* sp. were reported from coastal sand-dune

of Goa (Gaonkar *et al*., 2012) however, *Bacillus* sp. was found as predominant siderophore producing bacteria. Bacillus sp. has also been reported from coastal sand-dune ecosystem for their plant growth promoting activity (Shishido *et al*., 1996; Park *et al*., 2005; Canbolat *et al*., 2006; Godinho *et al*., 2010; Hong and Lee, 2014).

S.No.	**Bacterial isolate number**	**Identification**	**16S rRNA gene Accession numbers**
1	NAMR1.8	*Bacillus* sp.	KF500398
2	NAMR1.6	*Pseudomonas oryzihabitans*	KF470870
3	NAMR4.1	*Bacillus flexus*	HM026605
4	TMR1.9.1	*Bacillus vireti*	HQ897170
5	TMR1.9.2	*Bacillus vireti*	KF470871
6	TMR1.10.1	*Bacillus* sp.	HM035484
7	TMR1.22	*Bacillus endophyticus*	HQ897169
8	TMR1.3.2	*Bacillus megaterium**	GU984576
9	TMR4.3	*Bacillus megaterium**	ND
10	NAMR1.12	*Bacillus megaterium**	ND
11	NAMNR3.7	*Bacillus megaterium**	GU951917
12	TMR1.7	*Bacillus megaterium**	GU984577
13	TMR1.4	*Bacillus megaterium**	GU951918
14	TMNR1.5	*Bacillus megaterium**	GU984575
15	TMR1.28	*Bacillus megaterium**	ND
16	TMR1.3.1A	*Bacillus megaterium**	ND
17	TMR1.3.1B	*Bacillus megaterium**	GU906277
18	TMR1.26	*Bacillus megaterium**	GU906276
19	TMR2.4	*Bacillus megaterium**	ND
20	TMNR2.4	*Bacillus megaterium**	ND
21	TMNR1.3	*Paracoccus* sp.	HM035483
22	TMR3.1	*Paracoccus yeei*	GU906275

Table 2.7: Complete identification of PHA accumulating sand-dune bacteria

ND: not done; *: bacterial isolates showed amplification of *PhaC* gene of *B. megaterium* using Method I

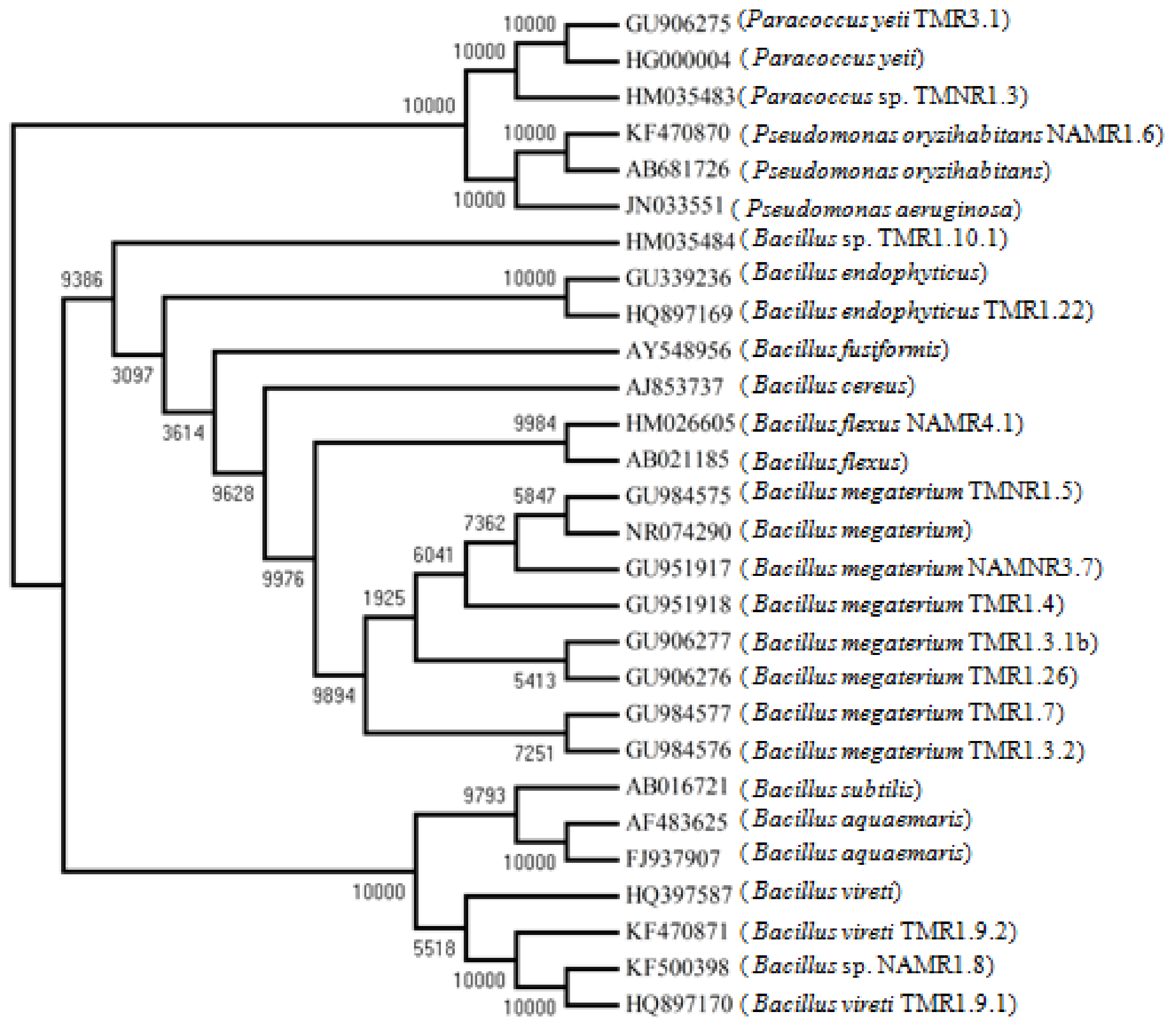


Fig. 2.12: Phylogenetic tree of 16S rRNA gene from selected bacterial isolates. Tree was constructed using 1000 seeds and 10000 bootstraps.

PHA accumulating bacteria has both ecological as well as industrial importance. With the screening of new PHA accumulating bacteria from different ecosystems and by understanding the role of intracellular PHA, it is clear that this polymer is not only a storage compound but also mobilized by intracellular PHA depolymerase produced by the organism and used as source of carbon and energy when carbon become limited (Kadouri *et al*., 2005; Zhao *et al*., 2007). In natural ecosystems intracellular PHA accumulated by bacteria enhances the survival of these organisms under environmental stress. PHA present in the environment can also be used for source of carbon and energy by other microorganisms producing extracellular PHA depolymerase. Bacterial cells containing higher amount of PHA survives longer time than those that do not contain PHA or have less PHA content, in changing environmental conditions (Dawes and Senior, 1973; Kadouri *et al*., 2002). Tal and Okon (1985) reported that when bacterial cells are exposed to stress conditions, such as ultraviolet irradiation, desiccation and osmotic pressure, cells with less PHB content died faster than cells with high PHB content. Ayub *et al*. (2004) demonstrated thermal and oxidative stress tolerance of *Pseudomonas* sp. 14-3 in PHA accumulating and non-accumulating conditions. The increase in levels of stress resistance was observed when PHA was accumulated by the isolate. They suggested that high PHB accumulation corresponds to high stress resistance in bacteria adapted to extreme environmental conditions. Arora *et al*. (2006) studied the effect of various salt concentrations on growth and PHB accumulation by *Sinorhizobium* strains. They obtained minimum PHB accumulation at low salt concentration and maximum PHB accumulation at higher salt concentration and suggested the role of PHB in cell protection in salinity conditions. Valappil *et al*. (2007) reported that maximum PHA was accumulated in *Bacillus cereus* and *Clostridium botulinum* just prior to spore formation and accumulated PHA was utilized during sporulation. They suggested that spore formation, spore germination and cyst production may be related to PHA synthesis and degradation.

Polyhydroxyalkanoates are biodegradable and possess properties similar to synthetic thermoplastics, is the reason behind its industrial interest to commercialize this polymer. PHAs are diverse polyesters produced naturally by many bacterial species under environmental stress. Approximately 150 different hydroxyalkanoates have been characterized as monomers of homopolymer or co-polymer of PHA (Steinbuchel and Lutke-Eversloh, 2003). The diverse type of PHA formation is mainly due to the broad substrate specificity of PHA synthases as well as the type of carbon source utilized by the microorganism for PHA accumulation (Sudesh and Doi, 2005). Based on the carbon chain

length, PHAs are of two types, short chain length (SCL) PHAs consisting of 3-5 carbon atoms and medium chain length (MCL) PHAs of 6-14 carbon atoms. Majority of the bacterial species such as, *Alcaligenes*, *Ralstonia*, *Bacillus*, *Rhizobium*, *Paracoccus*, *Burkholderia*, *Micrococcus*, *Chromatium*, *Halomonas*, *Vibrio* and *Streptomyces* are known for SCL-PHA production (Koller *et al*., 2010) but few isolates could accumulate co-polymers consisting of SCL or SCL and MCL PHAs (Steinbuchel and Hein, 2001; Reddy *et al*., 2009). MCL-PHAs are mainly produced by *Pseudomonads* belongs to the ribosomal RNA (rRNA) homology group I, these are *P. aeruginosa*, *P. oliovorans*, *P. putida*, *P. stutzeri*, *P. cichorii*, *P. guezennei* and *Pseudomonas* sp. (Kim *et al*., 2007; Simon-Colin *et al*., 2008; Narancic *et al*., 2012).

Chapter-III

Production of polymer

3.I.1 Introduction

Polyhydroxyalkanoates are biopolymers accumulated by many prokaryotic organisms as carbon and energy storage material. Depending on the cultivation condition, especially when there is a limitation of nitrogen and excess of carbon source present in the growth medium some bacterial strains accumulate PHA up to 90% of their dry cell weight (Anderson and Dawes, 1990). PHAs have attracted industrial interest as a biodegradable thermoplastic to replace synthetic plastics in the market. In addition to biodegradable thermoplastic nature, PHAs are biocompatible and produced from renewable carbon sources. The monomeric composition and quality of polymer depends on the bacterial strain and the carbon source they use for production of PHA (Doi, 1990). Polyhydroxybutyrate is a commonly produced and widely studied biopolyester among the homopolymer of PHA. Co-polymers like poly-3-hydroxybutyrate-co-3-hydroxyvalerate (P(3HB-co-3HV)) have more useful industrial and medical applications due to its superior quality than PHB.

Although PHAs are produced globally by various companies, its commercialization is still behind synthetic plastics because of its higher production cost. The major factors affecting the cost effectiveness of PHA production includes the bacterial strain, inexpensive carbon source, fermentation strategies for high cell density cultivation and downstream processes (Lenz and Merchessault, 2005; Hazer and Steinbuchel, 2007; Atlic *et al*., 2011). To overcome these limitations, several studies are undertaken for optimization of fermentation process for high cell density PHA production (Chen *et al*., 2001; Sun *et al*., 2006; Khanna and Srivastava, 2008; Ibrahim and Steinbuchel, 2010; Pradella *et al*., 2010; Cavalheiro *et al*., 2012; Kanjanachumpo *et al*., 2013).

Both Gram-positive and Gram-negative bacteria are known for PHA accumulation. Currently, only Gram-negative bacteria are used for industrial scale PHA production. These bacteria contain pyrogenic lipopolysaccharide (LPS) endotoxin, which get co-purified with PHA during extraction. PHA produced from these organisms requires additional purification steps resulting in higher production cost. Gram positive bacteria such as *Bacillus megaterium* do not contain LPS. In addition, this bacterium is aerobic, known for rapid growth using diverse cheaper carbon substrates and shows resistance to high osmotic pressure. These important properties of *Bacillus megaterium* can be exploited for industrial PHA production. So far,

there is only one report on high cell density PHB production using *B. megaterium* (Kanjanachumpo *et al*., 2013).

The following investigation is planned to check the ability of polymer accumulating bacterial isolates in shake flask condition for PHA production using glucose as sole source of carbon.

In Section I, these isolates were screened for their ability to accumulate PHA using organic acids as carbon source. Strain *B. megaterium* TMR 1.3.2 was selected for production of homo-polymer and co-polymer using glucose as carbon source and glucose with valeric acid as carbon sources.

In Section II, *B. megaterium* TMR 1.3.2 and *B. megaterium* Col1/A6 were selected for study on production of PHB in batch and fed-batch cultivation methods for high cell density production.

Section I

PHA production and polymer characterization

3.I.2 Materials and methods

3.I.2.1 Biomass and PHA production by sand-dune bacterial isolates

All PHA accumulating bacterial isolates were grown in shake flask conditions. A single colony of isolate grown on Nutrient agar for 24 h was inoculated in to 250 ml Erlenmeyer flask containing 100 ml of E2-mineral broth (Appendix A). Glucose (20 g/L) was used as sole source of carbon. The flask was incubated on shaker (170 rpm) for 48 h at 30 °C. The culture broth was processed for harvesting biomass and PHA extraction. The experiment was repeated for three times.

3.I.2.2 Analytical methods

3.I.2.2.I Biomass estimation

Twenty ml of culture broth was transferred to 50ml tube and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was discarded and the cell pellet was washed twice with de-ionized water. Cell pellet was dried at 80 °C until constant dry weight achieved.

3.I.2.2.II PHA estimation

Twenty ml of culture broth was taken in a 50 ml tube and centrifuged at 10,000 rpm for 10 min at 4 °C (Santimano *et al*., 2009). Supernatant was discarded and cell pellet was washed twice with de-ionized water. Washed cell pellet was suspended with 10 ml of sodium hypochlorite solution (2% available chlorine) and incubated at 30 °C on shaker at 170 rpm for 20 min. Equal volume of de-ionized water was added to the suspension and centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was discarded and the pellet was resuspended in distilled water and centrifuged again. Pellet was suspended in 10ml of chilled ethanol (95%) and centrifuged at 12,000 rpm for 20 minutes at 4 °C. The supernatant was discarded and the pellet of polymer was dried at 80 °C until constant dry weight achieved.

3.I.2.3 PHA accumulation using organic acids as carbon source

Organic acids such as pyruvic acid, succinic acid, propionic acid, valeric acid and octanoic acid were neutralized using sodium hydroxide and sterilized separately prior to addition in the medium. Twenty-two bacterial isolates were spot inoculated on E2-mineral medium agar plates containing respective organic acid as sole carbon source. The amount of organic acid

used is equivalent to 1% of glucose in the medium. The inoculated plates were incubated at 28 °C. Colonies on plates were stained with Nile blue A (as described in section 2.2.2 of Chapter II) and visualised under UV transilluminator.

3.I.2.4 Polymer production using glucose and valeric acid

Bacillus megaterium TMR1.3.2 was used for the study. The isolate was inoculated in 250 ml flask containing 100 ml of E2-mineral medium having different concentrations of valeric acid as sole carbon source and different combinations of glucose/valeric acid as carbon sources. These different combinations of valeric acid and glucose tested for polymer production can be seen in Table 3.2. The inoculated flasks were incubated at 30 °C on an Orbitek shaker (170 rpm) for 48 h. Biomass and polymer was determined as per the method described in section 3.I.2.2.I and 3.I.2.2.II.

3.I.2.6 Characterization of polymer produced by *Bacillus megaterium* TMR1.3.2

The *B. megaterium* TMR1.3.2 was inoculated in 250 ml flask containing 100 ml of E2-mineral medium having glucose 2% w/v as sole carbon source. In another flask the isolate was inoculated into E2 mineral medium containing combination of glucose (2% w/v) and valeric acid (0.8% w/v) as carbon sources. The flasks were incubated at 30 °C on an Orbitek shaker (170 rpm) for 48 h. After incubation, the polymer was extracted by the method described in section 3.I.2.2.II. The polymers obtained were characterized as follows.

3.I.2.6.I FTIR Spectroscopy

Polymer samples (10 mg) were dissolved in 200 µl of chloroform and made to thin film (Divyashree *et al*., 2009). The FTIR spectrum of the film of polymer was recorded at 400-4000 cm^{-1} in FTIR.

3.I.2.6.II ^{1}H NMR Spectroscopy

Polymer samples (10 mg) were dissolved in deutero-chloroform ($CDCl_3$) (0.5 ml) and analyzed at 400 MHz in AMX 400 (Bruker) spectrophotometer (Divyashree *et al*., 2009).

3.I.2.6.III ^{13}C NMR Spectroscopy

Polymer samples (20 mg) were dissolved in CDCl3 (0.5 ml) and analyzed at 400 MHz in AMX 400 (Bruker) spectrophotometer (Dai *et al*., 2008).

3.I.2.7 Characterization of polymer produced by all the bacterial isolates

The 22 bacterial isolates were grown in E2-mineral medium containing glucose (20 g/L) as sole source of carbon. The polymer extracted from these bacterial isolates was characterized by FTIR spectroscopy using the method described in the section 3.I.2.6.I.

3.I.3 Results and discussion

3.I.3.1 PHA production by sand-dune bacterial isolates

Production of biomass and PHA of all bacterial isolates using glucose as sole carbon source is shown in Fig. 3.1. At 48 h of cultivation time all the bacterial isolates showed PHA accumulation ranges between 21.8-71.2% of their dry cell weight. *Bacillus* sp. NAMR1.8 showed maximum (71.2% w/w) and *Paracoccus* sp. TMNR1.3 showed lowest (21.8% w/w) PHA accumulation among sand-dune bacterial isolates. However, *Bacillus megaterium* TMR1.3.2 showed over all maximum biomass (7.535±0.028 g/L) and PHA (3.526±0.011 g/L) accumulation followed by *Bacillus megaterium* TMR1.28 showed biomass (6.921±0.035 g/L) and PHA (3.185±0.024 g/L). This indicates the bacterium grew rapidly and accumulates PHA using glucose as carbon source. *Pseudomonas oryzihabitans* NAMR1.6, *Bacillus vireti* TMR1.9.1, *Bacillus vireti* TMR1.9.2 and *Bacillus endophyticus*TMR1.22 are being reported for the first time as PHA accumulating bacteria species.

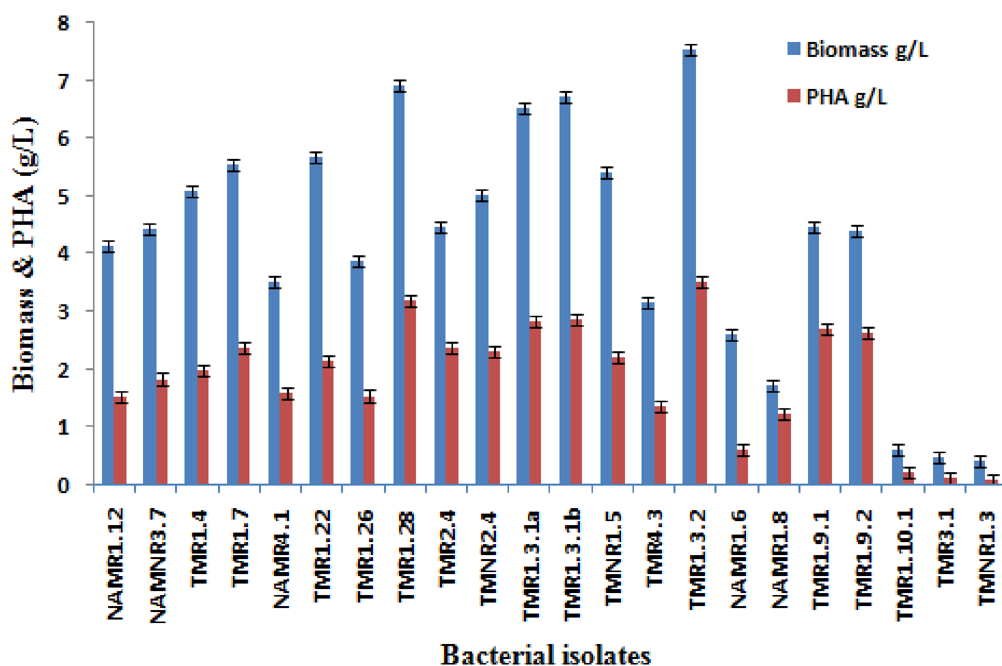


Fig. 3.1: Biomass and PHA production by the bacterial isolates obtained from sand dunes

Chen *et al*. (1991) demonstrated PHB production using different *Bacillus* species and reported PHB accumulation up to 50% of their dry cell weight. Shamala *et al*. (2003) reported isolation of different PHA accumulating *Bacillus* species from soil sample and showed PHA accumulation in between 10-40% of their dry cell weight using sucrose as carbon source. Yilmaz *et al*. (2005) have also been reported PHB accumulating *Bacillus* species such as *B. brevis*, *B. cereus*, *B. circulans*, *B. coagulans*, *B. licheniformis*, *B. megaterium*, *B. sphaericus* and *B. subtilis* from soil samples. Where all the bacterial isolates showed PHB accumulation between 1.06-41.67% of their dry cell weight and *B. brevis* M6 showed highest PHB accumulation (41.67%). Full *et al*. (2006) have reported 13 PHA accumulating Gram-positive and Gram-negative bacteria from different environmental samples, where *Bacillus* sp. CL1 showed maximum PHA accumulation 91% of DCW using glucose as carbon source. Rohini *et al*. (2006) demonstrated PHB production by *B. thuringiensis* R1 isolated from soil sample and reported 64.1% of dry cell weight as PHB using glycerol as carbon source. Lopez-Cortes *et al*. (2008) reported PHB accumulating bacteria *Bacillus* sp., *Staphylococcus* sp., *Paracoccus* sp., *Methylobacterium* sp., *Micrococcus* sp. and *Rhodococcus* sp. from polluted marine microbial mat. These bacterial isolates showed PHB accumulation between 0.04-66.5% of their dry cell weight and *Bacillus* sp. C18 showed maximum PHB accumulation (66.5% w/w). Different PHA accumulating bacteria such as *Bacillus* sp., *Pseudomonas* sp.,

Alcaligenes sp., *Aeromonas* sp. and *Chromobacterium* sp. were isolated by Reddy *et al*. (2008) from sludge samples of municipal sewage treatment plant. These bacterial isolates showed PHA accumulation between 17.71-64.32% of their dry cell weight using glucose as carbon source. Among these, *Bacillus* sp. 88D showed highest PHA accumulation of 64.32% of the dry cell weight. Prabhu *et al*. (2010) reported PHA accumulation by *B. megaterium* NQ-11/A6 isolated from marine sediment. The strain showed 61% of DCW as PHA using glucose as carbon source.

3.I.3.2 PHA production using organic acids as carbon source

Twenty-two bacterial isolates were tested for growth and PHA accumulation using various organic acids pyruvic acid, succinic acid, propionic acid, valeric acid and octanoic acid (Table 3.1). In the presence of pyruvic acid all the isolates showed PHA accumulation. Seventeen isolates showed accumulation using succinic acid, 9 isolates showed accumulation using propionic acid, 15 isolates showed accumulation using valeric acid and none of the isolates showed growth or PHA accumulation on octanoic acid. Seven bacterial isolates showed PHA accumulation using all the organic acids tested except octanoic acid. Interestingly these bacterial isolates were belongs to *Bacillus megaterium*. In compare to propionic acid, bacterial isolates showed more growth and PHA accumulation in the presence of valeric acid. Five bacterial isolates TMR2.4, TMNR1.3, TMR1.7, TMR1.3.2 and TMR3.1 showed average degree of PHA accumulation using valeric acid. Many researchers reported P(HB-co-HV) co-polymer production from *Alcaligenes eutrophus*, *Alcaligenes latus*, *Pseudomonas pseudoflava*, *Bacillus cereus*, *Micrococcus halodenitrificans*, *Bacillus thuringiensis* R-510, *Cupriavidus necator*, *Pseudomonas* sp., using propionic acid and valeric acid as precursor along with a major carbon source (Ramsay *et al*., 1990; Park *et al*., 1997; Madison and Huisman, 1999). Since these organic acids are toxic to bacterial cell, its presence even at lower concentrations inhibits cell growth (Fay and Farias, 1975; Ramsay *et al*., 1986). Byrom (1987) reported that in the presence of 0.1% (w/v) propionic acid the growth of *R. eutrophus* was inhibited. Park *et al*. (1997) studied co-polymer P(3HB-co-3HV) production from *B. thuringiensis* R-50 using glucose and propionic acid or propionic acid alone as carbon source. As the propionic acid concentration increased in the medium from 0 to 0.8% (w/v) the mole fraction of 3HV units in the co-polymer increased from 0 to 84%. However, only in the presence of propionic acid (0.1%) this strain produced poly(3HB-co-3HV) containing 42 mol% of 3HV. When the propionic acid concentration was increased the mol% of 3HV in polymer was decreased. Hu *et al*. (1997) have reported poly(3HB-co-3HV) production by a

sludge bacteria using butyric acid and valeric acid. When valeric acid (0.3%) used as sole carbon source, poly(3HB-co-3HV) produced was having 54 mol% of 3HV.

Isolates	Organic acids									
	Pyruvic acid		Succinic acid		Propionic acid		Valeric acid		Octanoic acid	
	Growth	PHA	Growth	PHA	Growth	PHA	Growth	PHA	Growth	PHA
NAMR1.6	+	+	-	-	-	-	+	-	-	-
NAMR1.8	+	+++	+	+++	+	-	+	-	-	-
NAMR1.12	+	+++	+	+++	+	+	+	+	-	-
NAMR4.1	+	++	+	-	+	-	+	+	-	-
NAMNR3.7	+	+++	+	++	+	+	+	+	-	-
TMR1.4	+	+++	+	-	+	-	+	+	-	-
TMR1.9.1	+	+++	+	+++	-	-	-	-	-	-
TMR1.9.2	+	+++	+	++	-	-	-	-	-	-
TMR1.22	+	+++	+	+++	+	+	+	-	-	-
TMR1.26	+	+++	+	++	+	+	+	+	-	-
TMR1.28	+	+++	+	++	+	-	+	+	-	-
TMR1.10.1	+	++	+	-	-	-	-	-	-	-
TMR2.4	+	++++	+	+	+	-	+	++	-	-
TMR1.3.1a	+	++	+	+	+	+	+	+	-	-
TMR1.3.1b	+	++	+	+	+	+	+	+	-	-
TMNR1.3	+	+++	+	+	+	-	+	++	-	-
TMNR2.4	+	++++	+	+++	+	-	+	+	-	-
TMNR1.5	+	+++	+	+	+	-	+	+	-	-
TMR4.3	+	+++	+	+	+	+	+	-	-	-
TMR1.7	+	++++	+	+	+	+	+	++	-	-
TMR1.3.2	+	++++	+	+	+	+	+	++	-	-
TMR3.1	+	+++	+	-	+	-	+	++	-	-

Table. 3.1: Extent of growth and PHA accumulation by the isolates on E2-mineral medium containing organic acids as sole sources of carbon. Where +: low; ++: average; +++: high; ++++: excellent; -: no.

Bacillus megaterium TMR1.3.2 was used for polymer production using different concentrations of valeric acid alone and in combination with glucose as carbon sources (Table 3.2). When valeric acid was used as the sole carbon source, no visible growth was observed irrespective of the valeric acid concentration (0.25 to 1% w/v). However, when valeric acid was used along with glucose (major carbon source), biomass and PHA accumulation was observed in all the combinations of carbon sources tested. The optimum condition obtained for polymer production was glucose (2% w/v) and valeric acid (0.8% w/v).

Glucose (%)	Valeric acid (%)	Growth	Biomass (g/L)	PHA (g/L)
0	1	-	-	-
0	0.8	-	-	-
0	0.5	-	-	-
0	0.3	-	-	-
0	0.1	-	-	-
0	0.05	-	-	-
0	0.025	-	-	-
2	1	+	2.478	0.75
2	0.8	+	2.713	0.91
1	1	+	2.265	0.66

Table. 3.2: Production of PHA by *B. megaterium* TMR1.3.2 with valeric acid as sole carbon source or in combination with glucose.

The polymers extracted from *B. megaterium* TMR1.3.2 grown in the presence of glucose and combination of glucose and valeric acid were characterized by FTIR, ^{1}HNMR and ^{13}CNMR. The FTIR spectra of both the polymers showed absorption peaks at similar positions (Fig. 3.2). The presence of absorption band at 1724-1750 cm^{-1} corresponds to carbonyl (C=O) stretching of polyester. The bands at 1381 and 1150-1320 cm^{-1} corresponds to CH_3 and CH_2 groups, respectively. Band at 2850-3050 cm^{-1} corresponds to CH group. The presence of these absorption bands confirms both the polymer as polyesters. The FTIR spectra obtained are in agreement with the reports on characterization of standard PHB by Xiao and Jiao (2011). Many researchers have reported characterization of PHA using FTIR (Divyashree *et al*., 2009; Shamala *et al*., 2009; Prabhu *et al*., 2010; Gumel *et al*., 2012).

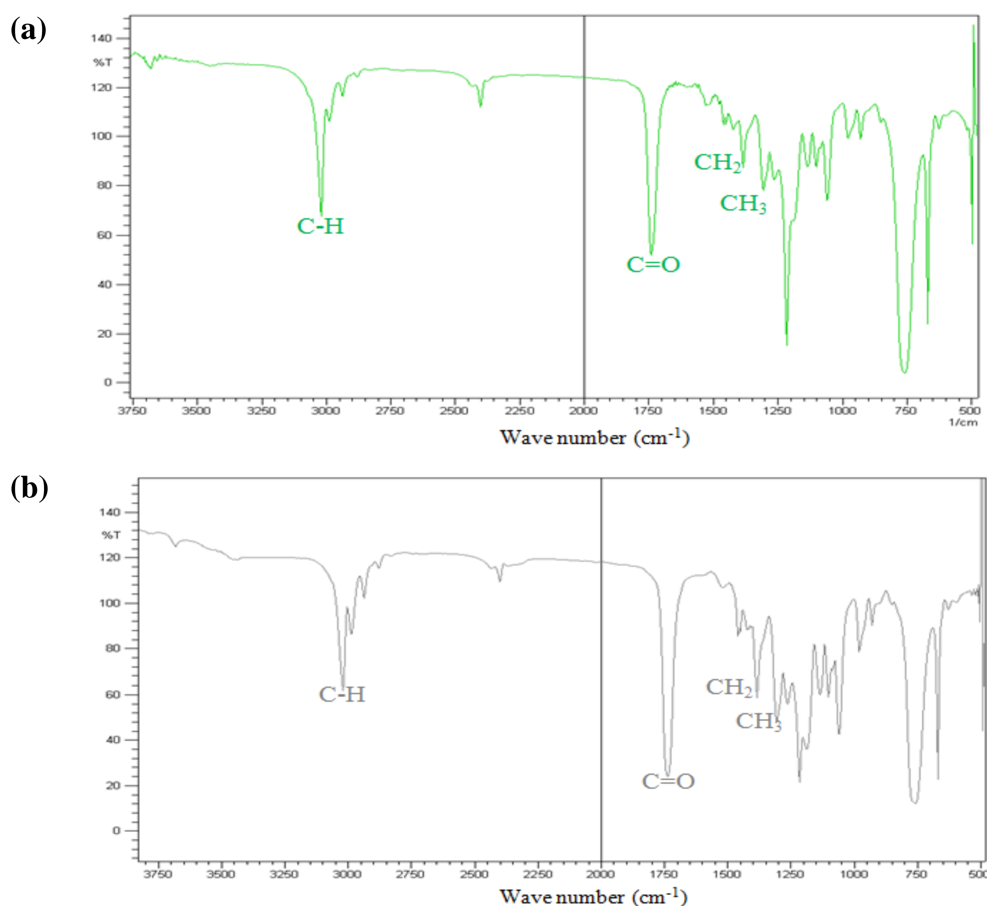


Fig. 3.2: The FTIR spectra of polymers extracted from *B. megaterium* TMR1.3.2 grew on **(a)** Glucose as sole source of carbon and **(b)** Combination of glucose and valeric acid as carbon sources.

The ^{1}H NMR analysis of both the polymers showed similar pattern of resonance spectra (Fig. 3.3). The resonance spectra at 1.28 ppm belongs to methyl ($-CH_3$) group, at 2.50 ppm corresponds to methylene ($-CH_2$) and at 5.30 ppm to methyne (-CH) group. The results obtained were compared with the ^{1}H NMR spectrum of standard PHB reported by Xiao and Jiao (2011) and confirmed as PHB. The ^{13}C NMR spectra of these polymers were obtained for additional confirmation. The chemical shifts of the resonances of corresponding carbon atoms of polymers are shown in Fig. 3.4. The chemical shift at 19.7 is of the methyl carbon, 40.7 ppm of the methylene carbon, 67.6 ppm of the methane carbon and 169.2 ppm of the carbonyl carbon. After comparing these results with the ^{13}C NMR spectrum of standard PHB reported by Chaijamrus and Udpuay (2008) confirmed the polymer as PHB. This *B. megaterium* TMR1.3.2 produces only PHB. Although this strain showed growth and PHA accumulation in the presence of glucose and valeric acid as carbon sources, it only accumulates polyhydroxybutyrate.

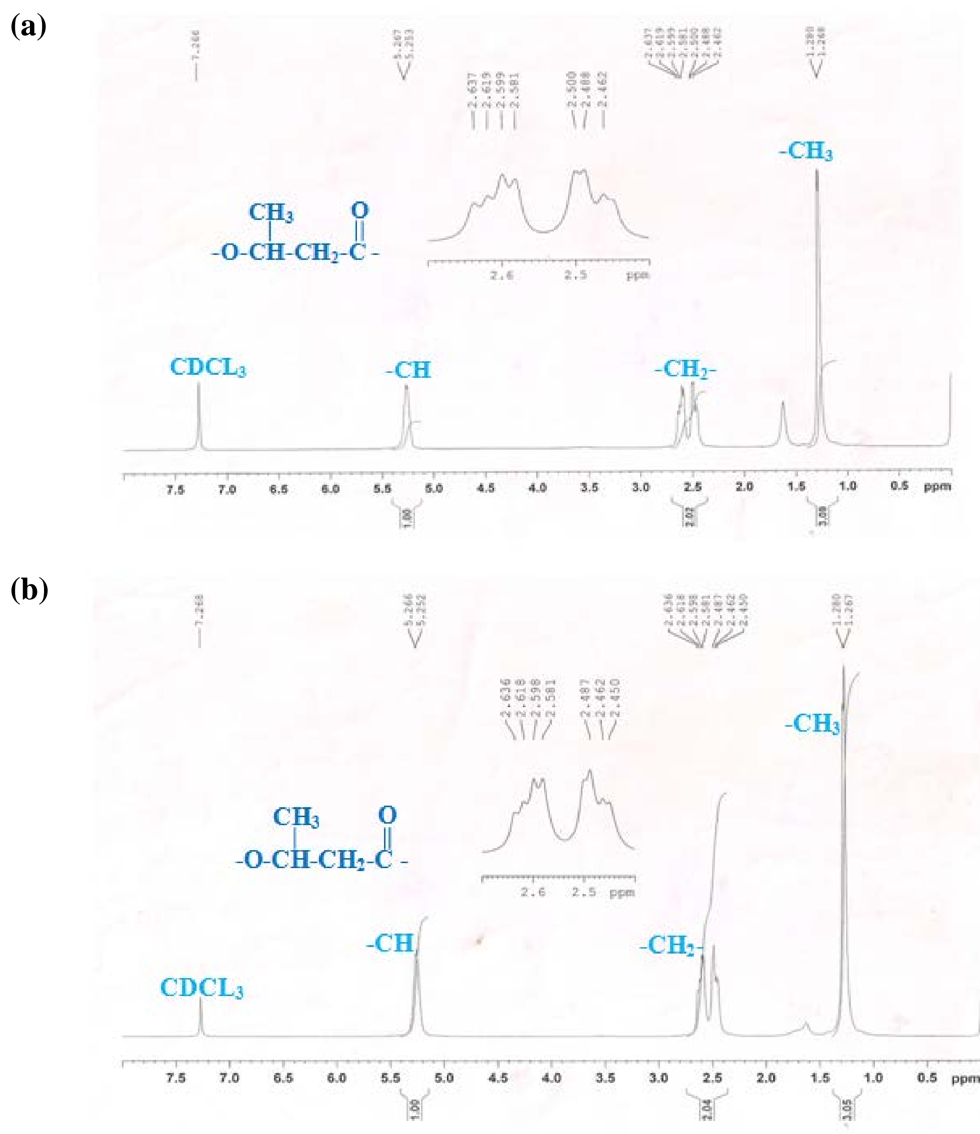


Fig. 3.3: ¹H NMR spectra of polymers extracted from *B. megaterium* TMR1.3.2 grew on **(a)** Glucose as sole source of carbon and **(b)** Combination of glucose and valeric acid as carbon sources.

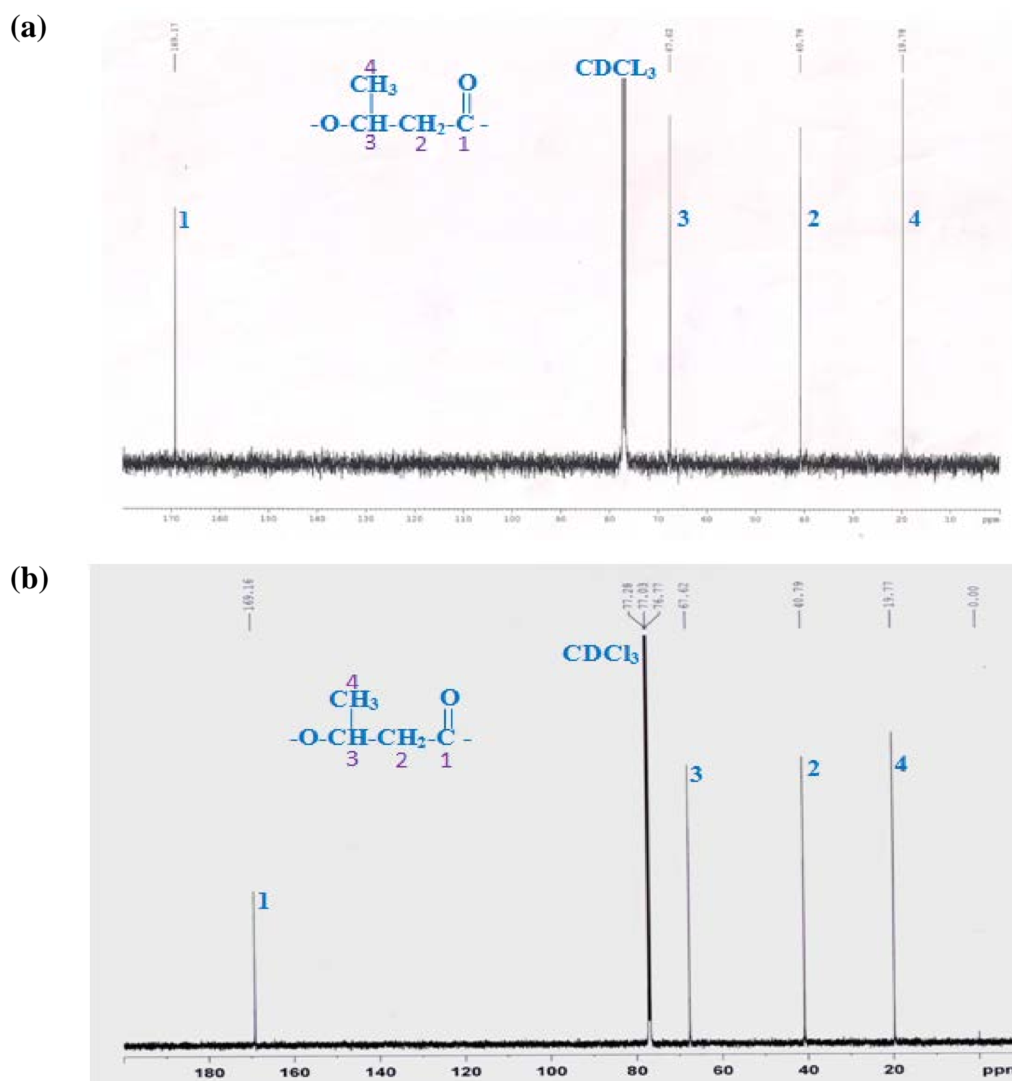


Fig. 3.4: ^{13}C NMR spectra of polymers extracted from *B. megaterium* TMR1.3.2 grew on **(a)** Glucose as sole source of carbon and **(b)** Combination of glucose and valeric acid as carbon sources.

The polymers extracted from 22 bacterial isolates of sand-dune ecosystem were characterized by FTIR spectroscopy (Fig. 3.5). The absorption bands of these polymers were observed similar to the polymer characterized from *B. megaterium* TMR1.3.2 and standard PHB reported by Xiao and Jiao (2011). This indicates all the PHA accumulating bacteria obtained from coastal sand-dunes produced polyhydroxybutyrate only.

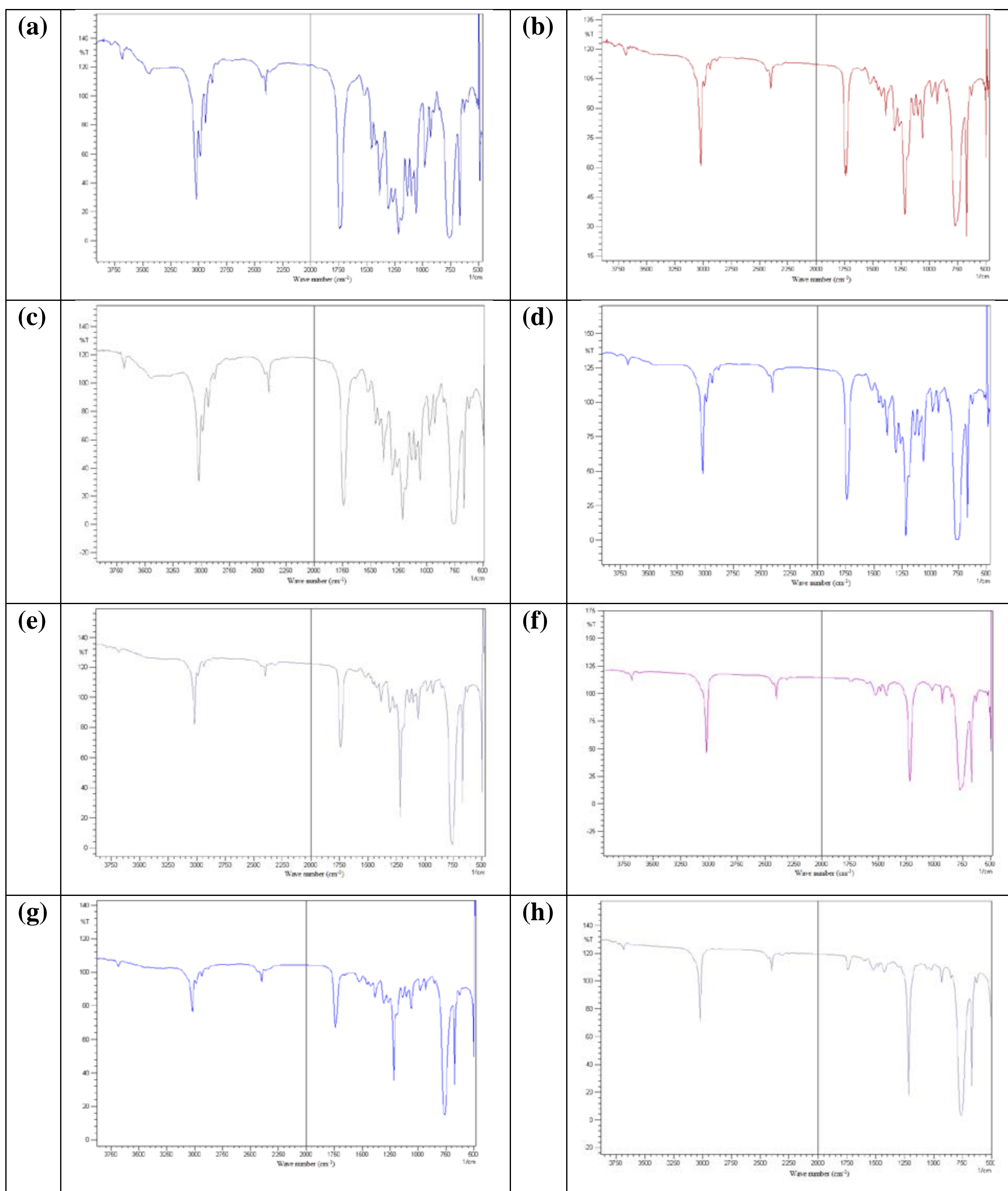


Fig. 3.5: FTIR spectra of the polymer obtained from isolates **(a)** TMR1.3.1a, **(b)** TMR1.3.1b, **(c)** TMR1.26, **(d)** TMR1.28, **(e)** TMR2.4,**(f)** TMR3.1, **(g)** TMR4.3 and **(h)** TMNR1.3 grown on E2-mineral medium containing glucose as carbon source.

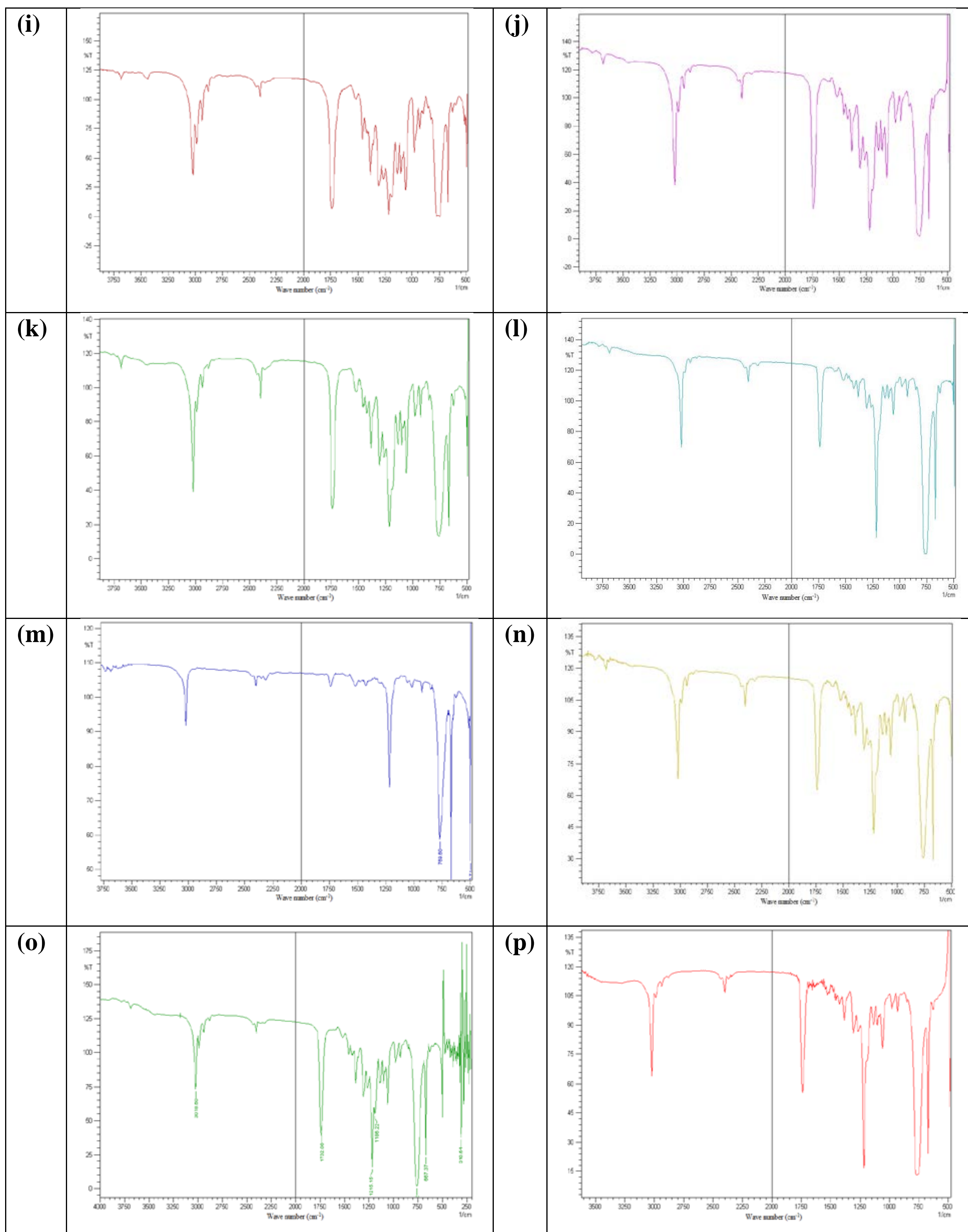


Fig. 3.5 contd: FTIR spectra of the polymer obtained from isolates **(i)** TMNR2.4, **(j)** NAMNR3.7, **(k)** NAMR1.12, **(l)** NAMR4.1, **(m)** TMR1.10.1, **(n)** TMR1.22, **(o)** TMR1.9.1 and **(p)** TMR1.9.2 grown on E2-mineral medium containing glucose as carbon source.

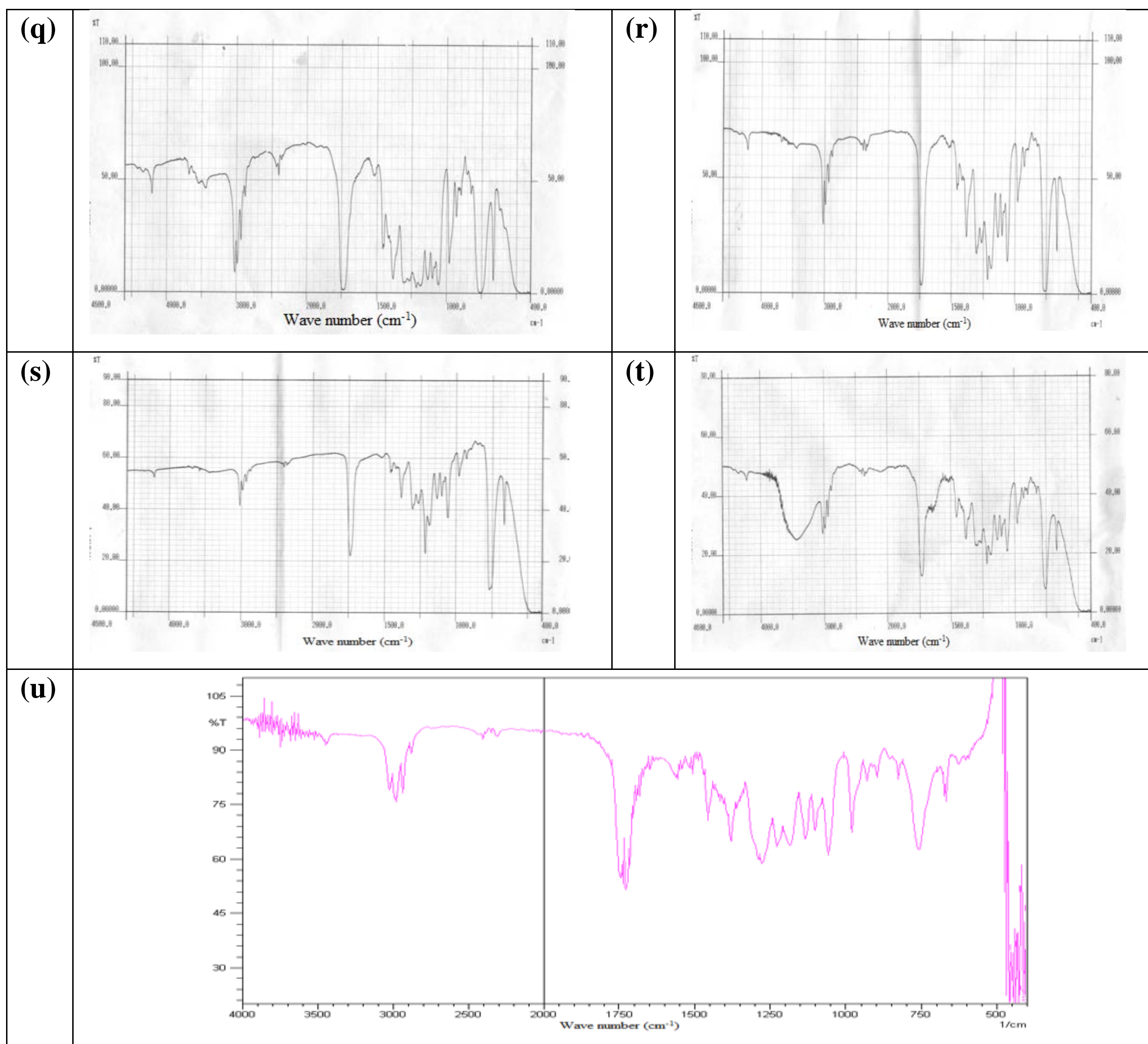


Fig. 3.5 contd: FTIR spectra of the polymer obtained from isolates **(q)** TMNR1.5, **(r)** NAMR1.8, **(s)** TMR1.17, **(t)** TMR1.4 and **(u)** NAMR1.6 grown on E2-mineral medium containing glucose as carbon source.

Section II

Batch and Fed-batch cultivations for PHB production

3.II.1 Materials and methods

3.II.1.1 Microorganisms and culture media

*Bacillus megaterium*TMR1.3.2 and *Bacillus megaterium* Col1/A6 previously isolated from coastal sand-dune ecosystem and humus sample respectively of Goa, India were used in this study. The strains were maintained on NA slants and stored at 4 °C. The modified E2-mineral medium was used for the PHB production, having (g/L) $KH_2PO_4.3H_2O$- 3.7; $K_2HPO_4.3H_2O$-7.5; NH_4Cl- 1.366; $MgSO_4.7H_2O$- 10 ml (0.1 M); yeast extract- 0.5; microelement stock solution- 1 ml ($FeSO_4.7H_2O$-2.78, $MnCl_2.4H_2O$-1.98, $CoSO_4.7H_2O$-2.81, $CaCl_2.2H_2O$-1.47, $CuCl_2.2H_2O$-0.17 and $ZnSO_4.7H_2O$-0.29). Glucose was used as sole source of carbon. The pH of the mineral broth was adjusted to 7.0.

3.II.1.2 Inoculum preparation

A single colony of 24 h grown *Bacillus megaterium* was inoculated in a 2 L Erlenmeyer flask containing 600 ml of mineral medium. Glucose 10 g/L was used as carbon source. The flask was incubated on an Orbitek environmental shaker (170 rpm) at 30 °C for 14 h.

3.II.1.3 Batch fermentation

The fermentation was carried out in a 14 L stirred fermentor (BioFlow 415, Bench top SIP, New Brunswick Scientific, USA) with 10 L of working volume. Batch cultivation with nitrogen and glucose limitation was carried out to understand the minimum carbon and nitrogen source required for growth and PHA accumulation by *Bacillus megaterium* TMR 1.3.2. In the batch cultivation with nitrogen limitation, the initial nitrogen and glucose concentration were 1.29 g/L and 24 g/L, respectively. For batch with glucose limitation, the initial glucose was 12 g/L and nitrogen was 3.9 g/L. The initial pH of the medium was adjusted to 7.0 and fermentation was carried without maintaining pH. Batch was continued until the substrates were completely utilized. Temperature and air-flow were maintained at 30 °C, and 1.0 vvm throughout the fermentation. The fermentation broth was inoculated with a 5% seed culture.

3.II.1.4 Fed-batch fermentation

Fed-batch cultivation was started with initial volume of 7 L of sterile mineral medium and inoculated with 10% seed culture of *Bacillus megaterium* TMR 1.3.2. The fed batch cultivation was started with initial glucose and nitrogen concentrations of 20 g/L and 12 g/L, respectively. Temperature, agitation, airflow and pH were maintained at 30 °C, 600 rpm, 1.2 vvm and 7.0, respectively throughout the fermentation. During fermentation, the pH was maintained by NaOH (4 N). The fermentor broth was fed with increasing substrate concentrations. Different feeding strategies were tested for high cell density PHB accumulation by the bacterial strain. The optimum fed batch cultivation method obtained was used for PHB accumulation using *Bacillus megaterium* Col1/A6. Broth samples were taken every one hour for determination of growth, PHB accumulation and residual glucose and ammonium chloride concentration in the fermentor broth.

3.II.1.5 Analytical procedures

3.II.1.5.I Determination of cell growth

Cell growth was observed as the dry cell weight (DCW) throughout the fermentation. The fermentor broth sample was centrifuged at 10,000 rpm for 10 minutes at 4 °C. The supernatant was collected in a separate tube for further analysis of the remaining glucose and ammonium chloride, the cell pellet obtained was washed twice with deionized water and dried at 80 °C until the constant weight was obtained. The biomass calculated from DCW was determined as

Biomass (g/L) = (Dry cell weight (g)/ sample volume)*1000

3.II.1.5.II PHB extraction

The washed cell pellet was suspended in sodium hypochlorite solution (4% available chlorine) (Santimano *et al.*, 2009). The suspension was incubated for 20 minutes on an orbitek environmental shaker (170 rpm) at 30 °C. The mixture was centrifuged at 12, 000 rpm for 20 minutes at 4 °C. The supernatant was discarded and the pellet was washed twice with deionized water. Pellet was resuspended in chilled ethanol (95%) and centrifuged at 12,000 rpm for 20 minutes at 4 °C. The supernatant was discarded and pellet was dried at 80 °C until the constant weight was obtained. PHB weight was calculated as

PHB (g/L) = (PHB dry weight (g)/ sample volume)*1000

3.II.1.5.III Residual glucose and ammonium chloride concentration in the fermentation broth

Total glucose present in the fermentation broth was determined by modified DNSA method (modified Miller's, (1959) method). The cell-free supernatant was diluted appropriately with deionized water. 1 ml of diluted supernatant was mixed with 1 ml of dinitrosalicylic acid (DNSA) solution and heated for 10 minutes in a water bath at 100 °C. After cooling, 0.4 ml of potassium sodium tartrate solution (33%) was added and absorption was taken at 540 nm against reagent blank.

The residual ammonium chloride concentration in the fermentation broth was determined by phenol-hypochlorite method (Grasshoff *et al.*, 1999). To 25 ml of appropriately diluted cell-free sample 1 ml of phenol reagent, 0.5 ml of citrate solution and 1 ml of hypochlorite reagent was added. The bottles were closed tightly and incubated for 30 minutes in a water bath at 37 °C. After incubation the bottles were kept at room temperature for 30 minutes and absorption was taken at 630 nm against reagent blank.

3.II.2 Results and discussion

3.II.2.1 Batch cultivation

Time-course of batch fermentation (Run 1) for production of biomass and PHB by *B. megaterium* TMR1.3.2 can be seen in Fig. 3.6a and utilization of glucose and ammonium chloride can be seen in Fig. 3.6b. The bacterial isolate started growing immediately after inoculation. The bacterial isolate grew exponentially until dissolved oxygen (DO) was available and there after the growth was slowed down for some time. At 12 h of cultivation, the dissolve oxygen (DO) of the fermentation broth was 1.3%. When the concentration of ammonium chloride and DO was limited, the PHB accumulation was slowed down for brief period. This is also reflecting in usage of glucose in the medium. Further, there was significant difference in the PHB obtained at 18 and 24 hours ($p<0.005$) indicating the continuous synthesis of polymer. Thereafter, rapid synthesis of PHB was recorded and continued until glucose became limited in the growth medium. The maximum biomass obtained was 6.7 g/L at 30 h. Maximum PHB was 2.26 g/L at 36 h, with the overall productivity of PHB as 0.062 g/L/h. At 36 h the PHB accounts for 33% of the dry cell weight.

The fermentation was further monitored for 72 h. After 42 h of incubation the amount of biomass and PHB accumulation declined, which indicates the utilization of accumulated PHB by the isolate *B. megaterium* TMR1.3.2. At the termination of the batch cultivation the biomass declined to 4.344 g/L and PHB to 0.504 g/L.

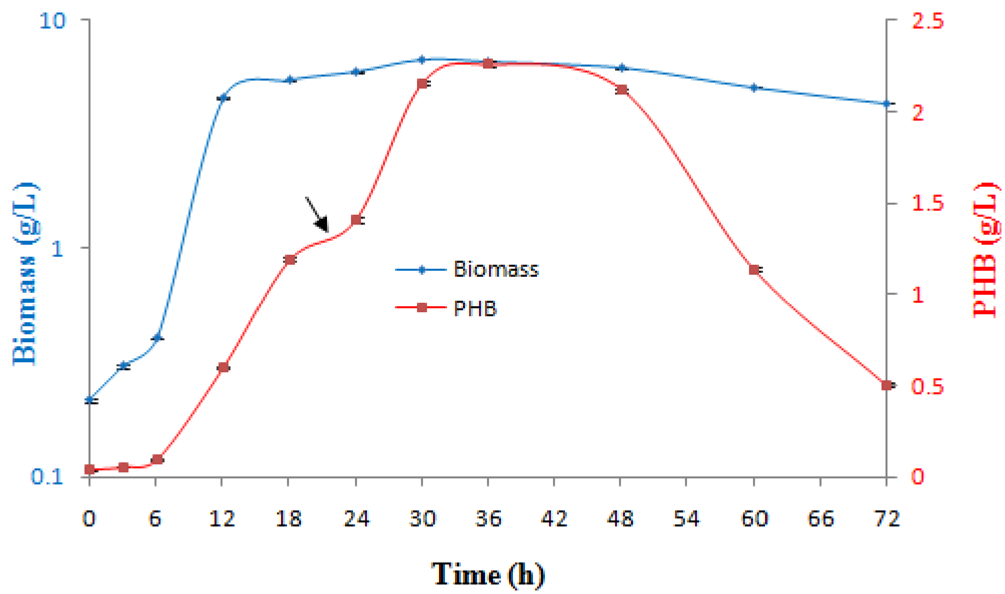


Fig. 3.6a: Time course of biomass and PHB production in batch fermentation (Run 1) using *B. megaterium* TMR1.3.2. Arrow indicates the slowed down of the PHB synthesis for brief period

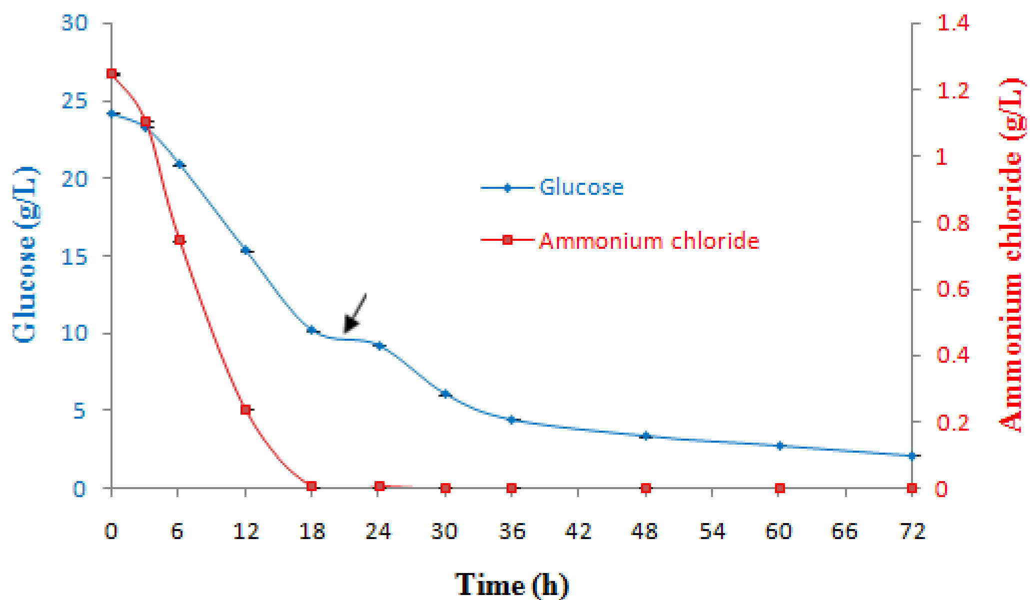


Fig. 3.6b: Time course of utilization of glucose and ammonium chloride during batch fermentation (Run 1). Arrow indication brief period of non-utilization of glucose

Batch cultivation (Run 2) was carried out under nitrogen limitation. Time-course for production of biomass and PHB by *B. megaterium* TMR1.3.2 can be seen in Fig. 3.7a and utilization of glucose and ammonium chloride in Fig. 3.7b. After inoculation the bacterial culture started growing, at 9 h of cultivation the DO, residual ammonium chloride and glucose were 47.9%, 0.156 g/L and 17 g/L, respectively. At 12 h of cultivation maximum biomass (9.092 g/L) and PHB (2.364 g/L) were recorded, with a 0.197 g/L/h of productivity of PHB. After 12 h, when concentration of ammonium chloride became limited, the DO started increasing. Biomass showed slight decline. Interestingly, although glucose was available PHB production did not continue and started showing declining. Further, glucose was marginally utilized till the termination of cultivation at 24 h of incubation. At this hour, the biomass and PHB present were 7.996 g/L and 1.272 g/L, respectively.

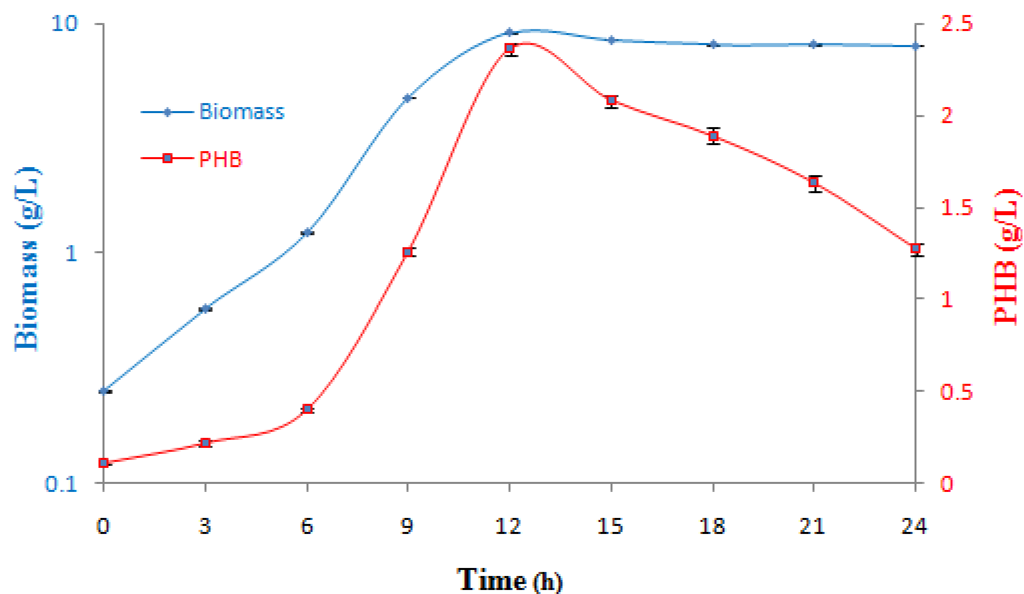


Fig. 3.7a: Time course of biomass and PHB production in batch fermentation (Run 2) using *B. megaterium* TMR1.3.2

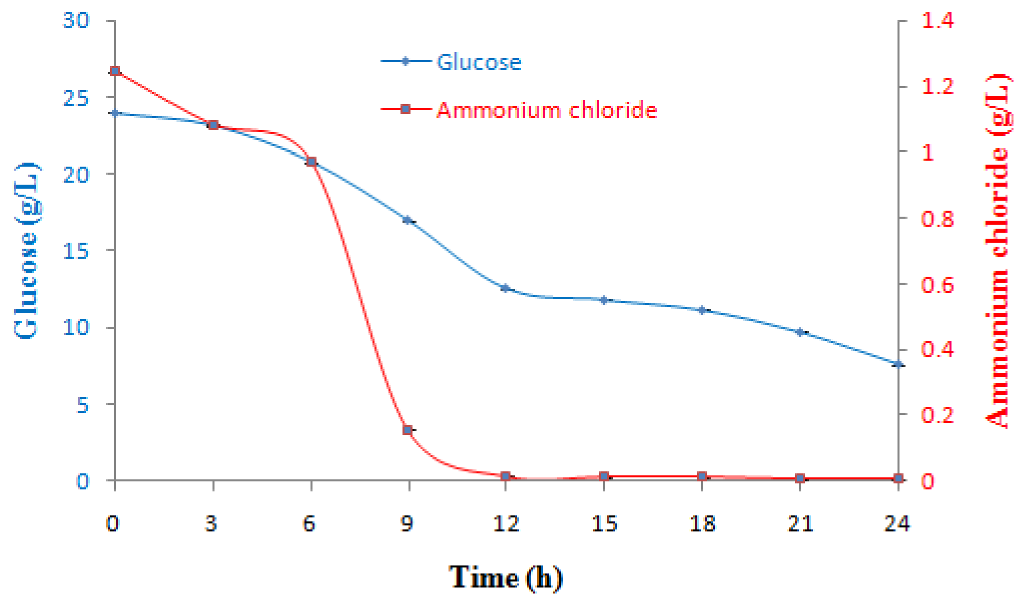


Fig. 3.7b: Time course of utilization of glucose and ammonium chloride during batch fermentation (Run 2)

Batch cultivation (Run 3) was performed under glucose limitation condition. Time-course of biomass and PHB production by *B. megaterium* TMR1.3.2 can be seen in Fig. 3.8a and utilization of glucose and ammonium chloride in Fig. 3.8b. The bacterial culture started growing immediately after inoculation. Maximum DCW obtained was 9.028 g/L at 14 h of cultivation. At this time the DO was 20.1% and residual ammonium chloride and glucose were 1.522 g/L and 1 g/L, respectively. Maximum PHB production was 2.4 g/L at 15 h of cultivation, with the overall productivity of PHB as 0.16 g/L/h. After 15 h of cultivation the residual glucose become limiting in the fermentation broth and further incubation resulted in decline of biomass as well as PHB production. The fermentation was carried out for 24 h. At the termination of batch fermentation the biomass present was 8.064 g/L and PHB was 1.976 g/L.

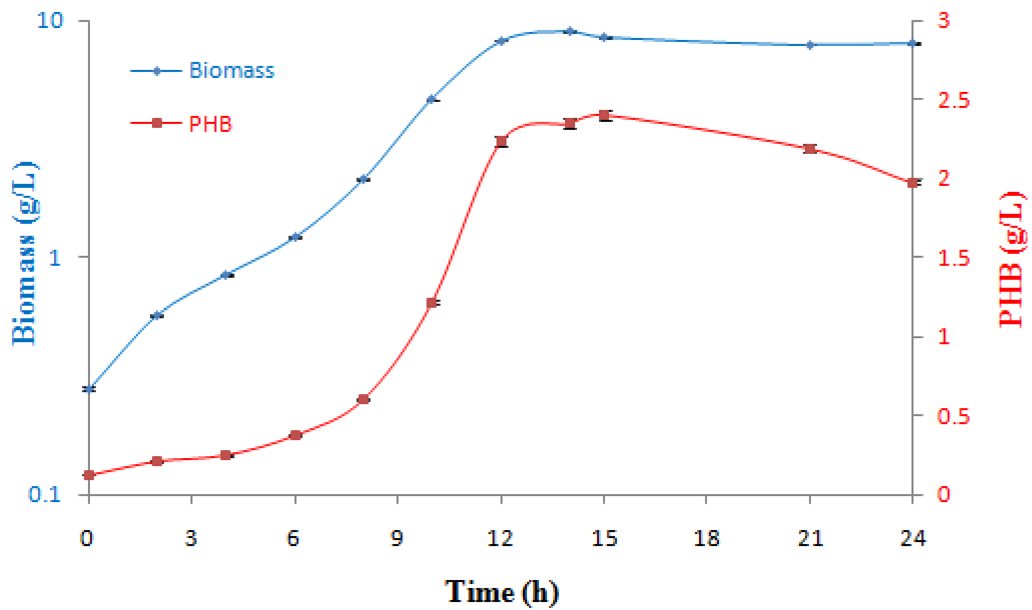


Fig. 3.8a: Time course of biomass and PHB production in batch fermentation (Run 3) using *B. megaterium* TMR1.3.2

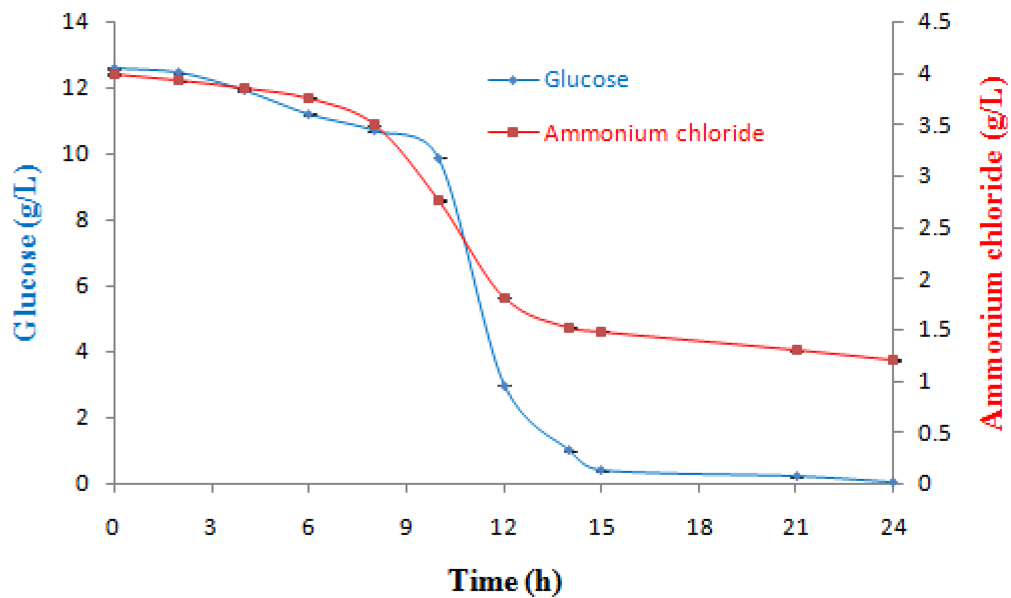


Fig. 3.8b: Time course of utilization of glucose and ammonium chloride in batch fermentation (Run 3)

The data obtained in batch fermentations were used to determine the kinetic parameters as per Monod's relationship. The residual biomass was used for calculating kinetic parameters. Kinetic parameters were $^{r}\mu_{max}$ = 0.56850 h^{-1}, $^{r}K_{G}$= 7.9 g/L, $^{r}K_{AC}$= 0.0875 g/L, $^{r}Y_{x/G}$= 1.33 g/g and $^{r}Y_{x/AC}$ = 3.215 g/g. Further these parameters were used for fed batch cultivation.

3.II.2.2 Fed-batch cultivation

Time-course of fed-batch fermentation (Run 4) for production of biomass and PHB using bacterial isolate *B. megaterium* TMR1.3.2 can be seen in Fig. 3.9a and utilization of glucose and ammonium chloride can be seen in Fig. 3.9b. The bacterial isolate started growing immediately after inoculation and at 7 h of fermentation there was no DO in the broth. At the 8 h of incubation, there was no residual glucose but the DO was increased to 76.1%. At this time, residual ammonium chloride was 1 g/L in the fermentation broth. As the presence of glucose became limited in the growth medium the biomass and PHB production was slowed down for a brief period of time. Immediately, after 10 h when the fermentor was fed with glucose and ammonium chloride solutions to obtain concentrations of 20 g/L and 4 g/L, respectively, the bacterial isolate resumed growth. The culture grew rapidly until both the substrates became limited in the fermentor broth. Major accumulation of PHB was seen during this phase. At the end of 14 h of fermentation the DCW and PHB production reached to 24.1 g/L and 7.832 g/L, respectively, with the PHB productivity of 0.559 g/L/h. When it was observed that the density of biomass production was not increasing further, then the fermentation was stopped. Fermentation was terminated at 15 h the biomass and PHB accumulated were 20.52 g/L and 7.824 g/L, respectively.

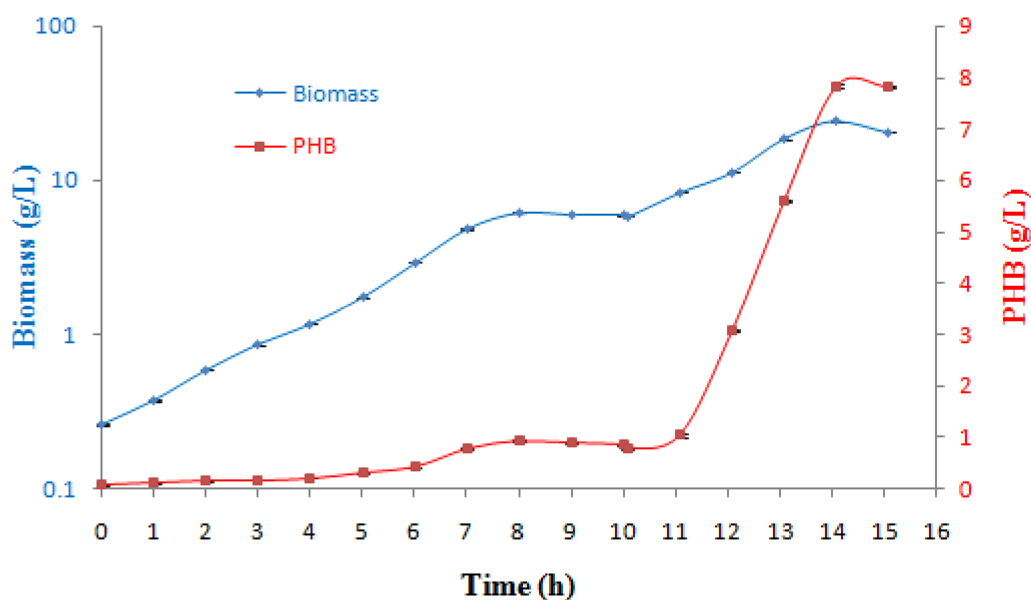


Fig. 3.9a: Time course of biomass and PHB production in fed-batch cultivation (Run 4) using *B. megaterium* TMR1.3.2

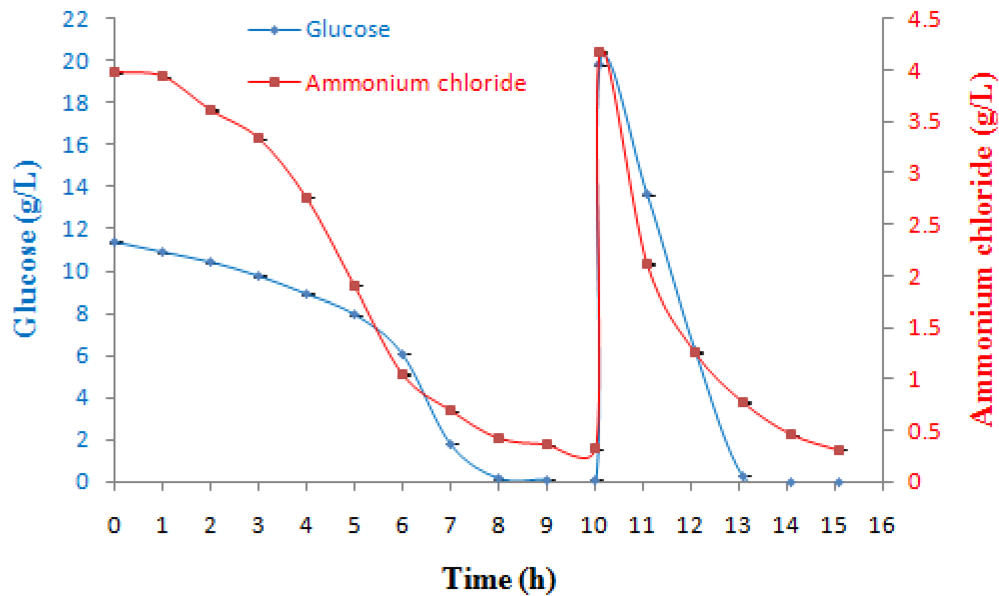


Fig. 3.9b: Time course of utilization of glucose and ammonium chloride during fed-batch fermentation by *B. megaterium* TMR1.3.2

In the fed-batch cultivation (Run 5) the culture broth was fed three times. Time-course of fed batch fermentation for biomass and PHB production using *B. megaterium* TMR1.3.2 can be seen in Fig. 3.10a and utilization of glucose and ammonium chloride can be seen in Fig. 3.10b. Initial concentrations of glucose and ammonium chloride were 30 g/L and 12 g/L, respectively. The bacterial isolate started growing immediately after inoculation. The fermentor was fed at 9.83 h with glucose and ammonium chloride solutions to obtain concentrations of 72 g/L and 11 g/L, respectively at the first feed. At 13 h the fermentor was fed with the second feed of glucose and ammonium chloride solutions to obtain concentrations of 100 and 14 g/L, respectively. After this feed, the biomass and PHB production was slowed down for a brief period of time and again growth and PHB production were resumed. At 19.583 h, fermentor was fed third time with only glucose solution to obtain 86 g/L. At the end of 21.583 h of fermentation the DCW and PHB production were reached to 104.68 g/L and 30.04 g/L, respectively, with the PHB productivity of 1.391 g/L/h. The maximum DCW of 107.41 g/L was obtained at 24.583 h. Further incubation resulted in a decline of the biomass and PHA production. Fermentation was terminated at 25.583 h.

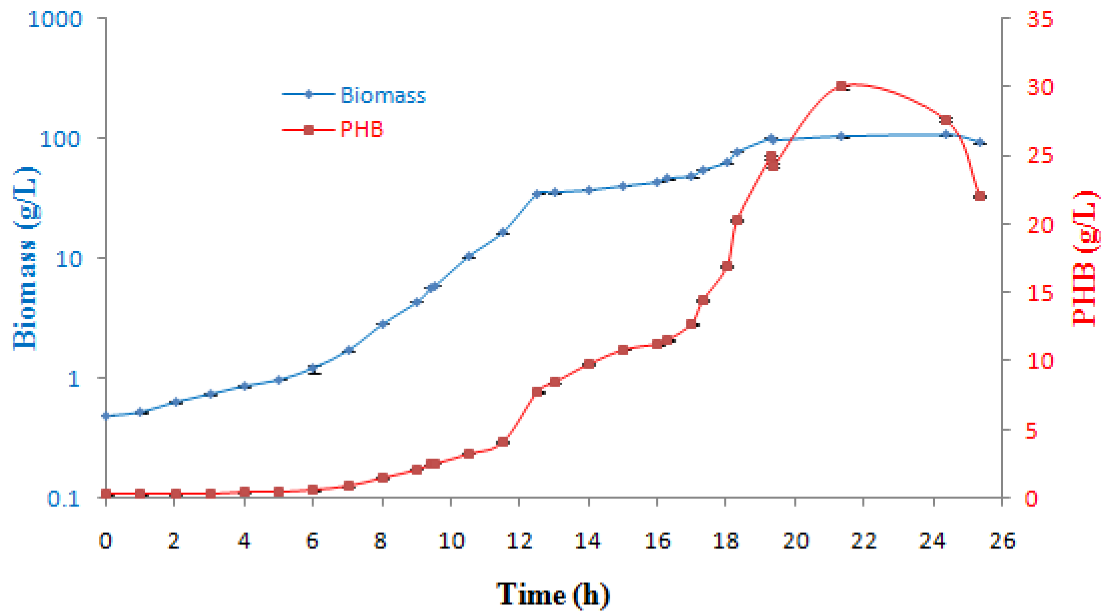


Fig. 3.10a: Time course of biomass and PHB production in fed-batch fermentation (Run 5) using *B. megaterium* TMR1.3.2

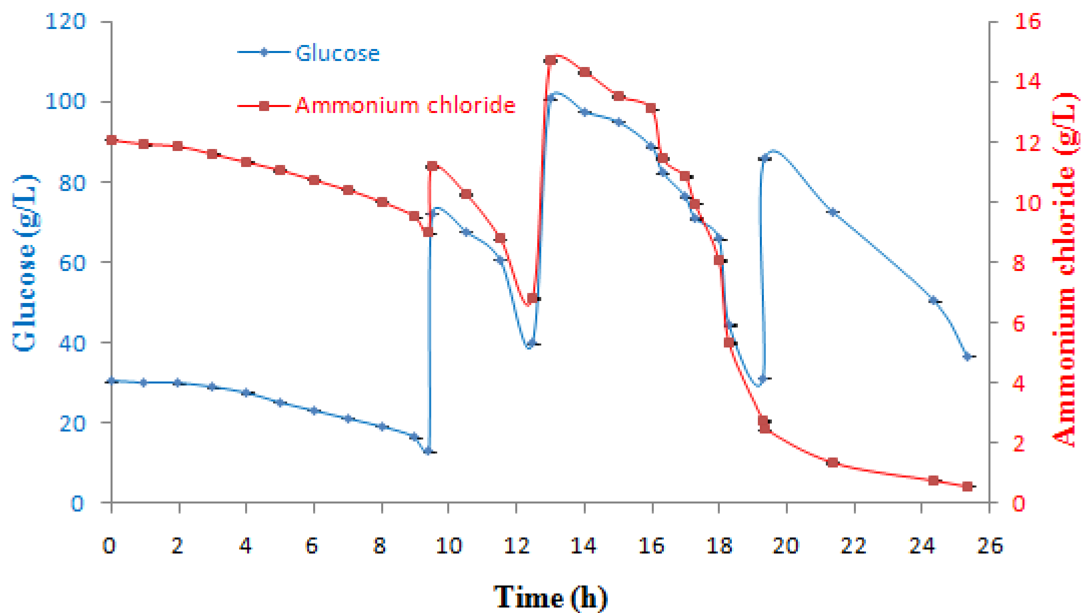


Fig. 3.10b: Time course of utilization of glucose and ammonium chloride during fed-batch fermentation (Run 5)

In this fed-batch cultivation (Run 6) an attempt was made to achieve high cell density along with high PHB production. The culture broth was fed several times with glucose and ammonium chloride solutions throughout the fermentation. Time-course of fed-batch fermentation for biomass and PHB production using *B. megaterium* TMR1.3.2 are shown in Fig. 3.11a and utilization of glucose and ammonium chloride in Fig. 3.11b. The initial

concentrations of glucose and ammonium chloride were 30 g/L and 12 g/L, respectively. The bacterial isolate started growing immediately after inoculation. After 10 h of cultivation the fermentation broth was fed with instant feedings of glucose and ammonium chloride solutions. The concentrations of glucose and ammonium chloride were maintained throughout the fermentation for exponential growth. The continuous increase in biomass and PHB production were achieved till the end of fermentation. The maximum DCW was 116.88 g/L and PHB was 50.61 g/L were obtained at 27.75 h, with the overall productivity of PHB as 1.823 g/L/h. Compared to fed-batch cultivation (Run 5), a two fold increase in PHB production was achieved in this fed-batch cultivation (Run 6).

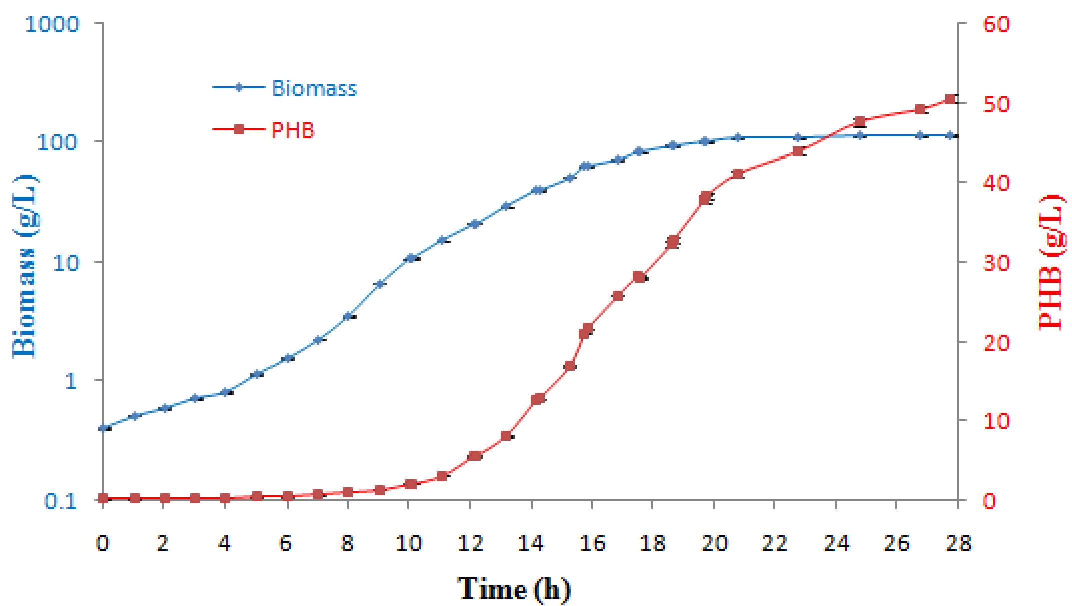


Fig. 3.11a: Time course of biomass and PHB production in fed-batch fermentation (Run 6) using *B. megaterium* TMR1.3.2

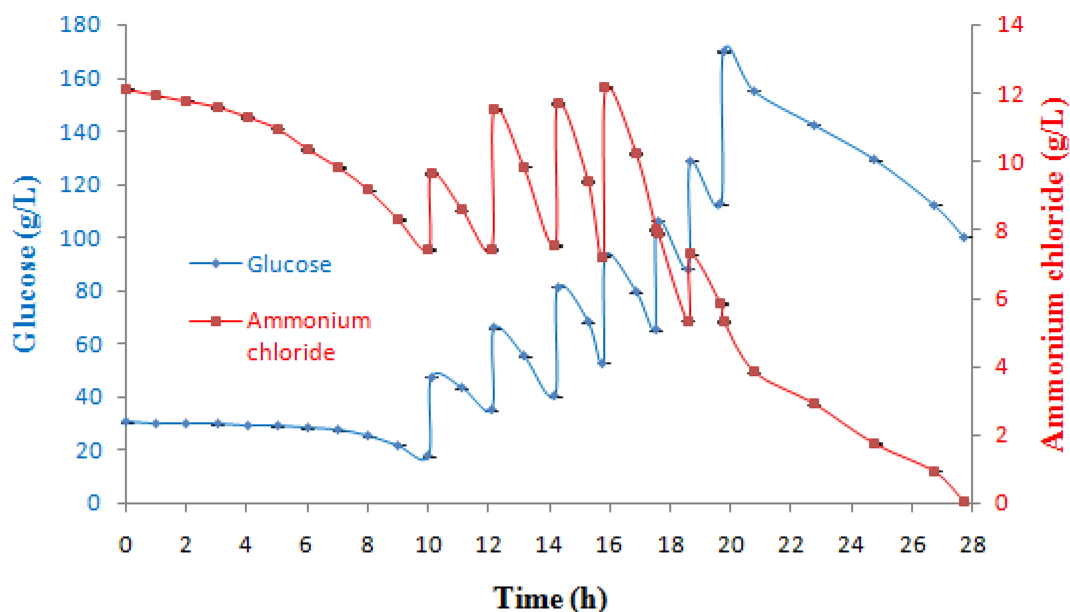


Fig. 3.11b: Time course of utilization of glucose and ammonium chloride during fed-batch fermentation (Run 6)

In fed-batch cultivation (Run 7) the *B. megaterium* Col1/A6 was used for biomass and PHB production. Time-course of fed batch fermentation for biomass and PHB production using *B. megaterium* Col1/A6 shown in Fig. 3.12a and utilization of glucose and ammonium chloride in Fig. 3.12b. The fermentation conditions and feeding strategy were used as per Run 6. The continuous increase in biomass and PHB production were achieved similar to Run 6 till the end of fermentation. The maximum DCW was 122.68 g/L and PHB was 65.76 g/L at 25 h, with the overall productivity of PHB as 2.63 g/L/h.

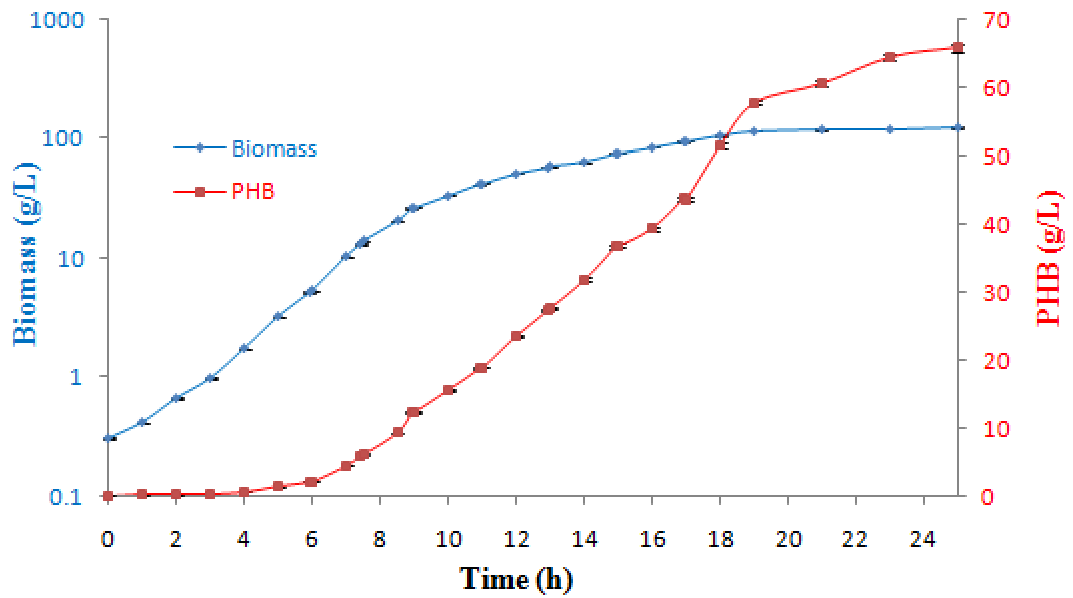


Fig. 3.12a: Time course of biomass and PHB production in fed-batch fermentation (Run 7) using *B. megaterium* Col1/A6

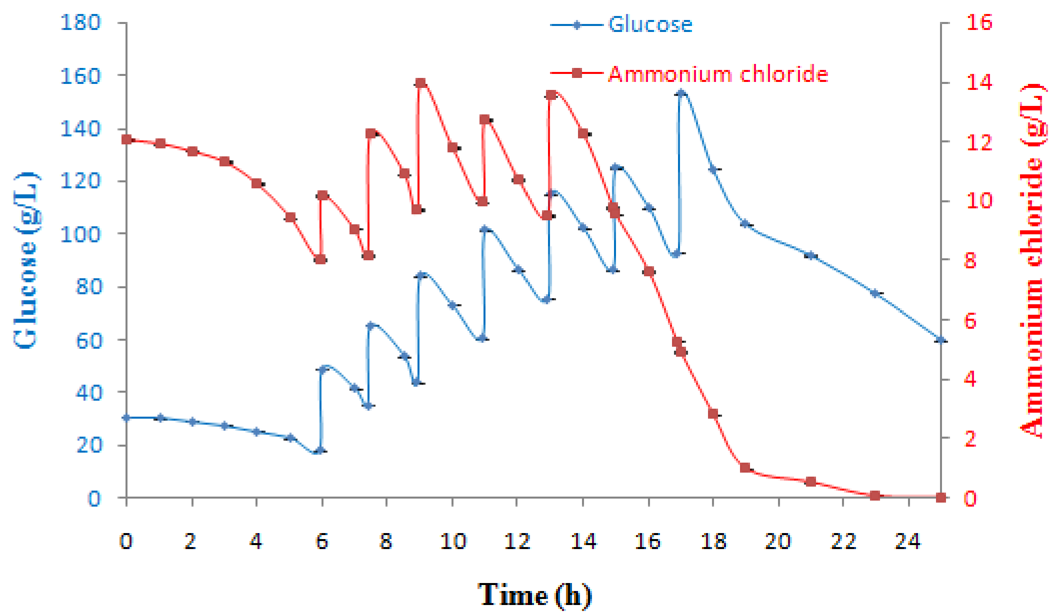


Fig. 3.12b: Time course of utilization of glucose and ammonium chloride during fed-batch fermentation (Run 7)

The batch and fed-batch cultivation methods are the main culture strategies used to achieve high cell density. In the present study *Bacillus megaterium* TMR1.3.2 and *Bacillus megaterium* Col1/A6 were used for PHB production from glucose. The comparison of results obtained from the batch and fed-batch fermentations can be seen in Table 3.3. In the batch fermentation (Run 1), the PHB synthesis was slowed down for a brief period of time when concentration of ammonium chloride became limited. Synthesis of PHB resumed again after this brief period. In this batch fermentation, both growth associated and non-growth associated PHB synthesis was observed. Further, incubation resulted in decline of PHB indicating utilisation of accumulated PHB by the isolate *B. megaterium* TMR1.3.2. Rodriguez-Contreras *et al*. (2013) reported that during fermentation of *B. megaterium* uyuni S29 with nitrogen limitation, maximum PHB production was achieved but further incubation without addition of nitrogen source could result in decrease in cell growth. Similarly in batch cultivation Run 2 with nitrogen limitation and Run 3 with glucose limitation, after the substrate was completely utilized in the medium, the biomass and PHB declined. This runs gave PHB productivity of 0.197 g/L/h and 0.160 g/L/h, respectively. In a batch fermentation of *B. megaterium* BA-019 using cane molasses as carbon source and urea as nitrogen source, biomass 8.78 g/L and PHB 5.41 g/L were achieved with the PHB productivity of 0.45 g/L/h after 12 h of fermentation (Kulpreecha *et al*., 2009). In an another batch cultivation using *B. megaterium* BA-019 Kanjanachumpol *et al*. (2013) reported biomass 32.5 g/L and PHB 8.8 g/L after 12 h of fermentation with overall productivity of PHB as 0.73 g/L/h.

The level of oxygen supply is one of the important factors which affects biomass and PHB production in *Bacillus* sp.. *Bacillus megaterium* being a strict aerobe requires controlled oxygen supply to achieve both cell growth and PHB production during fermentation (Hawthorne and Brusilow, 1986; Full *et al*., 2006). However, Faccin *et al*. (2009) demonstrated PHB production using *B. megaterium* in shake flask and fermentor conditions. They obtained higher PHB production in shake flask compared to the fermentation conditions and suggested that certain level of oxygen limitation is necessary to achieve higher PHB production. In the present study during batch fermentation it was observed that an increase in agitation and aeration increased cell growth. Further, during fed-batch cultivation the agitation and aeration were kept at 600 rpm and 1.2 vvm, respectively throughout the fermentation. Earlier Kanjanachumpol *et al*. (2013) reported the use of an agitation of 600 rpm and 1.0 vvm air flow to achieve high cell density fed-batch fermentation using *B. megaterium*.

Run	Batch/ Fed-batch	Volume (L)	Aeration (vvm)	Agitation (rpm)	Total Glucose Consumed at t_{Pmax} (g/L)	Total Ammonium Chloride Consumed at t_{Pmax} (g/L)	Run time [t_{Pmax}] (h:min)	X_{tPmax} (g/L)	PHB $_{tPmax}$ (g/L)	Biomass Yield (g/g)	PHB Yield (g/g)	Productivity (g/L/h)
1	Batch	10	1	300	19.740	1.244	36:00	6.652	2.260	0.337	0.114	0.063
2	Batch	10	1	600	11.475	1.229	12:00	9.092	2.364	0.792	0.206	0.197
3	Batch	10	1	600	12.201	2.498	15:00	8.516	2.400	0.697	0.196	0.160
4	Fed-batch	7	1.2	600	31.095	7.435	14:05	24.152	7.832	0.776	0.251	0.556
5	Fed-batch	7	1.2	600	131.640	20.911	21:35	104.680	30.040	0.795	0.228	1.392
6	Fed-batch	7	1.2	600	221	31.684	27:45	116.880	50.610	0.528	0.229	1.824
7	Fed-batch	7	1.2	600	251	29.352	25:00	122.680	65.760	0.488	0.261	2.630

Table 3.3: Comparison of biomass and PHB production by *Bacillus megaterium* obtained in different batch and fed-batch cultivation modes. The t_{Pmax} is the incubation time at which PHB amount was found maximum in the fermentation broth. Run nos 1-6 are carried out with *B. megaterium* TMR1.3.2 and Run no. 7 was carried out with *B. megaterium* Col1/A6. Productivity = PHB t_{Pmax} / t_{Pmax}

In fed-batch Run 4 the maximum biomass and PHB obtained were 24.1 and 7.832 g/L, respectively after 14 h of fermentation with the PHB productivity as 0.559 g/L/h. In fed-batch Run 5 the fermentation broth was exponentially fed with glucose and ammonium chloride solutions. Whereas at the end of 21.583 h of fermentation, the DCW and PHB reached to 104.68 g/L and 30.04 g/L, respectively with the PHB productivity of 1.391 g/L/h. In the fed-batch Run 6 the fermentation broth was fed instantly with glucose and ammonium chloride solutions. The maximum DCW was 116.88 g/L and PHB was 50.61 g/L were obtained at 27.75 h, with the overall productivity of PHB as 1.823 g/L/h. In fed-batch Run 7 the bacterial isolate *B. megaterium* Col1/A6 was used and fermentation broth was fed as per the feeding pattern of fed-batch Run 6. The maximum DCW was 122.68 g/L and PHB was 65.76 g/L were obtained at 25 h, with the overall productivity of PHB as 2.63 g/L/h. The biomass and PHB production by *B. megaterium* TMR1.3.2 and *Bacillus megaterium* Col1/A6 were compared with other microorganisms using batch and fed-batch fermentation (Table 3.4). Earlier Kanjanachumpol *et al*. (2013) demonstrated fed-batch fermentation for biomass and PHB production by *B. megaterium* BA-019. Using sugar cane molasses and urea as carbon and nitrogen sources respectively, they reported biomass 90.7 g/L and PHB 41.6 g/L with an overall PHB productivity of 1.73 g/L/h. The *B. megaterium* Col1/A6 showed the highest biomass and PHB production so far reported by any strains of *B. megaterium*. This is the first report of achieving a high cell density of more than 122 g/L and high PHB production of more than 65 g/L after 25 h of cultivation using *B. megaterium*. Interestingly these values obtained are close to *A. latus* DSM 1123 in fed-batch using sucrose as carbon source reported by Wang *et al*. (1997).

Microorganisms	Culture strategy	Carbon substrate	Culture Time (h)	DCW (g/L)	PHB (g/L)	PHB productivity (g/L/h)	PHB content (% wt)	Reference
A. eutrophus NCIMB11599	Fed-batch	Glucose	50	164.0	121.0	2.42	76	(1)
A. latus (DSM 1123)	Fed-batch	Sucrose	20	112.0	98.7	4.94	88	(2)
Recombinant *E. coli*	Fed-batch	Molasses	32	39.5	31.6	1.00	80	(3)
P. fluorescens A2a5	Batch	Sugarcane	96	32.0	22.0	0.23	70	(4)
A. chrococcum	Fed-batch	Starch	71	54.0	25.0	0.35	46	(5)
Wautersia eutropha	Fed-batch	Fructose	48	22.6	8.2	0.17	36	(6)
A. eutrophaus	Fed-batch	Tapioca	59	106.0	61.5	1.04	58	(7)
Zobellella Denitrificans MW1	Fed-batch	Glycerol	49.81	81.2	54.3	1.09	67	(8)
B. megaterium	Batch	Molasses	52.5	3.6	2.1	0.04	59	(9)
B. megaterium BA-019	Fed-batch (pH stat)	Molasses	24	72.6	30.5	1.27	42	(10)
B. megaterium	Batch	Glycerol	42	7.7	4.8	0.114	62.4	(11)
B. megaterium	Batch	Glucose	42	7.1	4.2	0.1	59.1	(11)
B. megaterium BA-019	Batch	Molasses	12	32.5	8.8	0.73	27	(12)
B. megaterium BA-019	Fed-batch	Molasses	24	73.0	31.3	1.30	43	(12)
B. megaterium BA-019	Fed-batch	Molasses	24	90.7	41.6	1.73	46	(12)
B. megaterium Uyuni S29	Fed-batch	Glucose	34	28.59	8.5	0.25	29.70	(13)
B. megaterium TMR1.3.2	Fed-batch	Glucose	27.75	116.88	50.61	1.82	43.3	This work
B. megaterium Col1/A6	Fed-batch	Glucose	25	122.68	65.76	2.63	53.6	This work

Table 3.4: Comparison of biomass and PHB production by various microorganisms from different batch and fed-batch cultivations
1: Kim *et al.* (1994); 2: Wang *et al.* (1997); 3: Liu *et al.* (1998); 4: Jiang *et al.* (2008); 5: Kim and Chang (1998); 6: Patwardhan and srivastava (2008); 7: Kim *et al.* (1995); 8: Ibrahim and Steinbuchel (2009); 9: Gouda *et al.* (2001); 10: Kulpreecha *et al.* (2009); 11: Naranjo *et al.* (2013); 12: Kanjanachumpol *et al.* (2013); 13: Rodriguez-Contreras *et al.* (2012).

Chapter-IV

Enzyme/protein associated with native polymer granules

4.1 Introduction

Bacteria synthesize polyhydroxyalkanoates (PHAs) as water insoluble granules inside the cell cytoplasm when there is excess of carbon source in the environment. PHA granules are of 0.2 to 0.5 nm in diameter. Proteins associated with these granules are involved in granule formation, PHA synthesis and depolymerization. It is envisaged that an understanding of the types of proteins present on the surface of granules will help in improving the knowledge of its regulatory mechanism and in turn the PHA production.

Griebel *et al*. (1968) for the first time analysed the PHB granules of *Bacillus megaterium.* They demonstrated that PHB granules contain approximately 97.5% of polymer, 2% of protein and 0.5% of lipid. Granules are composed of an amorphous PHA core, surrounded by a phospholipid monolayer embedded with granule associated proteins. Proteins associated with the granules play an important role in PHA metabolism (Potter and Steinbuchel, 2005). These proteins are PHA synthase (PhaC), PHA depolymerase (PhaZ), regulatory proteins (PhaR), phasins (PhaP) and many other. The PHA synthases are the only proteins reported covalently bound to the granules (Prabhu *et al*., 2010). Other proteins are non-covalently associated with it. The *in vivo* presences of these proteins on the surface of PHA granule are reported by many researchers. The localization of PhaP and PhaC of *Bacillus megaterium* and *Ralstonia eutropha* at the surface of PHA granule was demonstrated by fluorescence microscopy of translational fusion of PhaP and PhaC with green fluorescent protein and by immune-gold labelling in Transmission Electron Microscopy (Gerngross *et al*., 1993; McCool and Cannon, 1999; Neumann *et al*., 2008). Localization of PhaC, PhaP, PhaZ and PhaR of *Paracoccus denitrificans* at the surface of PHA granule was studied by Western blot analysis (Maehara *et al*., 1999, 2001). Presence of PHA depolymerase (PhaZ1) on the surface of PHA granule was studied by Western blot with anti-PhaZ1 antibodies (Potter and Steinbuchel, 2005). PHB synthase of *Ralstonia eutropha* PHB-4 fused with enhanced green fluorescent protein was localized on the surface of PHA granules (Peters *et al*., 2007). Phasins PhaP5, PhaP6 and PhaP7 fused with green fluorescent protein were localized with PHB granules in *Ralstonia eutropha* H16 (Pfeiffer and Jendrossek, 2012). SDS-PAGE profile of PHA granule associated proteins reveals the presence of a large number of proteins but only few proteins such as, PHA synthases, depolymerases, regulatory proteins and phasins are characterized so far. More than 400 different proteins were detected by proteome analysis of granule bound proteins from *Ralstonia eutropha* (Jendrossek and Pfeiffer, 2014).

Therefore, the present study was concentrated on isolation of proteins associated with native PHA granule from two bacterial strains namely, *Bacillus megaterium* TMR1.3.2 and *Bacillus megaterium* Col1/A6. Proteins bound to PHA granules were extracted, loaded in SDS-PAGE and digested using in-gel trypsin digestion. Peptic digests obtained were analysed by LCMS Q-ToF. Further, to understand the granule formation in *Bacillus megaterium*, both the bacterial strains were grown in E2-broth for 16 h and 24 h respectively. Cell biomass was treated with lysozyme for cell lysis. Cell lysates were processed for SEM-EDX analysis.

4.2 Materials and methods

4.2.1 Bacterial strains and growth conditions

The bacterial strain *Bacillus megaterium* TMR1.3.2 and *Bacillus megaterium* Col1/A6 were selected for this study. Cultures were inoculated in 250 ml flask containing 100 ml of E2-mineral broth (Appendix A) having glucose (20 g/L) as sole carbon source. The flasks were incubated at 30 °C on an Orbitek shaker (170 rpm) for 48 h. The culture broth was harvested at 7000 rpm for 10 min and the cell pellets were washed twice with sodium phosphate buffer (0.1 M, pH 7.0 (Appendix B)).

4.2.2 Isolation of native PHA granules

The cell pellets (1 g wet weight) obtained in section 4.2.1 were suspended in 5 ml of lysis buffer (Appendix B) and incubated for 1 h under shaking condition at 37 °C (Prabhu *et al.*, 2010). The lysate was centrifuged at 12000 rpm for 30 min at 4 °C. The pellet was washed with sodium phosphate buffer (Appendix B) and resuspended in a minimum volume of buffer. Suspension (0.5 ml) was loaded onto a sucrose density gradient consisting of 5 ml each of 40, 55 and 60% sucrose in 50 ml centrifuge tubes. The sealed tubes were centrifuged at 22000 rpm for 1 h at 4 °C. The inclusion bodies, which banded at the interphase between 40 and 55%, were collected in a fresh tube and suspended in 10 ml of sodium phosphate buffer. The suspension was centrifuged at 10000 rpm for 15 min. The pellet was resuspended in 0.5 ml of buffer and purified by using a second sucrose density gradient centrifugation. The inclusion bodies were collected in a fresh tube, washed three times with the buffer and stored at 4 °C for further use.

4.2.3 Extraction of proteins associated with PHA inclusion bodies

The PHA granules obtained in section 4.2.2 were suspended in modified STE buffer (Appendix B) (McCool and Cannon, 1999). The suspension was incubated at 4 °C for 30 min with intermittent vortexing occasionally. An equal volume of 2X sample buffer (Appendix D) was added and boiled at 100 °C for 5 min. Samples were centrifuged at 7000 rpm for 3 min at 4 °C. The supernatant was collected in a fresh tube and stored at 4 °C.

4.2.4 SDS-PAGE profile of PHA granule associated proteins

The proteins associated with granule obtained from both the bacterial strains were loaded on to 12% SDS-PAGE (w/v) polyacrylamide gel (Appendix D) (Sambrook *et al.*, 1989). Precision Protein Standards (Bio-Rad) were used as molecular weight markers (Sorrentino *et al.*, 2012). Electrophoresis run conditions were 100 volts for 3 h. The gel was stained with silver nitrate (Appendix D) (Blum *et al.*, 1987).

4.2.5 In-gel trypsin digestion of silver stained proteins

The entire gel was rinsed in ultrapure water for 1 h before processing. Silver stained protein bands were excised from the gel using blade and collected in 0.5 ml tubes. Gel pieces were destained with destaining solution (Appendix D). Then washed twice with washing solution (Appendix D) and dehydrated in acetonitrile. The gel pieces were rehydrated in 10 mM Dithiothreitol (DTT) solution (Appendix D) and incubated at 56 °C for 60 min. After incubation, the DTT solution was discarded. The gel pieces were resuspended with 55 mM Iodoacetamide (IAA) solution (Appendix D). The samples were incubated at room temperature for 30 min in dark. After incubation the IAA solution was removed and washed two times with washing solution. Then gel pieces were dehydrated again with acetonitrile. Gel pieces of each protein band was treated with trypsin (20 mg/ml) in 40 mM ammonium bicarbonate (Appendix D) and incubated at 37 °C for overnight. After incubation, the pH of the sample was adjusted between 2 to 3 with formic acid and acetonitrile (2:1) followed with vigorous vortexing for 45 min. The tryptic digest was collected in a fresh tube and stored at -20 °C till further analysis (Damare and Krishnaswamy, unpublished data).

4.2.6 LCMS Q-ToF analysis of proteins associated with granule

The tryptic digest was loaded in to LCMS Q-ToF. The samples on LCMS Q-ToF were injected with five internal repeats. The spectral data of each peptide were acquired by data acquisition software. The data obtained were compared with NCBI data base against redundant nucleotide/protein or *Bacillus megaterium* (strain ATCC 12872 / QMB1551) using

SpectrumMill software version 3.1. The proteins identified were compared for physical location of bands on the polyacrylamide gel according to their molecular weight.

4.2.7 SEM/EDX analysis of native PHA granules

Bacterial strain *B. megaterium* TMR1.3.2 and *B. megaterium* Col1/A6 were grown on E2-mineral broth for 16 h and 24 h respectively for early and late stages of PHA accumulation. Biomass was harvested by centrifugation at 7000 rpm for 10 min and washed twice with 0.1 M sodium phosphate buffer (pH 7) (Appendix B). Cell pellet was treated with lysozyme (10 mg/ml) and incubated at 37 °C for 15 min, 30 min and 45 min to obtain partial as well as complete cell lysis. Cell suspension was centrifuged at 1000 rpm for 10 min at 4 °C and washed with 0.1 M sodium phosphate buffer. The pellet was resuspended with require volume of 0.1 M sodium phosphate buffer. A drop of cell lysate was placed on cover slip, air dried and fixed with 2% (v/v) glutaraldehyde for overnight. The cover slip was washed with 0.1 M sodium phosphate buffer and dehydrated using increasing concentrations of acetone (30, 50, 70, and 90%) for 10 min each and finally in 100% acetone for 30 min and air dried (Rheims *et al*., 1999). The specimens were sputter coated with gold using an auto fine coater (JEOL JFC 1600) and visualized using SEM (JEOL JSM-6360).

4.3 Results and discussion

4.3.1 SDS-PAGE profile of PHB granule associated proteins from selected bacteria

Native PHB granules were isolated and purified from *B. megaterium* TMR1.3.2 and *B. megaterium* Col1/A6 by sucrose density gradient centrifugation. Whole PHB granule associated proteins were analysed by SDS-PAGE (Fig. 4.1). The PAGE profile of granule associated proteins revealed the presence of more than 25 distinctly visible protein bands with varying intensities. In both the bacterial strains similar pattern of visualized protein bands were observed. Stuart *et al*. (1998) have demonstrated the SDS-PAGE profile of PHA granule associated proteins from different genera of bacteria, where they observed that each genus has different protein profiles. McCool and Cannon (1999) analysed the PHA granule associated proteins of *B. megaterium* by electrophoresis on SDS-polyacrylamide gel. They have found at least 13 proteins with various intensities and reported that these proteins could be intrinsic structural components of PHA granules, enzymes involved in PHA metabolism, or possibly scaffolding components involved in assembly of PHA granules. The analysis of PHA inclusion body associated proteins of *B. megaterium* and bacterial strain HF-1 by SDS-PAGE

showed the presence of at least 20 proteins with different concentrations and reported the presence of two most abundant proteins of molecular weight approximately 20 and 40 kDa (Law *et al*., 2001). They also reported that in both the strains the patterns of protein bands after Coomassie blue and silver staining was similar except two bands so HF-1 is closely related to *B. megaterium*. Prabhu (2010) reported similar protein profiles of whole PHA granule associated proteins in different strains of *B. megaterium*, whereas different protein profiles was observed in case of bacterial isolates belongs to same genera but different species. The author reported the presence of 15 to 20 proteins with different intensities of granule associated protein profile obtained from the selected *Bacillus* sp.. Jendrossek and Pfeiffer (2014) have reported the presence of a large number of proteins on SDS-PAGE of PHA granule associated proteins from *R. eutropha* H16.

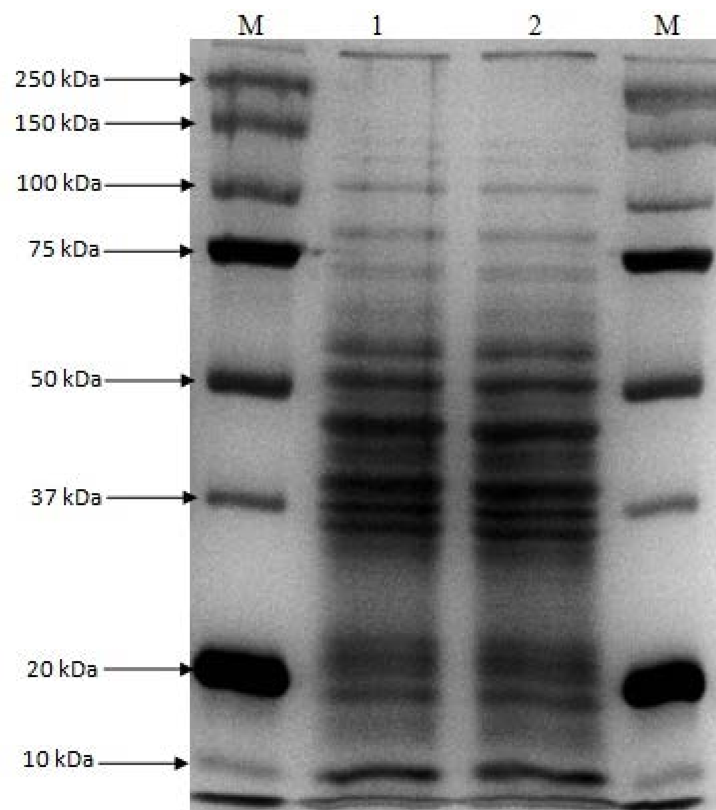


Fig. 4.1:SDS-PAGE profile of granule associated proteins, where Lane M-protein molecular weight marker; 1-*Bacillus megaterium* TMR1.3.2; 2-*Bacillus megaterium* Col1/A6.

4.3.2 Identification of PHB granule associated proteins

To identify the PHB granule associated proteins, the protein bands were excised from polyacrylamide gel (Fig. 4.1) and trypsin digests of these proteins were analyzed by LC-MS Q-ToF. The spectral files of the corresponding peptides acquired by data acquisition software were extracted and searched against *Bacillus megaterium* sub-set in NCBI database having 22,349 entries using SpectrumMill. More than 500 peptides corresponds to 60 proteins were identified from the similarity search with peptides of *Bacillus megaterium*. The number of proteins identified and their functions are listed in Table 4.1. Protein involved in PHA metabolism such as PhaC, PhaR, PhaA, PhaB, PhaP, ketol-acid reductoisomerase and 3-hydroxybutyryl-CoA dehydrogenase were identified. Among well-known PHB granule associated proteins PhaZ could not be detected in this study. Besides proteins of PHB metabolism other proteins such as, pyruvate dehydrogenase complex, acetyl-CoA metabolic process, citric acid cycle, fatty acid β-oxidation, fatty acid biosynthesis, cell wall synthesis, electron transport chain, protein biosynthesis, nucleic acid biosynthesis, amino acid biosynthesis and hypothetical proteins (10 numbers) were identified by proteome analysis of PHB granule associated proteins. This is the first report of proteome analysis of whole granule associated proteins from *B. megaterium* by LC-MS Q-ToF. So far the numbers of proteins/enzymes identified from PHA granules of different bacterial species, which are involved in PHA synthesis, are listed in Table 4.2.

Matsumoto *et al*. (2002) characterized PHA inclusion body associated proteins from *Pseudomonas* sp. 61-3 by SDS-PAGE and N-terminal amino acid sequencing. PHA synthase 1 (PhaC1), PhaF and PhaI were identified from P(3HB-co-3HA) inclusions and PHB synthase (PhbC) and 24-kDa unknown protein were identified from P(3HB) inclusions. Mee-Jung *et al*. (2006) attempted proteome analysis of PHB granule associated proteins from recombinant *E. coli* harbouring heterologous PHB biosynthesis genes by two-dimensional electrophoresis followed by LC-MS/MS. There are seven proteins such as elongation factor Tu (TufB), β-ketothiolase (PhbA), 16 kDa heat-shock protein B (IbpB), 16 kDa heat-shock protein A (IbpA) and hypothetical protein YbeD were identified from PHB granules. Two granule associated proteins IbpA/B were further characterized by immunoblotting and immunoelectron microscopic studies and confirmed their presence on the surface of PHB granule. Jendrossek and Pfeiffer (2014) have analyzed the granule associated proteins from *R. eutropha* H16 by LC-MS/MS, where they identified more than 400 polypeptides. Besides proteins of PHB metabolism (PhaC1, PhaP1, PhaP2, PhaP3, PhaP4 and PhaZa1), they also

found hypothetical proteins, outer membrane proteins and cellular proteins including enzymes of the tricarboxylic acid cycle.

McCool and Cannon (1999) reported the presence of three most abundant proteins having molecular mass 14, 20 and 41 kDa. However, N-terminal amino acid sequencing of these proteins followed by BLASTp search revealed that the 14-kDa protein was lysozyme, which was used for cell lysis and other two were unknown proteins. From their work it was confirmed that all the proteins which are co-purify with PHA granules during purification need not necessarily be associated them *in vivo*. Artificial binding of lysozyme and GroEL to PHB granules during the isolation of PHA granules has been reported by researchers (Liebergesell *et al*., 1992; Law *et al*., 2001). Jendrossek and Pfeiffer (2014) identified several proteins, other than well-known granule associated proteins. The authors suggested that the presence of these proteins might be due to artificial binding to PHB granules during cell lysis. However, in the present study the artificial binding of lysozyme to the PHB granules could not be detected. This indicates that the chances of artificial binding of cellular proteins to PHB granules are minimum.

Proteins identified from *B. megaterium*	**Function**
Polyhydroxyalkanoic acid synthase, PhaC subunit Polyhydroxyalkanoic acid synthase, PhaR subunit Acetoacetyl-CoA reductase, PhaB subunit β- ketothiolase, PhaA subunit Polyhydroxyalkanoic acid inclusion protein PhaP Ketol-acid reductoisomerase 3-hydroxybutyryl-CoA dehydrogenase	**PHA biosynthesis**
Pyruvate dehydrogenase complex E3 component, dihydrolipoamide dehydrogenase 2-oxoglutarate dehydrogenase, E2 component (dihydrolipoamide succinyltransferase Pyruvate dehydrogenase E1 component subunit alpha Pyruvate dehydrogenase E1 component subunit beta	**Pyruvate dehydrogenase complex**
Acetyl-CoA hydrolase/transferase family protein Acetyl-CoA carboxylase, biotin carboxylase	**Acetyl-CoA metabolic process**
Isocitrate dehydrogenase, NADP-dependent citrate synthase II	**Citric acid cycle**
Acyl-CoA dehydrogenase	**Fatty acid β oxidation**
MaoC like domain-containing protein	**Fatty acid biosynthesis**
1,4-alpha-glucan branching enzyme Methionine import ABC transporter methionine-binding protein MetQ Phosphomethylpyrimidine kinase Cell division ATPase FtsA ,Cell divisionandchromosome partionning Putative branched-chain amino acid ABC transporter permease Cell wall endopeptidase Auxin efflux carrier (AEC) family transporter N-acetylmuramic acid 6-phosphate etherase N-acetylmuramoyl-L-alanine amidase	**Cell wall synthesis**

Table 4.1: PHB granule associated proteins identified in *B. megaterium*

Proteins identified from *B. megaterium*	**Function**
NADH dehydrogenase Ndh ATP synthase F1 subunit beta Succinate dehydrogenase, flavoprotein subunit ATP synthase F1 subunit alpha ATP synthase F1 subunit gamma	**Electrone transport chain**
Glutamyl-tRNA(Gln) and/or aspartyl-tRNA(Asn) Amidotransferase subunit B 30S ribosomal protein S2 50S ribosomal protein L5 30S ribosomal protein S5 30S ribosomal protein S4 50S ribosomal protein L4 50S ribosomal protein L3 elongation factor Tu,	**Protein Synthesis**
Signal peptide peptidase SppA, 36K type	**Peptidase activity (Hydrolase)**
Hypothetical protein (10) Universal stress protein family domain-containing protein	**Unknown function**
Ribose-phosphate diphosphokinase	**Nucleic acid and amino acid biosynthesis**
Hydrolase HAD-superfamily hydrolase	**Esterases (Compound hydrolysis)**

Table 4.1 contd: PHB granule associated proteins identified in *B. megaterium*

Bacterial strain	Protein/Enzyme	Gene	Reference
Ralstonia eutropha H16	PHB synthase C1	*phaC1*	Schubert *et al.*, 1988; Peoples and Sinskey, 1989; Pfeiffer *et al.*, 2011; Cho *et al.*, 2012; Pfeiffer and Jendrossek, 2013
	PHB synthase C2	*phaC2*	Peplinski *et al.*,2010; Pfeiffer and Jendrossek, 2012
	Phasins	*phaP1*,*phaP2*, *phaP3*,*phaP4*	York *et al.*, 2001; Pötter *et al.*, 2004; Pfeiffer and Jendrossek,2011; 2012; 2013;Pfeiffer *et al.*, 2011
	Phasins	*phaP5*,*phaP6*, phaP7,*phaP8*	Pfeiffer and Jendrossek, 2011; 2012; 2013; Pfeiffer *et al.*, 2011; Wahl *et al.*, 2012
	Granule segregation factor	*phaM*	(Pfeiffer and Jendrossek, 2011; 2013; 2014; Pfeiffer *et al.*, 2011; Cho *et al.*, 2012; Wahl *et al.*, 2012
	PHB depolymerise	*phaZ1*	Saegusa *et al.*, 2001; Kobayashi *et al.*, 2003; Pfeiffer *et al.*, 2011; Brigham *et al.*, 2012a
	PHB depolymerise	*phaZ2*, *phaZ5*	York *et al.*, 2003; Brigham *et al.*, 2012a
	PHB depolymerise	*phaZ3*, *phaZ4*	Schwartz *et al.*, 2003; Brigham *et al.*, 2012a
	PHB depolymerise	*phaZ6*	Abe *et al.*, 2005
	Oligomer hydrolase	*phaY1*, *phaY2*	Kobayashi *et al.*, 2003; 2005; Uchino *et al.*, 2008
Pseudomonas putida GPo1, *Pseudomonas oleovorans* KT2442	PHA synthase	*phaC1*, *phaC2*	Foster *et al.*, 1996; Stuart *et al.*, 1996; Ren *et al.*, 2009a; 2009b
	PHB depolymerase	*phaZ*	Stuart *et al.*, 1996; de Eugenio *et al.*, 2007; 2010a; Ren *et al.*, 2009a
	Phasin	*phaI*	Klinke *et al.*, 2000; Sandoval *et al.*, 2007; Ren *et al.*, 2009b
	Granule segregation Factor, phasin	*phaF*	Moldes *et al.*, 2004; Sandoval *et al.*, 2007; Ren *et al.*, 2009b) (Prieto *et al.*, 1999; Klinke*et al.*, 2000; Galán *et al.*, 2011
	Transcriptional regulator	*phaD*	Klinke *et al.*, 2000; Sandoval *et al.*, 2007; De Eugenio *et al.*, 2010b
	Acyl-CoA synthetase	*acs1*	Ruth *et al.*, 2008) (Ren *et al.*, 2009b
Bacillus megaterium	PHB synthase	*phaC*, *phaR*	McCool and Cannon, 1999; 2001
	Phasin	*phaP*	McCool and Cannon, 1999; 2001
	Transcriptional regulator of phasin expression	*phaQ*	(McCool and Cannon, 1999; 2001) (Lee *et al.*, 2004)
	i-PHB depolymerase	*phaZ1*	Chen *et al.*, 2009
Rhodospirillum rubrum	PHB synthase	*phaC1*, *phaC2*, *phaC3*	Jin and Nikolau, 2012
	Phasin, activator of PHB depolymerase	*apdA*	Handrick *et al.*, 2004a,c
	PHB depolymerase	*phaZ1*, *phaZ2*, *phaZ3*	Handrick *et al.*, 2004b; Sznajder and Jendrossek, 2011
Paracoccus denitrificans	PHB synthase	*phaC*	Ueda *et al.*, 1996
	Phasin	*phaP*	Maehara *et al.*, 1999
	Transcriptional Regulator of phasing expression	*phaR*	Maehara *et al.*, 1999; 2002; Gao *et al.*, 2001; Yamada *et al.*, 2007
	i-PHB depolymerase	*phaZ*	Gao *et al.*, 2001
Halophilic Archaea	PHA synthase	*phaC*, *phaE*	Han *et al.*, 2007; Lu *et al.*, 2008; Cai *et al.*, 2012
	Phasin	*phaP*	Cai *et al.*, 2012

Table 4.2: PHA granule associated proteins involved in PHA metabolism from different bacterial species

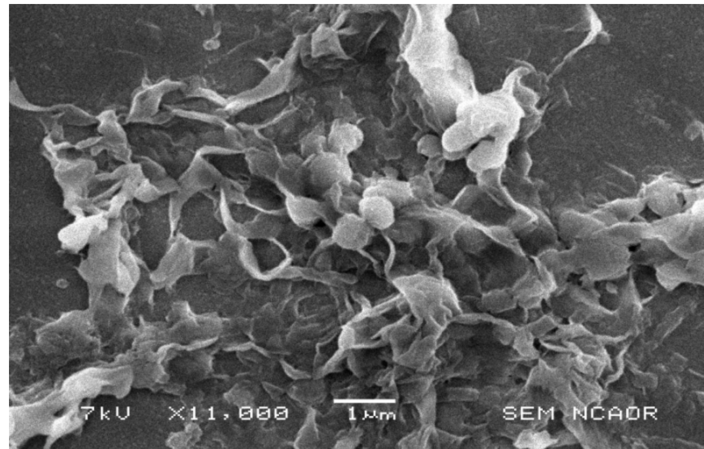
4.3.3 SEM/EDX analysis of native PHA granules

In order to understand the type of granule formation in PHA accumulating *Bacillus megaterium*, cells of two bacterial strains, *B. megaterium* TMR1.3.2 and *B. megaterium* Col1/A6 were analysed using SEM (Fig. 4.2, 4.3, 4.4 and 4.5). PHA granules were observed attached to bacterial cell in samples treated with lysozyme for 15 and 30 min. In the samples treated with lysozyme for 45 min the PHA granules were not bound with cell wall. Similar observations were recorded for both the strains irrespective of growth time. These granules were found in single and budding form. The size of PHA granules was found between 0.28 - 0.95 μm in *B. megaterium* TMR1.3.2 and 0.3 - 1.1 μm in *B. megaterium* Col1/A6. From this observation it can be concluded that PHA granules might be attached to the bacterial cell wall. During cell lysis for longer time with lysozyme these granules become free from cell wall. Further to differentiate between PHA granule and cell wall the EDX of both granule and cell wall was carried out (Fig. 4.6 and 4.7). The EDX of PHA granule showed the presence of C, N, O and P atoms. In the cell wall along with these atoms Al and Cu were also detected.

Prabhu *et al*. (2010) have purified PHA granules of *Bacillus megaterium* NQ-11/A2 and found that PHA granules were spherical, present in single or in budding form. Similar observations were reported earlier on SEM analysis of purified PHA granules from *B. megaterium* (Griebel *et al*., 1968; McCool and Cannon, 1996) and *B. thuringiensis* (Rohini *et al*., 2006).

The formation of PHB granules in *Caryophanon tenue* and *C. latum* was studied by Shekhovtsev and Zharikova (1978). Using light microscopy they found that in the early stages of granule formation, PHA granules were attached to the cytoplasmic membrane whereas at the later stages these granules were observed in the whole cell. In bacterial species such as *Ralstonia eutropha*, *Rhodospirillum rubrum* and *Azotobacter vinelandii* a close association of PHB granules with cell membrane was observed (Jendrossek, 2005; Hermawan and Jendrossek, 2007). Jendrossek *et al*. (2007) repeated the work on PHB granule formation in *C. latum* by using high resolution TEM. Although they could not observed any direct attachment of PHB granules to the cell membrane but confirmed that PHB granules were closely associated with cell membrane in the early stages of granule formation.

A



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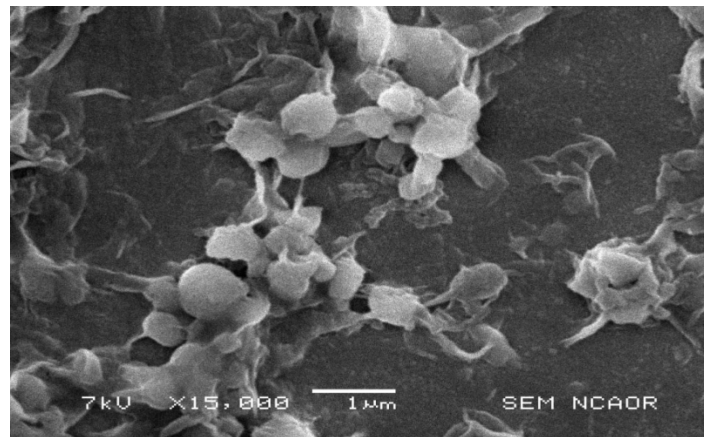

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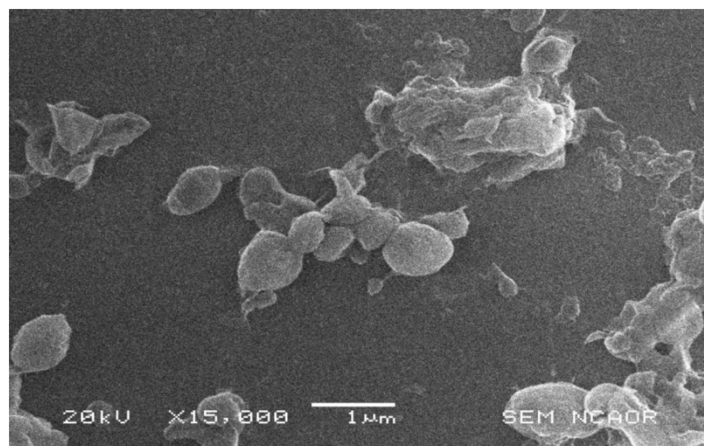

Fig. 4.2: SEM analysis of PHA granules isolated from *B. megaterium* TMR1.3.2 grown for 16 h with treatment of lysozyme for 15 min (A), 30 min (B) and 45 min (C)

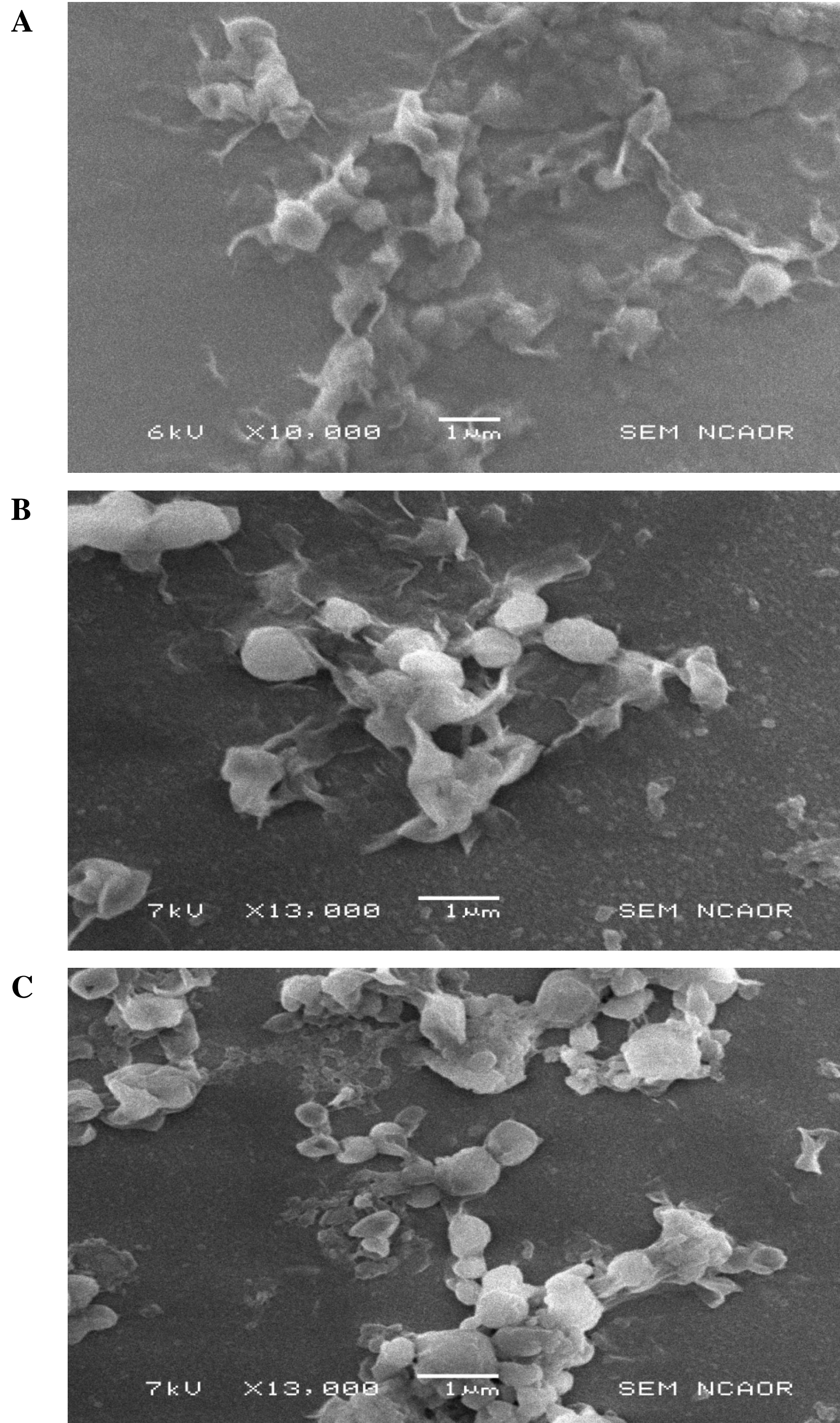

Fig. 4.3: SEM analysis of PHA granules isolated from *B. megaterium* TMR1.3.2 grown for 24 h with treatment of lysozyme for 15 min (A), 30 min (B) and 45 min (C)

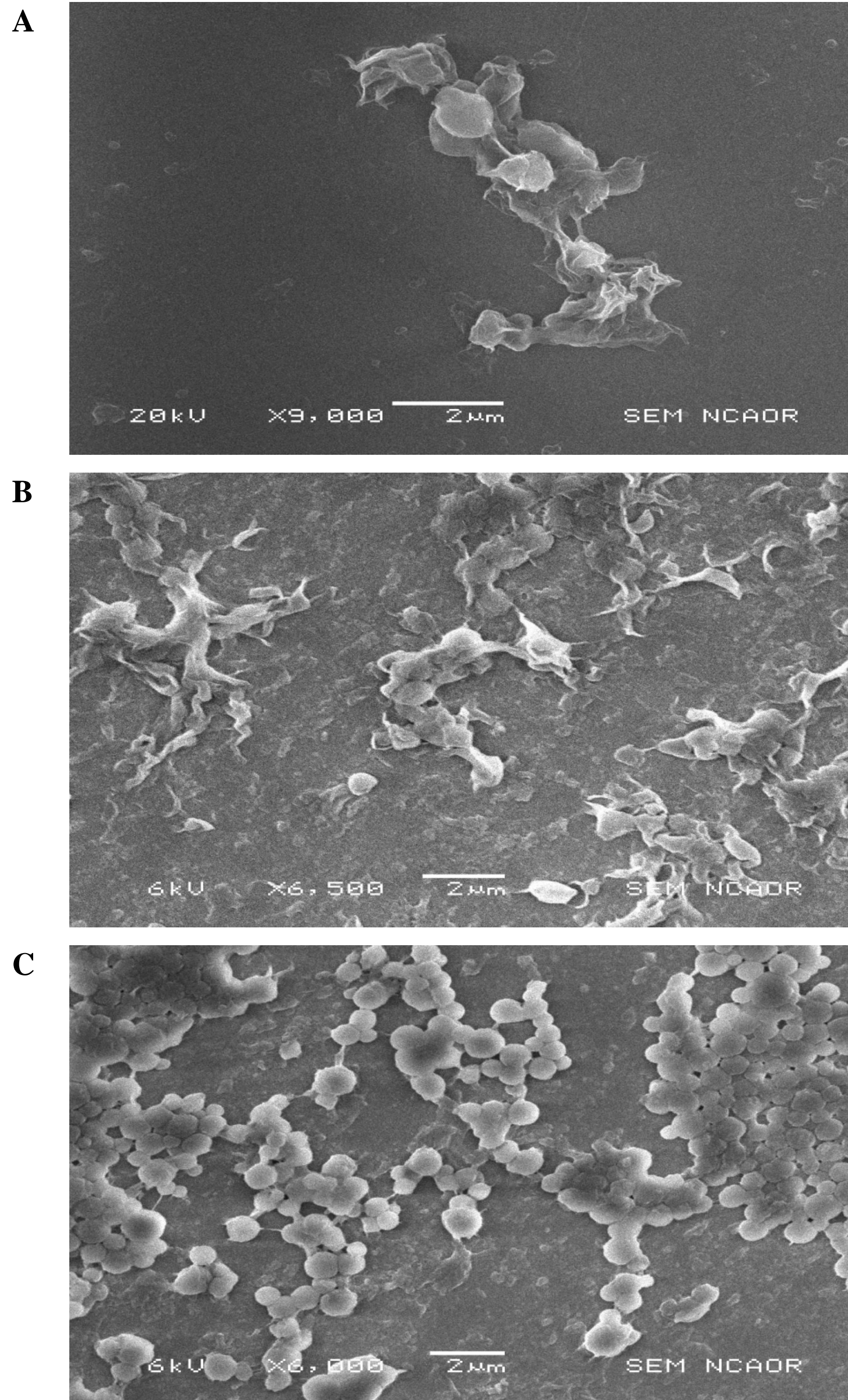

Fig. 4.4: SEM analysis of PHA granules isolated from *B. megaterium* Col1/A6 grown for 16 h with treatment of lysozyme for 15 min (A), 30 min (B) and 45 min (C)

A

B

C

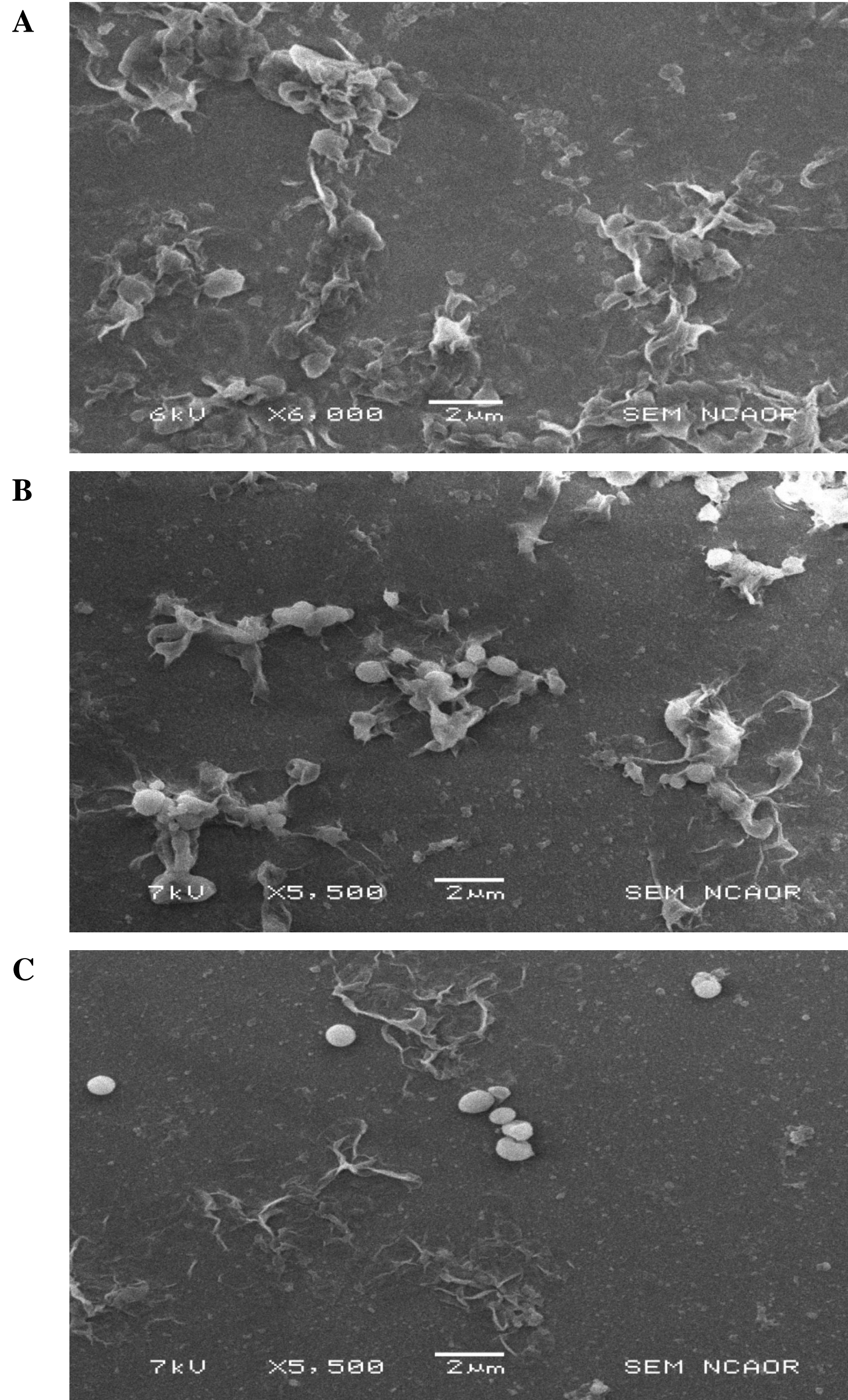

Fig. 4.5: SEM analysis of PHA granules isolated from *B. megaterium* Col1/A6 grown for 24 h with treatment of lysozyme for 15 min (A), 30 min (B) and 45 min (C)

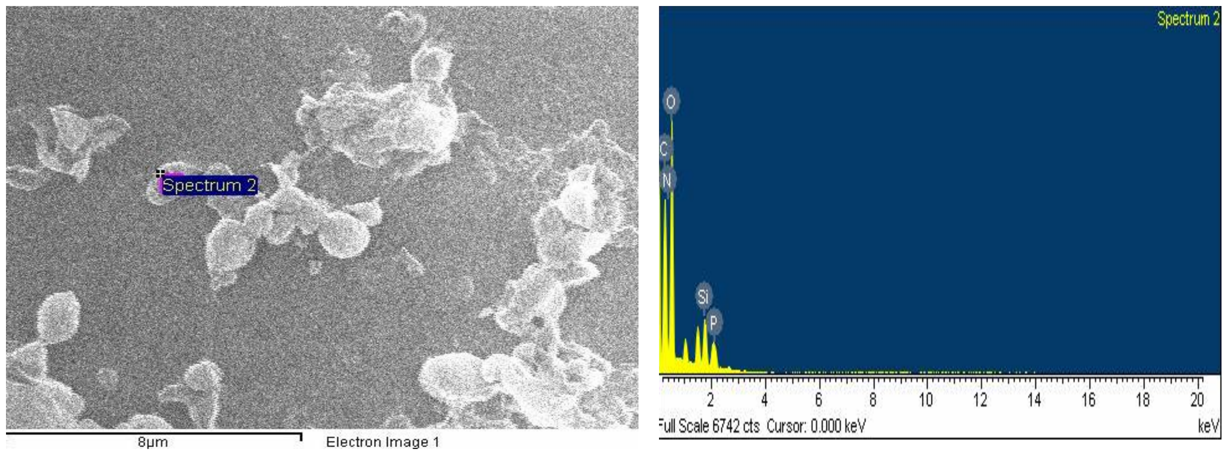

Fig. 4.6: SEM/EDX analysis of PHA granule isolated from *B. megaterium* TMR1.3.2

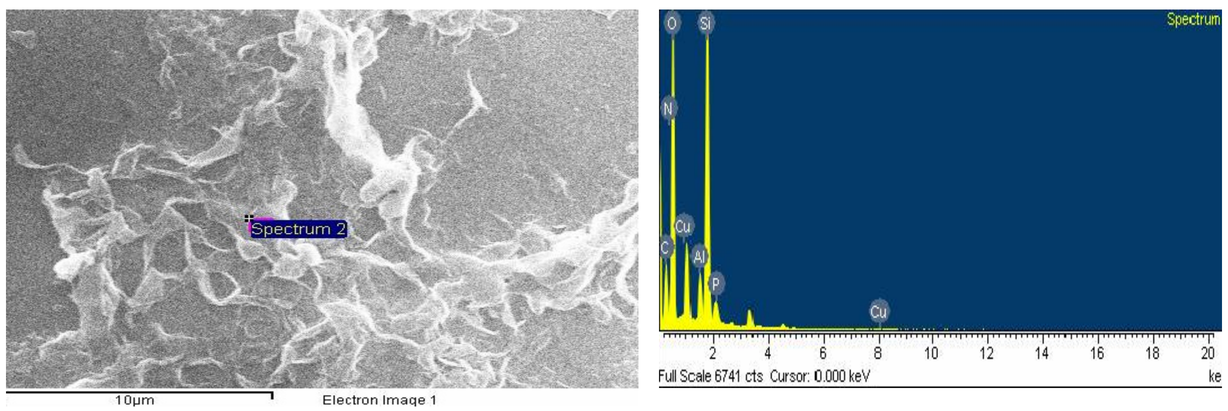

Fig. 4.7: SEM/EDX analysis of cell wall of *B. megaterium* TMR1.3.2

Summary

&

Future Prospects

Summary

The present investigation was carried out in order to isolate potential polyhydroxyalkanoate accumulating bacteria from costal sand-dune ecosystem of Miramar beach, Goa. The study dealt with isolation of heterotrophic bacteria from sand-dune ecosystem and screening for their ability to accumulate PHA. PHA accumulating bacterial isolates characterized phenotypically and genotypically for their identification. For polymer production, these bacterial isolates were grown in E2-mineral medium containing glucose as sole source of carbon. Biomass and polymer dry weight was carried out gravimetrically. All the bacterial isolates were screened for PHA accumulation using various organic acids as carbon sources. Polymers extracted were characterized by FTIR, ^{1}H NMR and ^{13}C NMR spectroscopy. Batch and fed-batch cultivation methods were investigated in fermentor for selected bacterial isolates. Native PHA granules were isolated from two bacterial isolates. Proteins associated with PHA granules were extracted and loaded in SDS-PAGE followed by silver staining. Individual protein bands were separately processed for in-gel trypsin digestion and tryptic digests obtained were loaded in LC/MS-QToF for identification.

The major outcomes of this study are as follows

- Highest heterotrophic bacterial counts were obtained from rhizosphere samples. Total 171 bacterial isolates were obtained, 77 were obtained on Nutrient agar and 94 were on Tryptone glucose yeast extract agar. Maximum numbers of bacterial isolate were also obtained from rhizosphere.
- Twenty-two isolates showed PHA accumulation on E2-mineral medium containing glucose as sole source of carbon. All the isolates showed PHA accumulation within 24 h. Among these, bacterial isolates TMR1.3.2, TMR1.26 and TMR1.28 showed maximum PHA accumulation at 48 h.
- Maximum numbers of PHA accumulating bacterial isolates were obtained from rhizosphere samples.
- PHA accumulating bacterial isolates was tentatively identified as per their phenotypic characteristics and clustering with the standard organisms in the phenogram. Thirteen isolates showed similarity with *B. megaterium*, one with *B. flexus*, one with *Pseudomonas oryzihabitans,* one with *Paracoccus yeei* and one as

Paracoccus sp.. Five isolates did not showed similarity with any standard species in the tree and as these were clustered near *B. megaterium* were identified as *Bacillus* sp..

- Two PCR methods were developed for rapid identification of PHA accumulating members of Bacillales. Method I for rapid identification of PHA accumulating *Bacillus megaterium* and Method II for rapid differentiation of PHA accumulating members of Bacillales.
- PCR amplification of *phaC* gene from sand-dune bacterial isolates was carried out using Method I. Out of 22 bacterial isolates screened, 13 bacterial isolates showed amplification of 900 bp amplicon and were identified as *Bacillus megaterium*.
- 16S rRNA gene of selected bacterial isolates were amplified and sequenced. Nucleotide sequences obtained were analyzed and deposited in Genbank. The phylogenetic tree and maximum sequence homology of the isolates identified these isolates as *B. megaterium* (7), *B.flexus* (1), *B. endophyticus* (1), *B. vireti* (2), *Bacillus* sp. (2), *P. oryzihabitans* (1), *Paracoccus yeei* (1) and *Paracoccus* sp. (1).
- The diversity of PHA accumulating bacteria observed from coastal sand-dune ecosystem includes both Gram positive and negative bacteria. Gram-positive bacteria were belonging to the genus *Bacillus* such as, *Bacillus megaterium* (13), *Bacillus flexus* (1), *Bacillus endophyticus* (1), *Bacillus vireti* (2), *Bacillus* sp. (2). Gram-negative bacteria were of genus *Pseudomonas* and *Paracoccus* which includes *Pseudomonas oryzihabitans* (1), *Paracoccus yeei* (1) and *Paracoccus* sp. (1).
- Production of biomass and PHA was carried out from all the bacterial isolates using glucose as sole source of carbon. Bacterial isolates showed polymer accumulation ranges between 21.8 and 71.2% of their dry cell weight.
- *Bacillus* sp. NAMR1.8 showed maximum and *Paracoccus* sp. TMNR1.3 showed lowest PHA accumulation among sand-dune bacterial isolates.
- *Bacillus megaterium* TMR1.3.2 showed maximum over all biomass and PHA accumulation at 48 h.
- *Pseudomonas oryzihabitans* NAMR1.6, *Bacillus vireti* TMR1.9.1, *Bacillus vireti* TMR1.9.2 and *Bacillus endophyticus* TMR1.22 were being reported for the first time as PHA accumulating bacterial species.

- *Bacillus mojavensis*, *Bacillus niacin*, *Bacillus simplex*, *Marinibacillus marinus* 21AIT and *Paenibacillus dendritiformis* 30A2 were also reported first time for PHA accumulation.
- Twenty-two bacterial isolates were screened for PHA accumulation using various organic acids. In the presence of pyruvic acid all the isolates showed PHA accumulation. Seventeen isolates showed accumulation using succinic acid, 9 isolates showed accumulation using propionic acid, 15 isolates showed accumulation using valeric acid and none of the isolates showed growth or PHA accumulation on octanoic acid.
- Seven bacterial isolates showed PHA accumulation using all the organic acids tested except octanoic acid. These bacterial isolates belong to *Bacillus megaterium*.
- *Bacillus megaterium* TMR1.3.2 was used for polymer production using glucose as sole source of carbon and combinations of glucose along with valeric acid as carbon sources. The polymers extracted were characterized by FTIR, ^{1}H NMR and ^{13}C NMR and identified as polyhydroxybutyrate.
- The polymers extracted from 22 bacterial isolates of sand-dune ecosystem using glucose as sole source of carbon were characterized by FTIR spectroscopy and identified as polyhydroxybutyrate.
- All the PHA accumulating bacteria obtained from coastal sand-dunes produces polyhydroxybutyrate only.
- *Bacillus megaterium* TMR1.3.2 and *Bacillus megaterium* Col1/A6 were selected for high cell density PHB production using batch and fed-batch cultivation.
- The kinetic parameters obtained using batch cultivation of *B. megaterium* TMR1.3.2 with glucose and nitrogen limitations were used for fed-batch cultivation.
- In fed-batch cultivation of *B. megaterium* TMR1.3.2 was carried out with exponential feeding of substrate concentrations. At 28 h of fermentation the DCW and PHB obtained were 116.88 g/L and 50.61 g/L respectively, with the overall productivity of PHB as 1.823 g/L/h.
- The same conditions were used for fed-batch cultivation of *B. megaterium* Col1/A6. At 25 h of cultivation the DCW and PHB obtained were 122.68 g/L and 65.76 g/L, respectively. The overall productivity of PHB was 2.63 g/L/h.

- The biomass and PHB obtained in fed-batch cultivation of *B. megaterium* TMR1.3.2 and *B. megaterium* Col1/A6 were higher than the earlier reports of high cell density PHB production using *B. megaterium*.
- Native PHB granules were isolated from *B. megaterium* TMR1.3.2 and *B. megaterium* Col1/A6. Proteins associated with PHB granules were extracted and loaded in SDS-PAGE followed by silver staining. The SDS-PAGE profile of granule associated proteins showed presence of 25-30 visible protein bands.
- Each protein bands were excised from the gel and processed for in-gel trypsin digestion. The tryptic digest extracts obtained were loaded in LC/MS QToF for protein identification. More than 60 proteins were identified on the proteome analysis of granule associated proteins.
- Protein involved in PHA metabolism such as PhaC, PhaR, PhaA, PhaB, PhaP, ketol-acid reductoisomerase and 3-hydroxybutyryl-CoA dehydrogenase were identified.
- Besides proteins of PHA metabolism other proteins such as, pyruvate dehydrogenase complex, acetyl-CoA metabolic process, citric acid cycle, fatty acid β-oxidation, fatty acid biosynthesis, cell wall synthesis, electrone transport chain, protein biosynthesis, nucleic acid biosynthesis, amino acid biosynthesis and hypothetical proteins were identified.
- This is the first report of proteome analysis of whole granule associated proteins from *B. megaterium* by LC-MS Q-ToF.
- To understand the type of granule formation, cells of two bacterial strain *B. megaterium* TMR1.3.2 and *B. megaterium* Col1/A6 were analyzed using SEM. PHA granules were observed attached to bacterial cell in samples treated with lysozyme for 15 and 30 min. In the samples treated with lysozyme for 45 min the PHA granules were not bound with cell wall.
- The sizes of PHA granules obtained were between 0.28 - 0.95 μm in *B. megaterium* TMR1.3.2 and 0.3 - 1.1 μm in *B. megaterium* Col1/A6.
- To differentiate between PHA granule and cell wall the EDX of both granule and cell wall was carried out. The EDX of PHA granule showed the presence of C, N, O and P atoms. In the cell wall along with these atoms Al and Cu were also detected.

Future Prospects

In the present study the fed-batch cultivation of *B. megaterium* TMR1.3.2 and *B. megaterium* Col1/A6 for PHB production showed promising results. Both the strains reached high cell density within less incubation time and showed PHB productivity of 1.824 g/L/h and 2.630 g/L/h, respectively. At present these are the highest PHB productivity reported by any *B. megaterium* strain. Pilot plant study for PHB production by *B. megaterium* TMR 1.3.2 and Col1/A6 could help to use these strains in the industry. These strains are capable of producing PHA using various low cost substrates. The process for PHA production by *B. megaterium* TMR1.3.2 and *Bacillus megaterium* Col1/A6 using low cost substrates will help in reducing the production cost.

More than 60 proteins associated with PHB granules of *Bacillus megaterium* were identified during proteome analysis. Besides PhaC, PhaZ and PhaP the role of other proteins on the surface of PHA granule are not yet clear. The study of the role of these granule associated proteins will help in better understanding of its metabolic processes. In the present study 10 hypothetical proteins were identified. The functional validation of these hypothetical proteins will be of great contribution in the field of PHA granule associated protein research.

Appendix

Appendix – A (Media)

A.1 Nutrient broth (pH-7.2) (NB/NA)

Peptone	10.0 g
Sodium chloride	5.0 g
Beef extract	1.5 g
Yeast extract	1.5 g
Distilled water (make final volume to)	1000 ml
*Agar (for solid medium)	20.0 g

A.2 Tryptone Glucose Yeast extract broth (pH-7.2) (TGY)

Tryptone	5.0 g
Yeast extract	3.0 g
Glucose	1.0 g
Distilled water (make final volume to)	1000 ml
*Agar (for solid medium)	20.0 g

The glucose was sterilized separately and added to the medium prior to use.

A.3 E2-mineral medium (pH-7.2) (Lageveen *et al*., 1988)

$NH_4H_2PO_4$	2.93 g
K_2HPO_4	7.5 g
KH_2PO_4	3.7 g
$MgSO_4.7H_2O$ (100 mM)	10 ml
Microelement (MT) stock	1.0 ml
Yeast extract	0.004 g
Glucose	20.0 g
Distilled water (make final volume to)	1000 ml
*Agar for solid medium	20.0 g

MT stock

$FeSO_4.7H_2O$	2.78 g

$MnCl_2.4H_2O$	1.98 g
$CoSO_4.7H_2O$	2.81 g
$CaCl_2.2H_2O$	1.47 g
$CuCl_2.2H_2O$	0.17 g
$ZnSo_4.7H_2O$	0.29 g
Distilled water (make final volume to)	1000 ml

The glucose, magnesium sulphate solution, yeast extract solution and microelement stock were sterilized separately and added to the medium prior to use.

A.11 Media for biochemical characterization (Sneath *et al*., 1986; Vos *et al*., 2009)

i) Motility agar (pH-7.2)

Peptone	5.0 g
Sodium chloride	5.0 g
Beef extract	3.0 g
Distilled water (make final volume to)	1000 ml
Agar	4.0 g

ii) Hugh-Leifson's medium (pH 6.9-7.0)

Peptone	2.0 g
Sodium chloride	5.0 g
K_2HPO_4	0.3 g
Bromothymol blue solution (1% w/v)	2.0 ml
Glucose (10% w/v)	100 ml
Distilled water (make final volume to)	1000 ml
Agar	4.0 g

iii) Indole production (pH 7.0)

Tryptone	10.0 g
Sodium chloride	5.0 g
Distilled water (make final volume to)	1000 ml

iv) Nitrate reduction broth (pH 7.2)

Peptone	5.0 g
Sodium chloride	5.0 g
Beef extract	3.0 g
KNO_3	1.0 g
Distilled water (make final volume to)	1000 ml

v) Voges-Proskauer broth (pH 6.9)

Peptone	5.0 g
K_2HPO_4	5.0 g
Glucose	5.0 g
Distilled water (make final volume to)	1000 ml

Glucose was sterilized separately and added to the medium prior to use.

vi) Medium for acid production from sugars (pH 7.0)

Diammonium hydrogen phosphate	1.0 g
Potassium chloride	0.2 g
Magnesium sulphate	0.2 g
Yeast extract	0.2 g
Sugar solution (10% w/v)	100 ml
Bromocresol purple (0.04% w/v)	15 ml
Distilled water (make final volume to)	1000 ml
Agar	20.0 g

Sugars used were glucose, sucrose, lactose, maltose, mannitol, trehalose and xylose. Sugar solutions were sterilized separately and added to the medium prior to use.

vii) Simmon'scitrate agar (pH 6.8)

$NH_4H_2PO_4$	1.0 g
K_2HPO_4	1.0 g
Sodium chloride	5.0 g
Sodium citrate	2.0 g
$MgSO_4$	0.2 g

Bromothymol blue solution (0.04% w/v)	20.0 ml
Distilled water (make final volume to)	1000 ml
Agar	20.0 g

Viii) Starch agar (pH 7.2)

Peptone	5.0 g
Beef extract	3.0 g
Sodium chloride	5.0 g
Soluble starch	20.0 g
Distilled water (make final volume to)	1000 ml
Agar	20.0 g

ix) Gelatin hydrolysis medium (pH 7.0)

Peptone	5.0 g
Beef extract	3.0 g
Gelatin	20.0 g
Distilled water (make final volume to)	1000 ml

x) Milk agar (pH 7.2)

Skimmed milk powder	100.0 g
Distilled water (make final volume to)	1000 ml
Agar	20.0 g

xi) Sodium chloride medium (pH 7.2)

Peptone	5.0 g
Sodium chloride	2 to 200 g
Beef extract	3.0 g
Distilled water (make final volume to)	1000 ml
Agar	20.0 g

xii) Christensen's urea medium (pH 6.9)

Peptone	1.0 g
KH_2PO_4	2.0 g

Sodium chloride	5.0 g
Urea	10.0 g
Glucose (10% w/v)	100 ml
Phenol red (0.2% w/v)	6.0 ml
Distilled water (make final volume to)	1000 ml
Agar	20.0 g

Sterile glucose and urea solutions were added to the medium prior to use.

xiii) Arginine hydrolysis medium (pH 7.2)

Arginine monohydrochloride	10.0 g
Peptone	1.0 g
Sodium chloride	8.0 g
K_2HPO_4	0.3 g
Phenol red (0.2% w/v)	6.0 ml
Distilled water (make final volume to)	1000 ml

Arginine was sterilized separately and added to the medium prior to use.

xiv) Esculin hydrolysis medium (pH 5.6)

Esculin	5.0 g
Ferric ammonium chloride	0.5 g
Sodium chloride	8.0 g
K_2HPO_4	0.4 g
Distilled water (make final volume to)	1000 ml

Esculin was sterilized separately and added to the medium prior to use.

Appendix – B (Stains, reagents and buffers)

B.1 Stains

B.1.1 Gram's staining

i) Crystal violet

Crystal violet	1.0 g
Ethanol (absolute)	10.0 ml
Distilled water (make final volume to)	100 ml

ii) Gram's Iodine solution

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water (make final volume to)	100 ml

iii) Decolourizer

Ethanol (absolute)	70 ml
Distilled water (make final volume to)	100 ml

iv) Safranin solution

Safranin	2.5 g
Ethanol (absolute)	10 ml
Distilled water (make final volume to)	100 ml

B.1.2 Endospore staining

i) Malachite Green solution

Malachite Green	5.0 g
Distilled water (make final volume to)	100 ml

ii) Safranin solution

Safranin	2.5 g
Ethanol (absolute)	10 ml
Distilled water (make final volume to)	100 ml

B.1.3 Nile Blue A stain

Nile Blue A	0.05 g
Ethanol (absolute)	100 ml

B.2 Reagents

B.2.1 Reagent for oxidase test

N,N,N',N'-Tetramethyl-*p*-phenylenediamine dihydrochloride	1.0 g
Distilled water (make final volume to)	100 ml

B.2.2 Reagent for catalase test

Hydrogen peroxide	10 ml
Distilled water	90 ml

B.2.3 Reagent for nitrate reduction test

Solution A:

Sulphanilic acid	0.8 g
Acetic acid (5 N)	100 ml

Solution B:

α-naphthylamine	0.5 g
Acetic acid (5 N)	100 ml

Acetic acid solution (5 N)

Glacial acetic acid	57.27 ml
Distilled water (make final volume to)	200 ml

B.2.4 Reagent for methyl red test

Methyl red	0.1 g
Ethanol (absolute)	300 ml
Distilled water	200 ml

B.2.5 Omeara's reagent for Voges-Proskauer test

Iso-amyl alcohol	150 ml
p-dimethyl-1-aminobenzaldehyde	10.0 g
HCl (concentrated)	50 ml

B.2.6 Kovac's reagent for Indole test

KOH	40.0 g
Creatine	0.3 g
Distilled water (make final volume to)	100 ml

B.2.7 Normal saline

Sodium chloride	0.85 g
Distilled water (make final volume to)	100 ml

B.3 Buffers

B.3.1 Sodium phosphate buffer (0.1 M, 100 ml, pH 7.0)

NaH_2PO_4 (0.1 M)	39.0 ml
Na_2HPO_4 (0.1 M)	61.0 ml

B.3.2 Tris-EDTA (TE) buffer (pH 8.0)

Tris-chloride (10 mM, pH 8.0)	60 ml
EDTA (1 mM, pH 8.0)	30 ml
Distilled water (make final volume to)	100 ml

B.3.3 Tris-acetate-EDTA (TAE) buffer (pH 8.0)

Tris-base	24.2 g
Glacial acetic acid	5.71 ml
EDTA (0.5 M, pH 8.0)	10 ml
Distilled water (make final volume to)	1000 ml

B.3.4 Lysis buffer (Native granule isolation)

Lysozyme	100 mg
Sodium phosphate buffer (0.1 M, pH 7.0)	1 ml

B.3.5SDS tris-EDTA (STE) buffer

Tris-chloride (10 mM, pH 8.0)	60 ml
EDTA (1 mM, pH 8.0)	30 ml
SDS	2.0 g
Distilled water (make final volume to)	100 ml

Appendix – C (Quantitative estimation methods)

C.1 Estimation of reducing sugars (modified Miller's (1959) method)

Reagents:

Dinitrosalicylic acid solution (DNSA)

NaOH	2.0 g
Phenol	0.2 g
DNSA	0.2 g
Distilled water (make final volume to)	100 ml

NaOH was dissolved in 50 ml of double distilled water. To this phenol and DNSA was added with constant stirring. The volume was finally made up to 100 ml with distilled water. Before using, 0.05 g of Sodium sulphite was added to 100 ml of DNSA solution and mixedthoroughly.

Tartarate solution

Potassium sodium tartarate	33.0 g
Distilled water (make final volume to)	100 ml

Protocol:

To 2 ml of appropriately diluted sample, 2 ml of DNSA solution was added and heated in a boiling water bath. After 10 minutes, 0.8 ml of tartarate solution was added and mixed thoroughly. Absorbance was recorded at 540 nm against reagent blank.

The standard calibration curve was constructed using glucose.

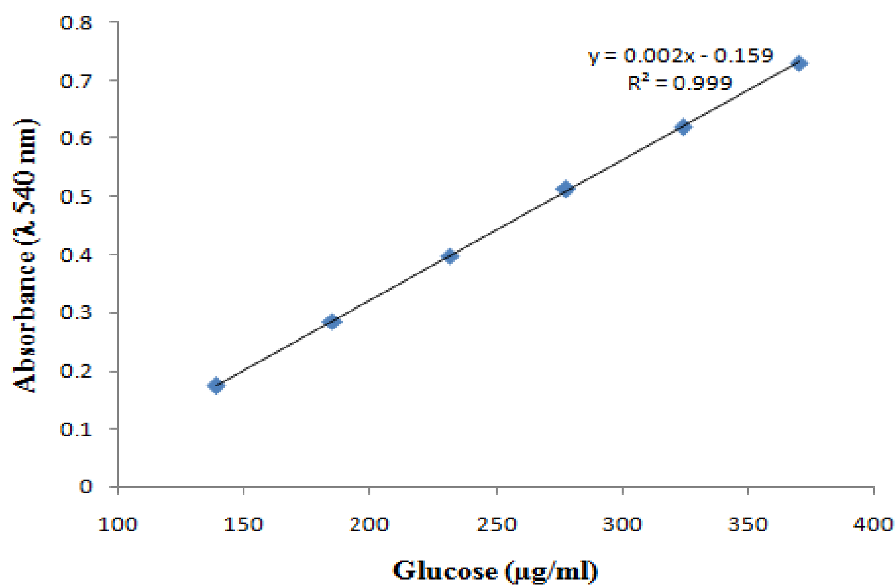

Fig. Standard curve for estimation of reducing sugar.

C.2 Estimation of Ammonia-nitrogen (Grasshoff *et al*., 1999)

Reagents:

Ammonia free water

MilliQ water was boiled at 100°C for 30 min to remove dissolved ammonia. Used to make all the reagents.

Sodium hydroxide solution

NaOH	4.0 g
Ammonia free water (final volume)	100 ml

Phenol reagent

Solution A:

Phenol	20.0 g
Ethanol (absolute)	75 ml
Ammonia free water	150 ml

Phenol was dissolved in 75 ml of ethanol. To this 150 ml of ammonia free water was added.

Solution B:

Disodium nitroprusside dihydrate	0.125 g
Ammonia free water	25 ml

Solution A and Solution B were mixed and stored in a tightly closed amber colour glass bottle at 4°C.

Hypochlorite reagent

Dichloro-s-triazine-2,4,6-(1H, 3H, 5H)-trione sodium salt	0.5 g
Sodium hydroxide solution	100 ml

Dichloro-s-triazine-2,4,6-(1H, 3H, 5H)-trione sodium salt was dissolved in 100 ml of sodium hydroxide solution. The hypochlorite solution was stored in a tightlyclosed amber colour glass bottle at 4°C.

Citrate solution

Trisodium citrate dihydrate	48.0 g
EDTA (disodium salt)	4.0 g
Ammonia free water	100 ml

Trisodium citrate and EDTA was dissolved in 100 ml of ammonia free water. To this 2 ml of sodium hydroxide solution was added and boiled until the volume reached below 100 ml. The solution was cooled and the final volume was adjusted to 100 ml using ammonia free water.

Protocol:

To 50 ml of appropriately diluted sample, 2 ml phenol reagent, 1 ml citrate solution and 2 ml of hypochlorite reagent were added and mixed thoroughly. The bottle was closed tightly and incubated for 30 min in a water bath maintained at 37°C. After incubation the bottle was allowed to cool for 30 min and the absorbance was recorded at 630 nm against reagent blank.

The standard calibration curve was prepared by using ammonium chloride.

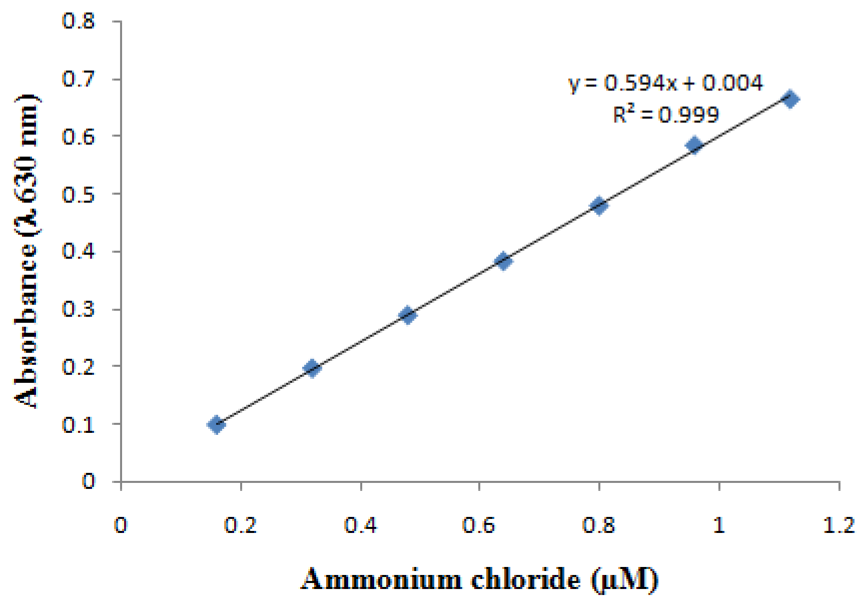

Fig. Standard curve for estimation of ammonia-nitrogen.

Appendix – D

Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

(Sambrook et al., 1989)

D.1 Stock solutions for SDS-PAGE

Acrylamide-*bis*-acrylamide solution (monomer solution)

Acrylamide	29.0 g
N,N' methylene *bis* acrylamide	1.0 g
De-ionized water (make final volume to)	100 ml

Acrylamide and N,N' methylene bis acrylamide was dissolved in 80 ml of warm de-ionized water. The pH of the solution was adjusted to 7.0. The final volume of the solution was made to 100 ml with de-ionized water. The solution was stored in amber colour bottle at room temperature.

Resolving gel buffer (Tris 1.5 M, pH 8.8)

Tris	18.171 g
De-ionized water (make final volume to)	100 ml

Tris was dissolved in 60 ml of de-ionized water. The pH of the solution was adjusted to 8.8 using 6N HCl and the final volume was made up to 100 ml with de-ionized water. The solution was stored at 4°C.

Stacking gel buffer (Tris 1.0 M, pH 6.8)

Tris	12.114 g
De-ionized water (make final volume to)	100 ml

Tris was dissolved in 60 ml of de-ionized water. The pH of the solution was adjusted to 6.8 using 6N HCl and the final volume was made up to 100 ml with de-ionized water. The solution was stored at 4°C.

Ammonium per sulphate (APS) (10% w/v)

Ammonium per sulphate	0.1 g
De-ionized water	1 ml

Sodium dodecyl sulphate (SDS) (10% w/v)

Sodium dodecyl sulphate	10.0 g
De-ionized water (make final volume to)	100 ml

Hydrochloric acid (6N)

Concentrated HCl	51 ml
De-ionized water (make final volume to)	100 ml

Bromophenol blue (1% w/v)

Bromophenol blue	0.1 g
De-ionized water (make final volume to)	10 ml

Tris-glycine electrophoresis buffer 5X (pH 8.3)

Tris base (25 mM)	3.02 g
Glycin (250 mM)	18.8 g
SDS (10% w/v)	10 ml
De-ionized water (make final volume to)	200 ml

Preparation of 1X tank buffer: 100 ml of 5X Tris-glycin electrophoresis buffer was made to 500 ml with de-ionized water.

Sample buffer 2X (10 ml)

Tris-HCl (1 M, pH 6.8)	1 ml
Glycerol	2 ml
Bromophenol blue (1% w/v)	2 ml
SDS (10% w/v)	4 ml
β-mercaptoethanol (200 mM)	284 μl
De-ionized water	716 μl

Preparation of resolving and stacking gel

Solution	Resolving gel 12% (10 ml)	Stacking gel 5% (4 ml)
Monomer	4.0	0.67
Tris (1.5 M, pH 8.8)	2.5	-
Tris (1.0 M, pH 6.8)	-	0.5
SDS (10% w/v)	0.1	0.04
APS (10% w/v)	0.1	0.04
De-ionized water	3.3	2.7
TEMED	0.004	0.004

Sample preparation:

Equal volume of protein sample and 2X sample buffer were mixed and boiled at 100°C for 5 min. After cooling, the protein sample was loaded in the gel.

D.2 Staining of SDS-PAGE gels

Silver staining (Blum *et al*., 1987)

Reagents:

Fixative solution (100 ml)

Methanol	50 ml
Glacial acetic acid	12 ml
Formaldehyde (37% w/v)	0.05 ml
De-ionized water	37.95 ml

$Na_2S_2O_3$ stock solution (2 ml)

$Na_2S_2O_3.5H_2O$	50 mg
De-ionized water	2 ml

Pre-treatment solution (100 ml)

$Na_2S_2O_3$ stock solution	0.8 ml
De-ionized water	99.2 ml

Silver solution (100 ml)

$AgNO_3$	0.2 g
Formaldehyde (37% w/v)	0.075 ml
De-ionized water	99.925 ml

Developing solution (100 ml)

Na_2CO_3	6.0 g
$Na_2S_2O_3$ stock solution	0.016 ml
Formaldehyde (37% w/v)	0.05 ml
De-ionized water	99.93 ml

Stop solution (100 ml)

Methanol	50 ml
Glacial acetic acid	12 ml
De-ionized water	38 ml

Procedure:

The gel was placed on a gel rocker and all the specified solutions were added and replaced in following steps.

S. no.	Step	Solution	Duration
1	Fixing	Fixative solution	at least 1 h
2	Washing	50% methanol	20 min
3	Washing	30% methanol	20 min
4	Pretreatment	Pretreatment solution	1 min
5	Rinsing	De-ionized water	3 times
6	Impregnate	Silver solution	20 min in dark
7	Rinsing	De-ionized water	3 times
8	Developing	Developing solution	Till protein bands appear
9	Stop	Stop solution	10 min
10	Washing	De-ionized water	30 sec

D.3 Reagents for in-gel trypsin digestion

Ammonium bicarbonate stock solution (500 mM)

Ammonium bicarbonate	40 mg
Nano-pure water	1 ml

Potassium ferricyanide stock solution (30 mM)

Potassium ferricyanide	10 mg
Nano-pure water	1 ml

Sodium thiosulphate stock solution (100 mM)

Sodium thiosulphate	16 mg
Nano-pure water	1 ml

Dithiothreitol (DTT) stock solution (2M)

Dithiothreitol	30 mg
Nano-pure water	100 µl

Destaining solution (1 ml)

Potassium ferricyanide solution (30 mM)	0.5 ml
Sodium thiosulphate solution (100 mM)	0.5 ml

Washing solution (1 ml)

Ammonium bicarbonate solution (50 mM)	0.5 ml
Acetonitrile	0.5 ml

Dithiothreitol (DTT) solution (1 ml, 10 mM)

Dithiothreitol solution (2 M)	0.05 ml
Ammonium bicarbonate solution (50 mM)	0.95 ml

Iodoacetamide (IAA) solution (1 ml, 55 mM)

Iodoacetamide	10 mg
Ammonium bicarbonate solution (50 mM)	1 ml

Trypsin preparation (20 mg/ml)

Solution A:

Ammonium bicarbonate	40 mg
Acetonitrile (9% v/v)	1 ml

Solution B:

Solution A	72 µl
Acetonitrile (9% v/v)	828 µl

Solution C:

Trypsin	20 mg
HCl (1 mM, chilled)	100 µl
Solution B	900 µl

Dissolve all the components and store at -20°C (Do not vortex).

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Publications

List of publications (article enclosed)

1. Nayak, P., Gaonkar, T., Mohanty, A., Kumar, A., Bhosle, S., Garg, S., 2013. Rapid identification of polyhydroxyalkanoate accumulating members of Bacillales using internal primers for *phaC* gene of *Bacillus megaterium*. ISRN Bacteriology. Article ID 562014, pp 1-12. doi:10.1155/2013/562014

2. Gaonkar, T., Nayak, P. K., Garg, S., Bhosle, S., 2012. Siderophore producing bacteria from a sand dune ecosystem and the effect of sodium benzoate on siderophore production by a potential isolate. The Scientific World Journal. Article ID 857249, pp 1-8. doi:10.1100/2012/857249

3. Nayak, P., Gaonkar, T., Mohanty, A., Kumar, A., Bhosle, S., Garg, S., 2013. Isolation and characterization of polyhydroxyalkanoates producing bacteria from coastal sand-dune ecosystem. In Microbial Diversity and its Applications. Barbuddhe *et al*. (Ed). NIPA, India. 7, 75-82.

Presentations at conferences

1. Nayak, P., Gaonkar, T., Mohanty, A., Kumar, A., Bhosle, S., Garg, S., (Poster GM-174). "Diversity of polyhydroxyalkanoates accumulating bacteria isolated from coastal sand-dunes" presented at the 50th Annual Conference of AMI December 15-18, 2009 at NCL Pune.

2. Nayak, P., Gaonkar, T., Mohanty, A., Kumar, A., Bhosle, S., Garg, S., (Poster -IP.04). Isolation and characterization of polyhydroxyalkanoates producing bacteria from coastal sand-dune ecosystem" presented at the National Symposium on Microbial Diversity and its applications in Health, Agriculture and Industry held on March 4-5, 2011 at ICAR Research Complex Goa.

3. Nayak, P., Palanker, N., Bhosle, S., Garg, S., (Poster EM-31). "Studies on polyhydroxyalkanoate accumulating heterotrophic bacteria from coastal sand-dunes of East-Coast of India" presented at the 52th Annual Conference of AMI November 3-6, 2011 at Panjab University, Chandigarh.