CHARACTERIZATION OF POLYHYDROXYALKANOATES ACCUMULATING VIBRIOS FROM MARINE BENTHIC ENVIRONMENTS AND PRODUCTION STUDIES OF POLYHYDROXYALKANOATES BY *VIBRIO* SP. BTKB33

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By

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CERTIFICATE

This is to certify that the research work presented in the thesis entitled **"Characterization of polyhydroxyalkanoates accumulating vibrios from marine benthic environments and production studies of polyhydroxyalkanoates by *Vibrio* sp. BTKB33"** is based on the original research work carried out by Mr. Raghul Subin S under my guidance and supervision at the Department of Biotechnology, Cochin University of Science and Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any degree.

SARITA G BHAT

DECLARATION

I hereby declare that the thesis entitled **"Characterization of polyhydroxyalkanoates accumulating vibrios from marine benthic environments and production studies of polyhydroxyalkanoates by *Vibrio* sp. BTKB33"** is the authentic record of research work carried out by me for my doctoral degree, under the supervision and guidance of Dr. Sarita G Bhat, Associate Professor and the Head, Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has previously formed the basis for the award of any degree or diploma, associateship or other similar titles or recognition.

Cochin - 682022 **Raghul Subin S**

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LIST OF ABBREVATIONS

%	Percentage
µg	microgram
µl	microliter
µM	micromolar
^{13}C	Radioactive Carbon
A_{260}	Absorbance at 260nm
A_{280}	Absorbance at 280nm
ANOVA	Analysis of Variance
ATP	Adenosine-5'-triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
CDW	Cell Dry Weight
CMCH	carboxymethyl chitosan
CoA	Coenzyme A
Da	dalton
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DSC	Differential Scanning Calorimetry
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
Fig.	Figure
FT-IR	Fourier-Transform infra red
g	gram
h	hours
HA	Hydroxyapatite

HB	hydroxybutyrate
HV	hydroxyvalerate
l	liter
LB	Luria Bertani
lcl	Long-chain-length
m	meter
M	Molar
MAR	Multiple Antibiotic Resistance
mcl	Medium-chain-length
min.	minutes
ml	milliliter
mM	millimolar
MOF	Marine oxidation fermentation
MPa	Megapascal
N	Normality
NA	Nutrient agar
NCBI	National Center for Biotechnology Information
ng	nanogram
nm	nanometer
NMR	Nuclear Magnetic Resonance
No.	Number
O.D.	Optical density
°C	Degree Celsius
°E	Degree East
°N	Degree north
ONPG	Ortho-nitrophenyl-β-D-galactopyranoside
P(3HB-3HHx)	Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
P(4HB)	Poly(4-hydroxybutyrate)

PB	Plackett-Burman
PCL	Polycaprolactone
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoate
PHB	Poly(3-hydroxybutyrate)
PHO	Poly(3-hydroxyoctanoate)
PP	polypropylene
ppm	parts per million
rDNA	Ribosomal Deoxyribonucleic acid
rpm	revolution per minute
RSM	Response Surface Methodology
s	seconds
scl	Short-chain-length
SDS	Sodium dodecyl sulphate
Sl. No.	Serial number
SmF	Submerged Fermentation
sp.	species
SSF	Solid state fermentation
TAE	Tris-acetate-EDTA
TCBS	Thiosulphate Citrate Bile salt Sucrose
TE	Tris-EDTA
TGA	Thermo gravimetric analysis
*T*m	Melting temperature
UV	Ultraviolet
UV-VIS	Ultraviolet-Visible
v/v	volume/volume
VP	Voges–Proskauer
w/v	weight/volume
µm	micromole

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1 Introduction

Petroleum based synthetic plastics have found various industrial and domestic applications worldwide for the past seventy years due to their versatility and durability (Ojumu *et al.*, 2004). Preliminary estimates by European market research & statistics had put plastic production to more than 230 million tons in 2005 (www.dodyplast.net). However, these synthetic plastics possess several negative attributes mainly with regard to their disposal, one being that plastics are xenobiotics showing recalcitrance to biodegradation (Flechter, 1993), production of toxic substances during incineration (Atlas, 1993) and higher waste accumulation in the landfills and marine environments. It is reported that plastic production also causes high oil consumption, e.g. about 254 million barrels of oil consumed in the US is used for plastic and chemical production (http://seekingalpha.com/article/54648-metabolix-profitable-plastic-production). The crude oil prices and natural gas prices drive the cost of synthetic petroleum derived plastics. Recently global demand for an alternative to these non-degradable petroleum derived plastics has increased tremendously. Over the past few years, bio-based plastics have been developed rapidly owing to rising petroleum prices and due to the possibility that petroleum supplies will be exhausted in the near decades (Chen, 2010). As an alternative to non-biodegradable plastics, eco-friendly biodegradable plastics can help overcome pollution problems. Increasingly, reduction of carbon dioxide emissions has become another reason for promoting bio-based plastics.

According to ASTM D 6400-99 (1976), biodegradable plastics are those plastics in which the degradation results from the action of naturally occurring microorganisms such as bacteria, fungi and algae. Bacteria and fungi play a pivotal role in the process of biodegradation in the natural world. The breakdown of materials provide them with precursors for cell components and energy for

energy-requiring processes. Biodegradation is therefore nothing more than catabolism. Biodegradable materials are usually the products of life itself. Some synthetic polymers can be microbially degraded (Pranamuda *et al.*, 1995: Steinbuchel, 1992), but the process is normally slow. Most have chemical compositions resisting enzymatic attack. This is not the case for polymers of biological origin, many of which have thermoplastic properties. Biodegradable plastics (bioplastics) are mainly categorized into three main types - photodegradable, semi-biodegradable and completely biodegradable plastics. Photodegradable plastics incorporate light sensitive groups into the backbone of the non degradable polymer as additives. Extensive UV radiation can cause disintegration of their polymeric structure and makes them susceptible for further bacterial degradation (Kalia *et al.*, 2000). Semi-biodegradable plastics include starch based plastics, protein (soybean protein) based plastics, cellulose-based plastics etc. They can also be blended with conventional plastics like polyethylene (PE), polypropylene (PP) etc. making these bio-based plastics only partially biodegradable, while the residual petroleum-based plastics remain as broken pieces, creating additional pollution. In addition, these plastics have intrinsic thermal and mechanical weaknesses, and they are now discouraged for applications (Chen, 2010).

The completely biodegradable plastic is of recent origin and promising, because of its complete utilization by microbes in nature (Reddy *et al.*, 2003). To produce biodegradable plastics resembling conventional plastics, bacteria are employed to make the building blocks for plastic polymers from renewable sources. Polyhydroxyalkanoates (PHAs), polylactic acid (PLA), polybutylenes succinate (PBS), polytrimethylene terephthalate and polyphenylene are the best studied polymers containing at least one monomer synthesized via bacterial transformation (Chen, 2010). Except for polymerization of hydroxyalkanoates which is conducted *in vivo*, all other monomers are polymerized *in vitro* by chemical reactions. Thus the PHA is completely natural in origin (Chen, 2010)

and have been reported to be truly biodegradable in both aerobic and anaerobic environments (Page, 1995) unlike many of the "so-called" biodegradable plastics made synthetically. In nature upon disposal, they are degraded completely into water and carbon dioxide under aerobic condition and into methane under anaerobic conditions by microorganisms in different environments (Santhanam and Sasidharan, 2010).

The advantages of PHAs and other bioplastics over petroleum-based polymers are manifold. PHAs and other bioplastics are natural polymers, showing diverse characteristics ranging from thermoplastic to elastic properties. They undergo degradation in presence of microbes within 5-6 weeks and aerobic degradation process results in production of carbon dioxide and water (Brandl *et al.,* 1990), which are environmentally friendly byproducts. The released carbon dioxide and water are absorbed during photosynthesis in nature. The synthesis and biodegradation of biopolymers are totally compatible with the carbon-cycle. The production of PHAs can be from renewable carbon resources, whereby it is unaffected by the depleting fossil fuels, or rise in crude oil prices, in turn resulting in their neutrality with regard to CO_2 emission , leading to conservation of finite fossil resources like mineral oil and coal. The wider use of bioplastics in daily life will solve the increasing problem of organic wastes and decrease the country's dependence for fossil fuels (Ceyhan and Ozdemir, 2011).

Polyhydroxyalkanoates (PHAs) are considered to be strong candidates for biodegradable plastics as their material properties are similar to various synthetic plastics currently in use and also because they show complete degradation in nature (Lee, 1996). PHAs are ubiquitous in nature as they are found in bacteria, several eukaryotic cells including plant and animal tissues. PHAs are polyesters accumulated by various bacteria under unbalanced growth conditions when the carbon substrate is in excess of other nutrients such as nitrogen, sulfur, phosphorus or oxygen (Madison and Huisman, 1999; Kim and Lenz, 2001; Reddy *et al.,* 2003). There have been reports of PHAs and their derivatives produced by and

derived from a variety of microorganisms, over 300 different bacteria, including Gram-negative and Gram-positive species. Until recently, there were only few reports on marine PHAs producing microorganisms (Arun *et al.*, 2009; Ayub *et al.*, 2004; Berlanga *et al.*, 2006; Chien *et al.*, 2007; Lopez *et al.*, 2008; Odham *et al.*, 1986; Rawte and Mavinkurve, 2004; Sun *et al.*, 1994; Weiner, 1997)

Biopolymers from marine prokaryotes, both bacteria and archaea, offer a number of novel material properties and commercial opportunities. Accumulation of PHA enhances the survival ability of microorganisms under adverse environmental conditions and the relation between PHA accumulation and stress were discussed by many researchers (Ayub *et al.*, 2004; Kadouri *et al.*, 2005; Lopez *et al.*, 1995, 1998; Lopez-Cortes *et al.*, 2008; Wang and Bakken, 1998; Zhao *et al.*, 2007). Microbial mats being a highly diverse and challenging environment, are considered important sources for isolation of novel PHA accumulating strains (Berlanga *et al.*, 2006; Lopez-Cortes *et al.*, 2008; Rothermich *et al.*, 2000).

When nutrient supplies are imbalanced, PHA accumulate as discrete granules in bacteria and act as carbon and reducing equivalents sink in microbes. This property helps bacteria to store excess nutrients *invivo* and the polymerization of these soluble intermediates into insoluble molecules prevents the leakage of this valuable nutrients out of bacterial cell (Peters and Rehm, 2005). For PHA accumulating bacteria, the "self-digestion" of PHAs occurs via mobilization which involves enzymes which are different from those responsible for extracellular degradation of PHAs.

PHAs and other bioplastics are biocompatible and hence suitable for medical applications. Some possible applications of PHAs and bioplastics include biodegradable carriers that demonstrate the ability to deliver drugs for a given time within the individual's body and can be used for surgical needles, suture materials, bone tissue replacement, etc. The major advantage of using biodegradable plastics is that it does not require surgical removal. Their

biocompatibility and low oxygen permeability allows for applications, like in the production of films and coatings, besides other special biomedicals like patch materials, stents, bone implants, drug delivery systems and scaffolds for tissue engineering (Ceyhan and Ozdemir, 2011). The other applications in medicine and pharmacy include osteosynthetic materials in the stimulation of bone growth, in bone plates, surgical sutures, blood vessel replacements and cardiovascular products (Chen and Wu, 2005; Galego *et al.*, 2000; Oeding and Schlegel, 1973; Philip *et al.*, 2007; Reddy *et al.*, 2003; Senior and Dawes, 1973; Williams and Martin, 2002). Due to their wider range of property ie., as thermoplastics to elastomers, they find several applications in the domestic (Glazer and Nikaido, 1994), agriculture (Hocking and Marchessault, 1994; Holmes, 1985; Dobbelaere *et al.*, 2001), marine (Asrar and Gruys, 2002) and industrial (Bucci and Tavares, 2005; Chen *et al.*, 2000) fields. The biodegradability of the PHAs can be modified with the incorporation of different monomers and also by structural modifications and these modified properties can be exploited for their application in various fields.

Despite the common practice of exploiting the diversity of bacteria in the environment for the industrial production of novel compounds, there are very few reports that have explored the potential of industrial production of PHAs by bacteria (Chen *et al.*, 2000; Reddy *et al.*, 2003). Steinbuchel (2005) reported more than 140 different monomeric units as constituents of PHA in bacteria that contribute to the differences in the physical and chemical characteristics of PHAs, which in turn is influenced by type of microorganisms, media ingredients, fermentation conditions, modes of fermentation and the recovery process (Keshavarz and Roy, 2010). Hence, there is a need for screening large number of organisms that accumulate PHA with varied combinations of monomers, which are high yielding and with the desirable trait. Isolation of diverse PHA producing bacteria via different enrichment techniques can help to identify novel and more efficient PHA producers.

Objectives of the study

Vibrios are a group of Gram negative, curved or straight motile rods that normally inhabit the aquatic environments. They can be found in their natural habitat as free living bacterium or in association with phyto or zoo plankton (Lipp *et al.*, 2003). Being ubiquitous in the aquatic environment, free living or in association with aquatic organisms, they occur in a wide range of aquatic environments, including estuaries, marine coastal waters and sediments, and aquaculture settings worldwide (Barbieri *et al.*, 1999; Heidelberg *et al.*, 2002; Thompson *et al.*, 2001; Urakawa *et al.*, 2000; Vandenberghe *et al.*, 2003; Venter *et al.*, 2004). Halophilic vibrios represent as much as 40 % of the total microbiota of subtropical coastal water (Chen *et al.*, 1986). Currently the genus *Vibrio* consists of 51 species of which at least 12 are known to be associated with human diseases.

Vibrios were among the first reported strains of PHAs producers from marine sediments (Baumann *et al.*, 1971; Oliver and Colwell, 1973) and were reported to be a dominant flora among the commensally heterotrophic bacteria in Cochin backwaters and near-shore areas of west coast of India (Chandrasekaran *et al.*, 1984). Boyandin *et al.* (2008) identified the ability of luminous bacteria of different taxa including *Vibrio* sp. to synthesize PHAs as storage macromolecules. PHA accumulation in *V. harveyi* (Boyandin *et al.*, 2008; Sun *et al.*, 1994), *Vibrio* sp. strain MK4 (Arun *et al.*, 2009), *Vibrio* sp. BM-1 (Wei *et al.*, 2011b), *V. natriegens* (Chien *et al.*, 2007), *V. fischeri* (Boyandin *et al.*, 2008) and *V. neries* (Rawte and Mavinkurve, 2004) were reported. Optimisation of different bioprocess variables influencing PHA production in *Vibrio* sp. were discussed by several workers (Arun *et al.*, 2009; Chien *et al.*, 2007; Rawte and Mavinkurve, 2004; Wei *et al.*, 2011b). Sun *et al.* (1994) discussed the role of lux autoinducer, N-(3-hydroxybutanoyl) homoserine lactone, in the regulation of PHB synthesis in

V.harveyi. It was interesting that majority of the reported PHA accumulating vibrios were sampled from marine environments (Arun *et al.*, 2009; Chien *et al.*, 2007; Rawte and Mavinkurve, 2004; Wei *et al.*, 2011b).

The present study therefore aimed at evaluating the occurrence of PHA accumulating vibrios inhabiting marine benthic environments; characterizing the potential PHA accumulators employing phenotypic and genotypic approaches and molecular characterization of the PHA synthase gene. The study also evaluated the PHA production in *Vibrio* sp. strain BTKB33, through submerged fermentation using statistical optimization and characterized the purified biopolymer.

The specific objectives are

1. **Screening for PHA producing vibrios from marine benthic environments.**
2. **Characterization of PHA producers employing phenotypic and genotypic approaches.**
3. **Optimization of bioprocess variables for PHA production by strain BTKB33 by submerged fermentation (SmF).**
4. **Characterization of the PHA produced by strain BTKB33.**

2 Review of literature

2.1 POLYHYDROXYALKANOATES (PHAs)

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

The homopolymer, polyhydroxybutyrate (PHB) was discovered by Maurice Lemoigne (1926) and is the first report on PHA. Since the discovery of PHB as storage inclusions in *Bacillus megaterium* in 1926 (Lemoigne, 1926), over 300 different bacteria, including Gram-negative and Gram-positive species, have been reported to accumulate various PHAs (Anderson and Dawes, 1990; Braunegg *et al.*, 1998; Berlanga *et al.*, 2006; Ciesielski *et al.*, 2006; Madison and Huisman, 1999; Steinbuchel, 1991; Steinbuchel, 1992; Steinbuchel and Valentin, 1995; Zinn *et al.*, 2001). Other than PHB, more than 140 different PHA constituents are known (Steinbuchel, 2005).

2.2 POLYHYDROXYALKANOATES IN NATURE

PHAs are accumulated in the cells as discrete granules, the size and number per cell vary depending on the different species. The granules appear as

highly refractive inclusions under electron microscopic observation. In *Alcaligenes eutrophus,* 8 to 13 granules were observed per cell, with diameter ranging from 0.2 to 0.5μm (Byrom, 1994). As PHAs are insoluble in water, the polymers are accumulated in intracellular granules inside the cells and the polymerization of these soluble intermediates into insoluble molecules prevents the leakage of valuable compound out of bacterial cell (Peters and Rehm, 2005). Phospholipids and proteins form a layer over the surface of a PHA granule and in the interface of a granule, the most dominant compound seen is Phasin, a class of proteins known to influence the number and size of PHA granules (Potter *et al.,* 2002; Potter and Steinbuchel, 2005).

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

The marine environment provides a virtually untapped resource for novel bacteria and possibly polymers. Occurrence of polyhydroxyalkanoates (PHA) accumulating microbes have been reported from various environments including mangroves (Rawte *et al.,* 2002), marine sediments (Arun *et al.,* 2009; Chien *et al.,* 2007; Findlay and White, 1983; Odham *et al.,* 1986; Rawte and Mavinkurve, 2004; Wang *et al.,* 2010; Wei *et al.,* 2011), antartic areas (Ayub *et al.,* 2004), soil (Chanprateep *et al.,* 2008; Sabat *et al.,* 1998; Wang and Bakken, 1998), estuarine detritus (Herron *et al.,* 1978), sewage sludges (Reddy *et al.,* 2009; Chee *et al.,* 2010), ponds (Yellore and Desai, 1998), palm-oil mill effluent pond (Alias and Tan, 2005; Redzwan, 1997) and integrated-farming pond (Redzwan, 1997).

Although several bacteria are reportedly known to synthesize and accumulate PHA, a few groups like the methanogenic bacteria and lactic acid bacteria are unable to synthesise these polymers. In most bacteria, PHA are

deposited as prokaryotic inclusion in the cell and these inclusions are only one among many other inclusions. In some bacteria like *E.coli*, the PHA were found as complexes of PHB-Ca^{2+}-polyphosphate molecules and this complex aids in transport Ca^{2+} ions out of the cell (Madison and Huisman, 1999).

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

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

2.3. PHA AND STRESS

Accumulation of reserve polymers such as PHAs increases survival under adverse environmental conditions (Ayub *et al.*, 2004; Kadouri *et al.*, 2005; Lopez *et al.*, 1995; Wang and Bakken, 1998). Several studies reported the relation between stress and PHA accumulation in microbes. Ayub and his co-workers (2004) reported the relation between stress resistance and PHAs accumulation, when better stress resistance was observed in *Pseudomonas* sp. 14-3 isolated from antarctic regions, a habitat normally exposed to extreme conditions than in wild type *Pseudomonas* sp.. Lopez *et al.* (1995) reported that accumulation of PHB

increases the survival capabilities of bacteria in homogeneous aquatic microcosms.

PHA accumulation in *A. hydrophila* 4AK4 provided improved resistance against several environmental stress factors, including heat and cold treatments, hydrogen peroxide, UV irradiation, ethanol and high osmotic pressure (Zhao *et al.*, 2007). *Cupriavidus necator* (previously called *Alcaligenes eutropha, Wautersia eutropha* or *Ralstonia eutropha*) and *Bacillus megaterium*, wild type strains survived better than their PHB-negative mutants under natural oligotrophic environments (Lopez *et al.*, 1995). The PHB accumulation increases survival of *Bacillus megaterium* in water (Lopez *et al.*, 1998). Furthermore, the spores in the polyhydroxyalkanoates negative mutant of *B. megaterium* needed heat shock for germination, which suggesting the possible role of polyhydroxyalkanoates or its degradation products in sporulation (Lopez *et al.*, 1995). The utilization of PHAs in *Azotobacter vinelandii* as a carbon and energy source during encystment has been reported (Segura *et al.* 2003).

The stress and PHA accumulation are inter-related and the application of stress during fermentation that induces PHA accumulation was reported in several studies (Ayub *et al.*, 2004; Obruca *et al.*, 2010; Wang *et al.*, 2005; Zhao and Chen, 2007). In non-endophyte strain *Azospirillum brasilense* Sp7, heavy metals induced enhanced PHB biosynthesis (Kamnev *et al.*, 2007). Addition of ethanol or hydrogen peroxide at the beginning of the stationary phase of *Cupriavidus necator* increases PHB yields (Obruca *et al.*, 2010). Molecular weight of PHB produced under stress conditions was significantly higher as compared to control cultivation (Wang, 2005). Rawte and Mavinkurve (2004) reported that lower aeration favours PHB synthesis in vibrios inhabiting marine environments. PHA accumulation in certain microbes was found to improve with reduced concentration of nitrogen and phosphorous (Belova, 1997; Rawte and Mavinkurve, 2004; Wang, 1997).

2.4. UTILIZATION OF PHAS BY BACTERIA

Nutrient deficiency is common in natural environments (Dawes, 1985), leading to starvation of bacteria. It is therefore very important for bacteria to develop survival ability during starvation to maintain the size of their population. In PHA accumulating bacteria, 'self-digestion' of PHAs is important to restore carbon in bacterial cells under carbon limiting conditions (Merrick and Doudoroff, 1964) and is called mobilization (Handrick *et al.,* 2000). Mobilization involves enzymes which are different from those responsible for extracellular degradation (Merrick *et al.,* 1999). Extracellular degradation is the utilization of PHA granules which are released from other bacteria after cell lysis. The extracellular degradation enzymes are able to degrade denatured PHAs which are released from dead bacterial cells. In contrast, enzymes involved in mobilization can only hydrolyze native amorphous granules.

During the absence of carbon source, mobilization of these previously accumulated storage materials is essential for continuous survival. PHB will be broken down to form acetyl-CoA (Sudesh *et al.,* 2000) and these mobilized products are then oxidized via tricarboxylic acid cycle to synthesize ATP which would enhance the survival and resistance of bacteria in stressful environments (Ruiz *et al.,* 2001). The studies on mobilization of PHAs on several bacterial strains like *Cupriavidus necator* (Handrick *et al.,* 2000), *Legionella pneumophila* (James *et al.,* 1999) and certain *Pseudomonas* sp. (Ayub *et al.,* 2004; Ruiz *et al.,* 2001) showed that mobilization is important to improve survival during carbon starvation. This consumption of accumulated PHB also induced the synthesis of guanosine tetraphosphate (ppGpp) and RpoS (a starvation or stationary phase sigma factor) molecules and hence, improved the resistance towards stress agents such as ethanol and heat (Ayub *et al.,* 2004; Ruiz *et al.,* 2001). It is proposed that the biosynthesis of PHAs with the presence of RpoS increased the survival of *Aeromonas hydrophila* under various stress conditions (Zhao *et al.,* 2007). This is because the biosynthesis and mobilization of PHA are active processes which

proceed at the same time (Uchino *et al.,* 2007) and hence the study of PHA mobilization is also important especially for process optimization studies during large scale production of PHAs.

2.5. PROPERTIES OF PHA

2.5.1. CHEMICAL PROPERTIES

PHAs are a family of optically active biological polyesters, containing (R)-3-hydroxyalkanoic acids (HA) monomer units (Anderson and Dawes, 1990). The 3-HA are all in the R configuration due to the stereospecificity of the polymerizing enzyme, PHA synthase.

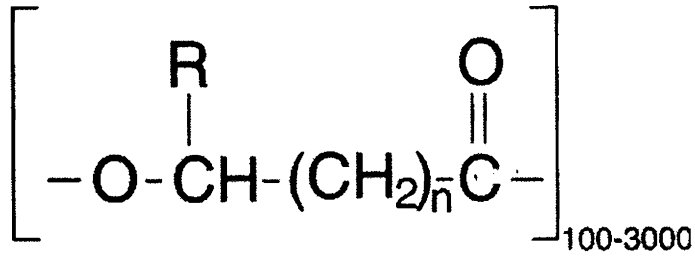
The chemical structure of PHAs produced in bacteria are represented in Fig. 2.1. The pendant group (R in Fig. 2.1.) varies from methyl (C1) to tridecyl (C13) (Poirier *et al.,* 1995). The carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighbouring monomer. This polymerization reaction is catalyzed by the host's PHA synthase.

There is enormous variation possible in the length and composition of the side chains of the PHAs and these variations make the PHA polymer family suitable for an array of applications (Doi, 1990). At the C-3 or β position is placed an alkyl group, which can vary from methyl to tridecyl component. This alkyl chain can be saturated, aromatic, unsaturated, halogenated, epoxidized or with branched monomers (Abe *et al.,* 1990; Arkin *et al.,* 2000; Choi and Yoon, 1994; Curley *et al.,* 1996; Doi and Abe, 1990; Fritzsche *et al.,* 1990; Garcia *et al.,* 1999; Hazer *et al.,* 1994; Kim *et al.,* 1991, Kim *et al.,* 1992; Song and Yoon, 1996).

PHAs are divided mainly into two groups depending upon the number of carbon atoms present in the side chain,:

(A) Short-chain-length PHAs (scl-PHAs)

(B) Medium-chain-length PHAs (mcl-PHAs)



n = 1	R = hydrogen	Poly(3-hydroxypropionate)	PHP
	R = methyl	Poly(3-hydroxybutyrate)	PHB
	R = ethyl	Poly(3-hydroxyvalerate)	PHV
	R = propyl	Poly(3-hydroxycaproate)	PHC
	R = butyl	Poly(3-hydroxyheptanoate)	PHH
	R = pentyl	Poly(3-hydroxyoctanoate)	PHO
	R = hexyl	Poly(3-hydroxynonanoate)	PHN
	R = heptyl	Poly(3-hydroxydecanoate)	PHD
	R = octyl	Poly(3-hydroxyundecanoate)	PHUD
	R = nonyl	Poly(3- hydroxydodecanoate)	PHDD
n = 2	R = hydrogen	Poly(4-hydroxybutyrate)	P(4HB)
n = 3	R = hydrogen	Poly(5- hydroxybutyrate)	P(5HB)

Fig. 2.1 Chemical structure of PHA (figure adapted from Poirier *et al.*, 1995)

Scl-PHAs consists of 3–5 carbon atoms, whereas 6–14 carbon atoms are present in mcl-PHAs. The difference lies in the substrate used and specificity of the enzyme PHA synthase to bring hydroxyalkanoates to a certain range. There is a third group of PHAs named long-chain-length PHAs (lcl-PHAs) comprised of 15 and above carbon atoms, which are given less consideration compared to the rest (Anderson and Dawes, 1990; Luengo *et al.*, 2003; Steinbuchel and Valentine 1995).

PHAs vary in their mechanical properties depending on the composition of the monomeric units. The scl-PHAs are generally considered as thermoplastics, whereas mcl-PHAs are elastomers. PHB, the main candidate among scl-PHAs displays a similar degree of crystallinity and melting point (*T*m) as polypropylene

(PP), but is stiffer and more brittle than PP. Its copolymerization with hydroxy valerate (HV) monomers reduces its stiffness and increases its toughness, giving a product with desirable properties for commercial applications. The mcl PHAs are semicrystalline elastomers with a low *T*m, low tensile strength and high extension to break and can be used as biodegradable rubber. Hence, it is important to study the mechanical and physical properties of such commercially important polymers before their use in the industry.

2.5.2. PHYSICAL PROPERTIES

Biosynthethic PHAs can occur as homopolyesters, copolyesters and as blends. The physical properties of the PHAs are similar to conventional plastics like PP (Marchessault and Yu, 2004; Sato *et al.*, 2005; Tsz-Chun *et al.*, 2005).

Table 2.1 Properties of PHAs and polypropylene (PP). PHBV contains 20% 3 HV-monomer, PHB4B contains 16% 4 HB-monomers, PHBHx conatins 10% 3HHx-monomers (Tsuge, 2002).

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

The properties of PHB (homopolymer of Poly-3-hydroxybutyrate), PHBV (PHB + 20% hydroxyvalerate monomers) and PHB4B (PHB + 16% 4-hydroxybutyrate monomers) and PHBHx (PHB + 10% mcl monomers) are compared with PP and is represented in Table 2.1 (Tsuge, 2002).

PHAs show a high degree of polymerization, as high as 30,000. They are generally lipophilic substances and are insoluble in water. They are soluble in

chlorinated hydrocarbons such as chloroform, methylene chloride, 1,2-dichloroethane or 1,1,2-trichloroethane and in propylene carbonate. In certain solvents, their solubility may vary according to the chemical composition eg. PHB is insoluble in petroleum ether where as PHO is soluble in this organic solvent (Timm *et al.,* 1990). They are optically active and piezoelectric in nature. The molecular weight of the polymers is in the range of 2×10^5 to 3×10^6 daltons, based on the type of microorganism and growth conditions (Byrom, 1994).

2.6. BIODEGRADABILITY OF PHA

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

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

Madison and Huisman, (1999) reported that the degradation rate of a piece of PHB varies from a few months (in anaerobic sewage) to years (in seawater). Studies have shown that 85% of PHAs were degraded in seven weeks (Flechter, 1993; Johnstone, 1990). UV light is found to accelerate the degradation of PHAs (Shangguan *et al.*, 2006). They are compostable over a wide range of temperatures, at a maximum of around 60°C, with moisture content of 55%. PHA is also reported to degrade in aquatic environments (Lake Lugano, Switzerland) within 254 days at temperatures not exceeding 6°C (Johnstone, 1990). Within mammals, the polymer is hydrolysed only slowly (Pouton and Akhtar, 1996). Even more important than biodegradability of PHAs is the fact that their production is biological and based on renewable resources (Braunegg *et al.*, 2004).

2.7. PHA BIOSYNTHESIS IN EUCARYOTIC CELLS

PHAs being ubiquitous are also reported in several plant and animal tissues. The synthesis of PHB in *Saccharomyces cerevisiae* has been demonstrated by expressing the PHB synthase gene from *Cupriavidus necator* (Leaf *et al.*, 1996). PHB production in *Saccharomyces cerevisiae* cells required only the PHB polymerase gene from *C. necator* introduced into the cells. However, in case of recombinant *E.coli,* the introduction of the complete PHB pathway was necessary for PHA formation to occur. The PHA production was also found to be very low ie, only 0.5% of the CDW and is very low compared to bacterial production of PHAs. Poirier *et al.* (2001) introduced a modified *phaC1* gene from *Pseudomonas aeruginosa* into *S. cerevisiae.* Zhang *et al.* (2006) engineered the synthesis of PHA polymers composed of monomers ranging from 4 to 14 carbon atoms in *S. cerevisiae.*

PHA production in an insect cell was reported in cabbage looper cells by the introduction of *phbC* gene from *R. eutropha* using a Baculovirus system (Williams *et al.*, 1996). PHA production in an insect, *Spodoptera frugiperda* cells using a baculovirus has also been reported (Williams and People, 1996). Though

PHA production in insect cells has been reported, the percentage of PHA accumulation is found to be very low compared to that of the microbial system (Madison and Huisman, 1999).

Synthesis of PHA in plants was first demonstrated in 1992 by the accumulation of PHB in the cytoplasm of cells of *Arabidopsis thaliana* (Poirier *et al.*, 1992). In order to produce PHA in large quantities from cheap resources, several researchers have been investigating the possibility of producing PHB in transgenic plants. Synthesis of PHAs in crops is also an excellent way of increasing the value of the crops (Poirier, 1999; Somerville and Bonetta, 2001). Since β-ketothiolase, the first enzyme of PHA synthesis is present in the cytoplasm of higher plants, only the reductase and the PHA synthase are required to synthesize PHA in plant cells (Poirier *et al.*, 1992). Even though transgenic *Arabidopsis thaliana* plants harboring the *C. necator* PHA biosynthesis genes was constructed, these plants accumulated low level of PHB granules in the nucleus, vacuole and cytoplasm. Poirier *et al.* (1992) has reported that the accumulation of PHB in the nucleus of plant cell could affect its growth. The plastid was suggested to be the ideal location for the PHB accumulation because it is the location of high flux of carbon through acetyl-CoA (Nawrath *et al.*, 1994). Recently, the genetically engineered genes of *Cupriavidus necator* were successfully targeted to the plant plastids and the enzymes were active in the plastids (Nawrath *et al.*, 1994).

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

PHB has been produced in Rape seed (*Brassica napus*) by expressing the PHB genes of *C. necator* (Houmiel *et al.*, 1999; Valentin *et al.*, 1999).

2.8. MICROBIAL SYNTHESIS OF PHA

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

Approximately 140 different hydroxyalkanoic acids are known to be incorporated into PHAs (Steinbuchel, 2005), with microbial species from over 90 genera being reported to accumulate these polyesters (Zinn *et al.*, 2001). The different bacteria accumulating PHA in their natural environment is presented in Table 2.2.

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

Table 2.2. Some natural bacterial PHAs accumulators

GENUS	*SPECIES*	REFERENCE
Bacillus	*B. megaterium*	Lopez *et al.*, 1998; Lemoigne, 1926; Lopez *et al.*, 1995; Omar *et al.*, 2001; Ram *et al.*, 2010
	B. megaterium ATCC 6748	Chaijamrus and Udpuay, 2008
	Bacillus sp. CFR 256	Vijayendra *et al.*, 2007
	B. sonorensis	Shrivastav *et al.*, 2010
	B. sphaericus NCIM 2475	Otari and Ghosh, 2009.
	B. sphaericus NCIM 5149	Ramadas *et al.*, 2009.
	Bacillus sp.	Valappil *et al.*, 2007; Thirumala *et al.*, 2010
	Bacillus sp. CFR 256	Vijayendra *et al.*, 2007
	B. cereus M5	Yilmaz and Beyatli, 2005
	B. firmus NII 0830	Deepthi *et al.*, 2011
	Bacillus sp. PS1	Shukla *et al.*, 2011
Lactobacillus	*L. acidophilus; L. helveticus; L. bulgaricus; L. casei; L. lactis; L. plantarum; L. brevis*	Yuksekdag *et al.*, 2008
Burkholderia	*Burkholderia sp. USM*	Chee *et al.*, 2010
	B. cepacia IPT 048	Silva *et al.*, 2004
	B. sacchari IPT 101	Silva *et al.*, 2004
Staphylococcus	*Staphylococcus sp.*	Wang *et al.*, 2007
Streptococcus	*S. thermophilus Ba21S Strain*	Yuksekdag and Beyatli, 2008
Streptococcus	*S. thermophilus; S. durans*	Yuksekdag and Beyatli, 2008
Halomonas	*Halomonas sp. KM-1 Strain*	Yoshikazu and Sei-ichi, 2010
	Haloferax mediterranei	Lillo and Valera, 1990; Castillo *et al.*, 1986
	Halomonas hydrothermalis	Shrivastav *et al.*, 2010
	H. volcanii	Castillo *et al.*, 1986
Methylobacterium	*Protomonas extorquens sp.* strain K	Suzuki *et al.*, 1988
	M. extorquens	Bourque et al., 1992
	Methylobacterium sp. ZP24	Yellore and Desai, 1998
Aeromonas	*A. hydrophila 4AK4*	Zhao *et al.*, 2007
Chromobacterium	*C. violaceum*	Rawte and Mavinkurve, 2004
Pseudoaltermonas	*Pseudoaltermonas sp.* SM9913	Wang *et al.*, 2010
Zobellella	*Z. denitrificans* MW1	Ibrahim H and Steinbuchel, 2009
Delftia	*D. acidovorans* DS-1	Lee *et al.*, 2007
Comamonas	*Comamonas sp.*EB172	Zakaria *et al.*, 2010
Sinorhizobium	*S. fredii*	Liangqi *et al.*, 2006
Klebsiella	*Klebsiella sp.*	Wang *et al.*,2007
Azospirillum	*A.brasilense Sp7*	Kamnev *et al.*, 2007
Rhizobium	*Rhizobium DDSS-69*	Natarajan *et al.*, 1995
Rhodobacter	*R. sphaeroides* ES 16	Sangharak and Praserstan, 2008
	R. sphaerodies	Hassan, 1996
Rhodopseudomonas	*R. palustris* SP5212	Mahuya *et al.*, 2005
	R. palustris KU003	Ramchander *et al.*, 2010
Listeria	*Listeria sp.*	Rawte and Mavinkurve, 2004
Enterobacter	*Enterobacter aerogenes* 12BI	Ceyhan and Ozdemir, 2011

GENUS	*SPECIES*	REFERENCE
Thermus	*T. thermophilus HB8*	Pantazaki *et al.*, 2009
Paracoccus	*P. seriniphilus E71*	Lopez-Cortes *et al.*, 2010
	Paracoccus sp. Strain E33	Lopez-Cortes *et al.*, 2008
Azoarcus	*Azoarcus sp.*	Lemos *et al.*, 2008
Amaricoccus	*Amaricoccus sp.*	Lemos *et al.* 2008
Paracoccus	*Paracoccus sp.*	Lemos *et al.* 2008
Thauera	*Thauera sp.*	Lemos *et al.* 2008
Pseudomonas	*P.oleovorans*	Brandl *et al.*, 1988; Santhanam and Sasidharan ; 2010; Timm *et al.*, 1990; Tsuge *et al.*, 2005
	P. aeruginosa IFO3924	Marsudi *et al.*, 2008
	P. guezennei biovar. tikehau	Simon *et al.*, 2008
	P. hydrogenovora	Koller *et al.*, 2008
	P. putida KT2442	Ribera *et al.*, 2001
	Pseudomonas sp. LDC-5	Saranya and Shenbagarathai, 2010
Vibrio	*V. natriegens*	Chien *et al.*, 2007
	V. harveyi.	Sun *et al.*, 1994; Boyandin *et al.*, 2008
	V. neries	Rawte and Mavinkurve, 2004
	Vibrio sp. strain BM-1	Wei *et al.*, 2011b
	Vibrio sp. strain MK4	Arun *et al.*, 2009;
	V. fischeri	Boyandin *et al.*, 2008
Azotobacter	*A.vinelandii*	Segura *et al.*, 2003
	A.vinelandii UWD	Cho *et al.*, 1997
	A.chroococcum H23	Pozo *et al.*, 2002
	A.chroococcum	Khanafari *et al.*, 2006
Alcaligenes	*A. latus*	Palleroni and Palleroni, 1978; Yamane *et al.*, 1996; Santhanam and Sasidharan, 2010
	A.latus ATCC 29712	El-Sayed *et al.*, 2009
	Alcaligenes latus DSM 1124	Yu *et al.*, 1999
Cupriavidus	*Cupriavidus necator*	Kim *et al.*, 1994; Santhanam and Sasidharan ; 2010; Taidi *et al.*, 1994; Obruca *et al.*, 2010; Lopez *et al.*, 1995 Vandamme and Coenye, 2004; Vaneechoutte *et al.*, 2004 ; Koutinas *et al.*, 2007; Fukui and Doi, 1997; Cavalheiro *et al.*, 2009; Yu *et al.*, 199
	Cupriavidus sp. USMAA2-4	Kek *et al.*,2010
	C. taiwanensis	Wei *et al.*, 2011a
	Cupriavidus sp. KKU38	Sangyoka *et al.*, 2001
	C. necator DSM 545	Cavalheiro *et al.*, 2009

** Cupriavidus necator=Ralstonia eutropha=Alcaligenes eutrophus= Wautersia eutropha.*

2.8.1. Recombinant microbial PHA Producers

Several studies have reported the use of rDNA technology in PHA production. The effects of additional copies of *pha* genes on the formation of polymer by the wild-type organism has been reported and although elevated levels

of PHA were occasionally found, no significant effects of high-copy-number *pha* genes on PHA metabolism was observed (Madison and Huisman, 1999). Studies have shown that the *pha* genes from *P. oleovorans* introduced into itself or into *P. putida* does not cause any increase in PHA synthesis. However, an additional *P. denitrificans phaC* gene on a plasmid doubled PHV levels in parent strain (Ueda *et al.,* 1996).

The *phb*CAB operon from *R. eutropha* was expressed in several strains of *Pseudomonas* like *P. aeruginosa*, *P. putida*, *P. oleovorans*, *P. syringae*, and *P. fluorescens*, that normally do not accumulate PHB. In contrast, *P. stutzeri* considered as a non PHA producer was unable to synthesize PHB with the recombinant *C. necator* genes (Steinbuchel and Schlegel, 1991)

E.coli normally does not synthesize PHAs since it lacks the PHA biosynthetic genes. However, recombinant *E.coli* having PHA biosynthetic genes from *C. necator* has been reported to accumulate PHB. The major advantage of this recombinant *E. coli* is that all the genetic engineering principles that apply to this organism can be utilized in optimizing the production of PHB and other PHAs (Pouton and Akhtar, 1996). High molecular weights of polyhydroxyalkanoates can also be achieved in this organism as *E. coli* does not contain PHA depolymerase enzymes (Sim *et al.,* 1997). There are also potential advantages in terms of ease of extraction of the polymer and purity of the product (Pouton and Akhtar, 1996). Additionally, a PHB synthesizing mutant of *E. coli* has been developed from which the polymer can be extracted by mild heat treatment rather than by chemical extraction techniques (Pool, 1989). A number of recombinant microorganisms other than *E.coli* are also being developed for the economical production of PHAs (Pries *et al.,* 1990; Zhang *et al.,* 1994) and/or for the production of unusual PHAs (Liebergesell *et al.,* 1993; Steinbuchel *et al.,* 1994).

2.9. IMPORTANCE OF MICROBIAL SYNTHESIS OF PHAs

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

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

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

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

amount is necessary as the chemical composition of PHAs depends mainly on the bacterial strain.

2.10. BIOSYNTHESIS OF PHAs

PHA synthase is considered as the crucial enzyme in all PHA synthesis pathways (Anderson and Dawes, 1990; Madison and Huisman, 1999; Rehm and Steinbuchel, 1999). The first two enzymes involved in the synthesis, β-ketothiolase (EC 2.3.1.9) and acetoacetyl-CoA reductase (EC 1.1.1.36), have been studied in detail for several bacteria (Haywood *et al.,* 1988 a & b; Nishimura *et al.,* 1978; Oeding and schlegel, 1973; Saito *et al.,* 1977).

During normal bacterial growth, the β-ketothiolase, first important enzyme in PHA biosynthesis, will be inhibited by free coenzyme-A, coming out of the Krebs cycle. But when entry of acetyl-CoA into the Krebs cycle is restricted, during stressed condition of nutrient limitation, the surplus acetyl-CoA is channeled into PHA biosynthesis (Ratledge and Kristiansen, 2001).

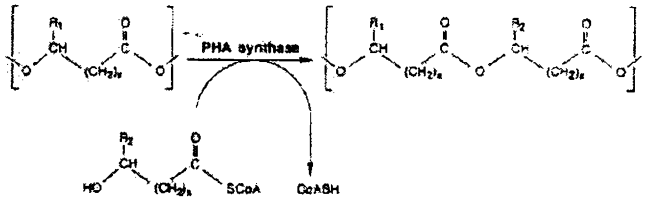


Fig. 2.2. Synthesis of PHA. A hydroxyacyl-CoA thioester is initially linked to another thioester or linked onto a growing chain of PHA by the enzyme PHA synthase, releasing coenzyme A (Steinbuchel *et al.,* 1992)

PHA synthases are a class of highly versatile enzymes, as they are not specific to any one type of hydroxyalkanoic acid (Steinbuchel *et al.,* 1992). The synthesis of PHA is represented in the Fig. 2.2.

The PHA synthases can be broadly categorized into three different types based on their primary amino acid sequences and *in vivo* substrate specificities (Rehm and Steinbuchel, 1999). Type I PHA synthases are preferentially active

towards coenzyme A thioesters of various scl 3-HA comprising three to five carbon atoms. These were also found to incorporate 4- and 5-HA, such as 4-hydroxybutyric acid (4HB) (Kunioka *et al.*, 1988), 4- hydroxyvaleric acid (4HV) (Valentine, 1992) and 5HV (Doi *et al.*, 1987) into PHA. Type II PHA synthases (represented by *Pseudomonas aeruginosa*) are preferentially active towards coenzyme A thioesters of various mcl 3HA comprising at least five carbon atoms. Both types of PHA synthases are encoded by the same phaC gene (Trotsenko and Belova, 2000).Type III PHA synthases (*Chromatium vinosum*) comprise of two different types of subunits, the PhaC subunit and the PhaE subunit (Trotsenko and Belova, 2000). These PHA synthases prefer coenzyme A thioesters of scl 3-HA. Type III PHA synthase has been extensively investigated in a wide variety of organisms including phototrophic bacteria, *Synechocystis* (Hai *et al.*, 2001; Hein *et al.*, 1998), *Chlorogleocapsis fritschii* (Hai *et al.* 2001) and *Ectothiorhodospira shaposhnikovii* (Zhang *et al.*, 2004). Certain bacteria, like *Aeromonas caviae* (Fukui *et al.*, 1998) and *Rhodococcus ruber* (Haywood *et al.*, 1991) are reported to have PHA synthases that exhibit specificity for both scl and mcl HAs.

The PHA synthases of *C. necator* can only polymerize scl 3HAs while that of *Pseudomonas oleovorans* polymerizes only mcl 3HAs. For scl PHAs, the monomer units are oxidized at positions other than the third carbons while for mcl PHAs, all the monomer units are oxidized at the third position except in few cases (Valentin *et al.*, 1994). PHAs (mcl) containing various functional groups such as olefins, branched alkyls, halogens, aromatic and cyano have been reported (Fritzsche *et al.*, 1990; Hazer *et al.*, 1994; Huijberts *et al.*, 1992; Kim *et al.*, 1992). This flexibility of PHA biosynthesis makes it possible to design and produce related biopolymers having useful physical properties ranging from stiff and brittle plastic to rubbery polymers (Anderson and Dawes, 1990).

2.11. BIOSYNTHETIC PATHWAYS OF PHAs

The synthesis of PHAs requires the enzyme PHA synthase (PhaC), which uses β-hydroxyacyl-coenzyme A substrates for polymerization (Madison and Huisman, 1999). The production of such substrates can occur by a variety of pathways (Madison and Huisman, 1999), including the simplest using the enzymes β-ketothiolase (encoded by phaA) and acetoacetyl-CoA reductase (encoded by phaB), β-oxidation (Page, 1995) and a fatty acid *de novo* synthesis pathway (Rehm *et al.*, 1998). Four different pathways have been elucidated so far for the biosynthesis of PHA (Byrom, 1994; Doi and Abe, 1990; Steinbuchel and Schlegal, 1991; Poirier *et al.*, 1995).

2.11.1. Biosynthetic pathway in *Cupriavidus necator*

This pathway, also known as PHB biosynthetic pathway, consists of three enzymatic reactions catalyzed by three distinct enzymes is represented in Fig. 2.3.

The 1st reaction is the condensation of two acetyl coenzyme A (acetyl-CoA) molecules into acetoacetyl-CoA by β-ketoacyl- CoA thiolase (encoded by *phbA*). It is followed by the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl-CoA dehydrogenase (encoded by *phbB*). Finally, the (R)-3-hydroxybutyryl-CoA monomers are polymerized into PHB by PHB synthase (encoded by *phbC*) (Anderson and Dawes 1990). PHA synthase in *Cupriavidus necator* reacts with a narrow range of substrates, with chain length of 3-5 C atoms and prefers C4-substrates (Steinbüchel and Schlegel, 1991). Therefore, PHAs obtained by this pathway contain scl monomers. Though PHB is prominent in bacteria, a wide range of other PHAs are also synthesized in them (Kim and Lenz, 2001; Luengo *et al.*, 2003). A number of PHAs with different C3 to C5 monomers have been produced in several bacteria including *R. eutropha* through alterations in the type and relative quantity of the carbon sources in the growth media (Dias *et al.*, 2006; Steinbuchel and Schlegel, 1991).

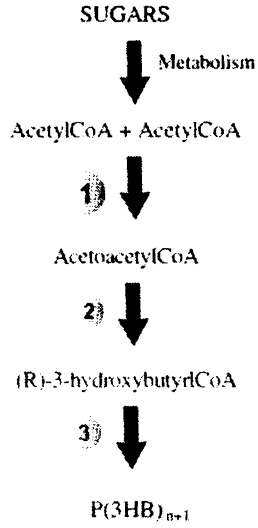


Fig. 2.3 Biosynthesis of PHA from *Cupriavidus necator* (figure adapted from Naik *et al.*, 2008). (1) Ketothiolase (2) NADPH dependent acetoacetyl CoA reductase (3) PHA synthase.

2.11.2. Biosynthetic pathway in *Rhodopseudomonas rubrum*

This is the second type of pathway, which is found in *Rhodopseudomonas rubrum, where* β-oxidation of fatty acid leads to the biosynthesis of PHA. The first reaction remains the same as that for *R. eutropha* leading to the formation of acetoacetyl CoA, which is then reduced into L-(+)-3-hydroxybutyryl CoA by a NADH dependent reductase. In the final step L-(+)-3-hydroxybutyryl CoA is converted into D-(-)-3-hydroxybutyryl CoA by two enol CoA hydratase enzymes (Khanna and Srivastava, 2005).

2.11.3. Biosynthetic Pathway in *Pseudomonas* group I

This is the third biosynthetic pathway of PHA, which is found in most *Pseudomonas* species, belonging to rDNA homology group-I e.g. *P. oleovorans.* In these organisms fatty acid β-oxidation of alkanes, alkanols or alkanoic acids leads to the synthesis of mcl-PHAs (Naik *et al.*, 2008).

2.11.4. Biosynthetic pathway in *Pseudomonas* group II

This is the fourth type of PHA biosynthetic pathway found in *Pseudomonas* belonging to rDNA homology group-II. Synthesis of PHA results from denovo fatty acid synthesis pathway which involves the synthesis of copolymers of mcl-PHAs from acetyl CoA. (Naik *et al.*, 2008).

2.12. GENES INVOLVED IN PHA BIOSYNTHESIS

Madison and Huisman (1999) reported that more than 20 kinds of PHA synthesis operons have been cloned and analysed from various bacteria and that the proteins required for PHA biosynthesis pathways have diverged considerably. The organization of the genes and enzymes involved in the biosynthesis of PHA varies from organism to organism. Mainly seven types of arrangements have been observed for PHA synthesizing genes (Madison and Huisman, 1999, McCool and Cannon, 2001). The PHA synthase genes and genes for other proteins related to the metabolism of PHA are often clustered in the bacterial genomes.

Genes related to the enzymes for scl PHAs formation have been designated as *phb*, and those related to enzymes for mcl PHAs formation as *pha*. Genes coding for proteins involved in the biosynthesis of PHA will be referred in alphabetical order as *phaA* (ketothiolase), *phaB* (acetoacetyl-CoA reductase), *phaC* (PHA synthase), *phaG* (3- hydroxyacyl-acyl carrier protein-CoA transacylase), *phaJ* (enoyl-CoA hydratase) and so on. Since the genes are arranged in clusters, their organization /arrangements differ from species to species. The genes required for the degradation are referred in reverse alphabetical order such as *phaZ* for PHA depolymerases, *phaY*, *phaX*, *phaW* etc. The genes for phasins are referred to as *phaP*. The gene product i.e protein is indicated with first letter in upper case e.g β-ketothiolase is written as *PhaA* (Rehm and Steinbüchel 1999).

After the cloning of the PHA synthase operon of *Ralstonia eutropha*, as many as 54 different PHA synthases (plus one partial PHA synthase gene from *Pseudomonas* sp.) from a total of 44 microorganisms were cloned, and the primary

structures of 44 different PHA synthases are available. The strategies successfully employed to clone these genes and the organization of the PHA synthase genes and other genes encoding proteins related to PHA metabolism has been reviewed extensively (Rehm and Steinbuchel,1999).

2.13. ORGANISATION OF PHA BIOSYNTHESIS GENES

The loci for genes related to PHA biosynthesis are considerably diverged due to the increased diversity of PHA biosynthetic pathways and these genes have been characterized in 18 different species (Madison and Huisman, 1999). All pathways are not completely elucidated in these strains since *pha* and *phb* genes are not necessarily clustered and the gene organization varies considerably from species to species. Other genes possibly related to PHA metabolism may be linked to the essential *pha* and *phb* genes. Genes specifying enzymes for scl-PHA formation are designated *phb*, and those specifying enzymes for mcl-PHA formation are designated *pha* (Madison and Huisman, 1999).

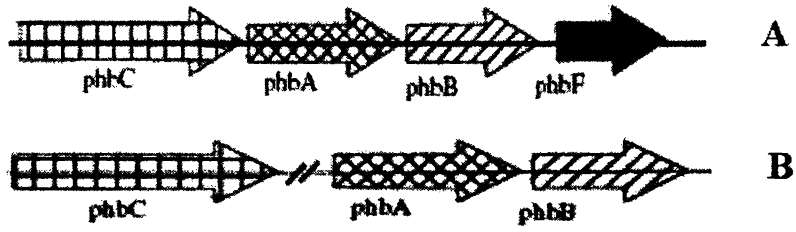


Fig. 2.4 Molecular organization of PHA synthase genes, which encoding PHA synthases of type I (figure adapted from Madison and Huisman, 1999). *phbC*, gene encoding PHA synthase; *phbA*, gene encoding b-ketothiolase; *phbB*, gene encoding acetoacetyl-CoA reductase.

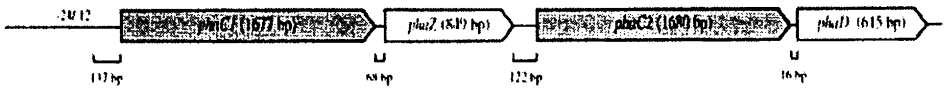
In *Alcaligenes latus, P. acidophila*, and *C. necator* the *phb*CAB genes are arranged tandemly on the chromosome, whereas in *Acinetobacter* sp., the *phb*CAB gene are not present in the same order, however all of them consist of complete CAB operon (Peoples and Sinskey, 1989) and is represented in Fig. 2.4A. The molecular organization of PHA synthase genes in these organisms represents PHA

synthase genes of type I , which preferentially utilize coenzyme A thioester of various 3HA-scl comprising 3-5 carbon atoms and is represented in Figure 4 A&B. Approximately 4kbp downstream of this operon, a second β-ketothiolase gene was identified (Slater *et al.*, 1998). Besides *C. necator*, *Alcaligenes latus; Burkholderia* sp. DSMZ9242, *Chromobacterium violaceum* and *Comamonas acidovorans* also seem to possess a *phb*CAB operon, whereas in *Acinetobacter* sp., *Pseudomonas* sp. and *V. cholerae* these genes though clustered, are organized in a different array.

In bacteria like *Zoogloea ramigera, Paracoccus denitrificans and Rhizobium meliloti* the *phbC* and *phbAB* loci are unlinked or interrupted i.e. *phbA* and *phbB* together form an operon whereas *phbC* is located elsewhere in the chromosome. (Lee, 1996; Tombolini *et al.*, 1995) (Fig. 2.4B). The only exception is in *Aeromonas caviae,* where the gene encoding an enoyl-CoA hydratase is located downstream of *phbC*, a PHB polymerase. The *phbC* is flanked by *phbJ*, which provides monomers for the enzyme (Fukui *et al.*, 1998). In *Methylobacterium extorquens, Nocardia corallina, Rhizobium etli* and *Rhodococcus ruber,* an additional gene *phbP* is present with an unknown function in the PHB regulation. Here also *phbCP* and *phbAB* are present in the same locus but with different orientations and therefore only the PHB polymerase encoding gene *phbC* has been identified so far (Hustede and Steinbuchel, 1993).

In *Pseudomonas oleovorans* and *P. aeruginosa* the *pha* loci is reported to have two *phaC* gene i.e. *phaC1* and *phaC2*. These two genes are separated by an additional gene *phaZ*, which encodes intracellular PHA depolymerase enzyme. The enzymes encoded by *phaC1* and *phaC2* are identical in their primary structure and have almost similar substrate specificity (Timm *et al.*, 1990). The molecular organization of the PHA synthase genes in these organisms is represented in Fig. 2.5. The type II PHA synthases produced preferentially utilize coenzyme A thioester of various 3HA-mcl comprising at least five carbon atoms.

Pseudomonas oleovorans



Pseudomonas aeruginosa

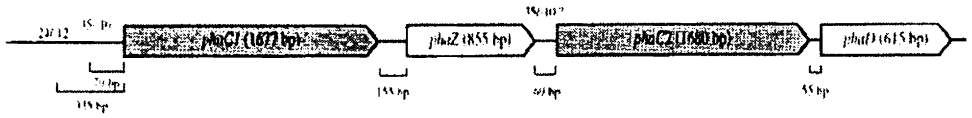
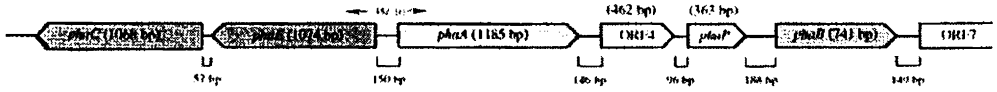


Fig. 2.5 Molecular organization of PHA synthase genes (encoding type II PHA synthases) which are co-localized with PHA depolymerase genes (figure adapted from Rehm and Steinbuchel, 1999). *phaC1:C2*, genes encoding PHA synthase; *phaZ*, gene encoding PHA depolymerase; *phaD* and ORF, open reading frames with unknown function.

Chromatium vinosum



Thiocystis violacea

Thiocapsa pfennigii

Synechocystis sp. PCC6803

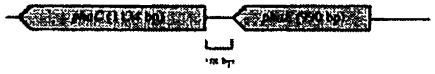


Fig. 2.6 Molecular organization of genes encoding PHA synthases of type III, which are composed of two different subunits (figure adapted from Rehm and Steinbuchel, 1999). *phaC*, gene encoding subunit of PHA synthase; *phaE*, gene encoding subunit of PHA synthase; *phaA*, gene encoding b-ketothiolase; *phaB*, gene encoding acetoacetyl-CoA reductase; ORF, open reading frame with unknown function.

The molecular organization of PHA synthase genes in organisms like *Chromatium venosum, Thiocustis violacea, Thiocapsa pfennigii* and *Synechocystis* sp. represents PHA synthase genes of type III consisting of two different types of subunits- *phaC* subunit exhibiting amino acid sequence similarity from 21 to 28% to type I and II PHA synthases and the *phaE* subunit with no similarity to PHA synthases and these PHA synthases prefer coenzyme A thioester of 3HA-scl. Molecular organization of genes encoding PHA synthases of type III is represented in Fig. 2.6.

2.14. PHA PRODUCTION BY FERMENTATION

The suitability of a bacterium for industrial production for PHAs in large scale depends on several factors including stability and safety of the organism, growth and PHA accumulation rates, achievable cell densities and PHAs content, extractability of the polymer, molecular weight of accumulated PHA, range of utilizable carbon sources, cost of carbon sources and other components of the medium and occurrence of the byproducts (Byrom, 1992).

Depending on the culture conditions that favor PHA accumulation, bacteria that are used for the production of PHA can be classified into two groups. The first group of bacteria requires limitation of essential nutrients such as nitrogen, oxygen and presence of excess carbon source for the efficient synthesis of PHA. The representative bacteria belonging to this group include *C. necator*, *Protomonas extorquens* and *Protomonas oleovorans*. However, the second group of bacteria does not require nutrient limitation for PHA synthesis and can accumulate PHA during exponential growth phase eg. *Cupriavidus necator* (Khanna and Srivastava, 2005; Lee, 1996).

Batch and fed-batch fermentations are widely used in the industrial fermentation processes. Fed-batch culture is suitable for bacteria belonging to the first group. A two-stage cultivation method is most often employed, which was initially adopted by ICI for the industrial production of P(3HB-*co*-3HV) (Byrom,

1987). In the first stage, the bacterial cells are grown until a pre-determined cell mass concentration is reached without nutrient limitation. The cells are then transferred to the second stage medium with limiting nutrients and the carbon substrates fed are utilized by the cells to make PHA. The wider range of PHAs produced with varying characteristics, low productivity and high costs compared to the traditional mineral based plastics remains a hindrance for the extensive industrial application of PHAs (Choi and Lee, 1999; Chen *et al.*, 2001; Reddy *et al.*, 2003). Unfortunately, despite the reported low productivity in most cases, the versatility of these biopolymers has made them ideal candidates as their high-value low-volume products, find application in medicine and pharmacy.

Major problem associated with the industrial production of PHAs from bacteria are its cultural optimization studies. Several reports exist on the influence of various factors involved in PHA production. Major physical parameters to be considered in PHA production studies are incubation temperature and pH. The effect of incubation temperature on PHA production varies from one genus to another (Grothe *et al.*, 1999; Lillo and Valera, 1990; Wei *et al.*, 2011a). Microbial PHA production is highly influenced by pH of cultivation medium (Arun *et al.*, 2009; Lillo and Valera, 1990; Palleroni and palleroni, 1978; Ramadas *et al.*, 2009; Rawte and Mavinkurve, 2004; Sangkharak and Prasertsan, 2008; Wei *et al.*, 2011b). The pH of the medium also has a strong influence on the PHA composition (Villano *et al.*, 2009).

Optimization of the suitable carbon source in the medium is an important criteria for high production of PHA (Santhanam and Sasidharan, 2010). By changing the carbon source and bacterial strains and also the concentration of carbon source used in the fermentation process, it is possible to produce different PHAs with varied monomer content ranging from thermoplastic to rubbery polymers (Anderson and Dawes, 1990). A major point to consider while checking the suitability of the bacteria for efficient industrial production of PHAs is the ability of the cell to utilize an inexpensive carbon source. Since a major

contributor of higher cost in production of PHA is the media composition (Salehizadeh and VanLoosdrecht, 2004) major efforts are pointed towards finding a cheap growth media. Significant reduction in cost will be achieved if cheap media are found with the necessary requirements for production of PHAs with high productivity (Ojumu *et al.*, 2004; Lee, 1996). The choice of media also depends on type of microorganism, either wild type or recombinant and its need for nutrient limiting conditions (Reddy *et al.*, 2003; Lee, 1996). Several workers have extensively studied the PHA production using cheap carbon sources including media containing agricultural or industrial wastes and this is presented in Table 2.3.

Table 2.3 Examples of bacterial fermentation studies for production of PHAs using renewable resources.

Strains	Substrates	Reference
Alcaligenes latus DSM 1124	Soya waste, malt waste	Yu *et al.*, 1999
Bacillus megaterium	Beet molasses, date syrup	Omar *et al.*, 2001
Burkholderia sp. USM (JCM 15050)	Palm oil derivatives, fatty acids, glycerol	Chee *et al.*, 2010
Cupriavidus necator	Bagasse hydrolysates	Yu *et al.*, 2008
Cupriavidus necator DSM 545	Waste glycerol	Cavalheiro *et al.*, 2009
Pseudomonas aeruginosa IFO3924	Palm oil	Marsudi *et al.*, 2008
Pseudomonas aeruginosa NCIB 40045	Waste frying oil	Fernandez *et al.*, 2005
Pseudomonas guezennei biovar. tikehau	Coprah oil	Simon *et al.*, 2008
Thermus thermophilus HB8	Whey	Pantazaki *et al.*, 2009
Bacillus cereus M5	Sugar cane and beet molasses	Yilmaz and Beyatli, 2005
Ralstonia pickettii 61A6	Sugar cane liquor	Bonatto *et al.*, 2004
Pseudomonas fluorescens A2a5	Sugar cane liquor	Jiang *et al.*, 2008
Burkholderia cepacia IPT 048	Cellulose hydrolysates	Silva *et al.*, 2004
B. sacchari IPT 101	Cellulose hydrolysates	Silva *et al.*, 2004
Pseudomonas hydrogenovora	Dairy whey	Koller *et al.*, 2008
Methylobacterium sp. ZP24	Dairy whey	Nath *et al.*, 2008
Bacillus sp. CFR 256	Corn steep liquor	Vijayendra *et al.*, 2007
Bacillus megaterium ATCC 6748	Corn steep liquor and molasses	Chaijamrus and Udpuay, 2008
Cupriavidus necator	Corn syrup	Daneshi *et al.*, 2010
Cupriavidus necator	Plant oils	Fukui and Doi, 1998
Cupriavidus sp. USMAA2-4	Crude palm kernel oil	Kek *et al.*, 2010

Strains	Substrates	Reference
Cupriavidus necator	Fatty acids and waste glycerol	Kahar *et al.*, 2004
Pseudomonas aeruginosa NCIB 40045	Fatty acids and waste glycerol	Fernández *et al.*, 2005
Cupriavidus necator	Fatty acids and waste glycerol	Cavalheiro *et al.*, 2009
Bacillus megaterium	Dairy waste and sea water	Ram *et al.*, 2010
Azotobacter vinelandii UWD	Swine waste liquor	Cho *et al.*, 1997
Bacillus sp.	Waste activated sludge	Thirumala *et al.*, 2010
Pseudomonas putida KT2442	Waste water from olive oil	Ribera *et al.*, 2001
Azotobacter chroococcum H23	Waste water from olive oil	Pozo *et al.*, 2002
Pseudomonas hydrogenovora	Sunflower cake, soy bran and olive mill	Koller *et al.*, 2008
Bacillus sp.	Waste activated sludge	Thirumala *et al.*, 2010
Pseudomonas putida KT2442	Waste water from olive oil	Ribera *et al.*, 2001
Azotobacter chroococcum H23	Waste water from olive oil mills	Pozo *et al.*, 2002
Cupriavidus necator	Industrial wastes (sesame oil)	Arun *et al.*, 2006
Bacillus sphaericus NCIM 5149	Enzyme hydrolysate of jackfruit seed powder	Ramadas *et al.*, 2009
Halomonas sp. KM-1	biodiesel waste glycerol	Yoshikazu and Sei-ichi, 2010
Ralstonia eutropha	biodiesel waste glycerol	Taidi *et al.*, 1994
Vibrio sp.	biodiesel waste glycerol	Chien *et al.*, 2007; Wei *et al.*, 2011a
Rhodobacter sphaeroides	palm oil mill effluent	Hassan *et al.*, 1996
Bacillus sonorensis	Jatropha biodiesel byproduct	Shrivastava *et al.*, 2010
Halomonas hydrothermalis	Jatropha biodiesel byproduct	Shrivastava *et al.*, 2010
Cupriavidus sp. KKU38	Cassava starch manufacturing wastewater	Sangyoka *et al.*, 2001
Enterobacter aerogenes 12Bl	Domestic wastewater	Ceyhan and Ozdemir, 2011

* *Cupriavidus necator=Ralstonia eutropha=Alcaligenes eutrophus= Wautersia eutropha.*

The utilization of industrial wastes as cheap C source can help to convert a low value product into high value biodegradable polymers. Glycerol was considered as one of the most important industrial waste that could be used as a carbon source for microbial production of PHAs in industrial microbiology. Glycerol as an ideal carbon source for PHA production in certain vibrios (Chien *et al.* 2007, Wei *et al.*, 2011), *Halomonas* sp. KM-1 Strain (Yoshikazu and Sei-ichi, 2010) and *Ralstonia eutropha* (Taidi *et al.*, 1994) have already been reported. In certain cases, fatty acids from the fermented fruit and vegetable residues also can

be supplemented as a carbon source for the microorganisms to reduce the production cost (Nonato *et al.,* 2001).

The NaCl concentration of the medium is reported to be crucial in PHA accumulation in *Vibrio* sp., as PHA production is enhanced by the increase in concentration of NaCl in medium (Arun *et al.,* 2009; Chien *et al.,* 2007). However, the studies of Wei and coworkers (2011) on PHA production in *Vibrio* sp. BM-1 inferred that lower NaCl concentration favours PHA production.

Mahuya and group (2005) investigated the effect of nutrient limitation on PHB accumulation in their studies on *Rhodopseudomonas palustris* SP5212. Nutrient limitation is necessary to trigger PHA production and generally ammonia is used as the critical control factor for uncoupling the growth of cells and PHA production (Wang and Lee, 1997). The studies on different nitrogen sources in PHA production were discussed by many researchers (Arun *et al.,* 2009; Grothe *et al.,* 1999; Khanafari *et al.,* 2006; Kim *et al.,* 2003; Koutinas *et al.,* 2007; Liangqi *et al.,* 2006; Ramachander *et al.,* 2010; Sangkharak and Prasertsan, 2008; Saranya and Shenbagarathai, 2010; Wei *et al.,* 2011). Among the media ingredients, the inorganic salts play an important role in PHA accumulation (Lee *et al.,* 2007) and role of various inorganic salts in PHA production were reported (Lee *et al.,* 2007; Saranya and Shenbagarathai, 2010; Wei *et al.,* 2011b). Among the various factors affecting PHA production, C:N ratio is also important (Khatipov *et al.,* 1998).

Incubation period is another crucial factor in industrial production of PHAs and depends on the characteristics of the strain and growth rate. PHA accumulated by organisms are utilized as a carbon source for their survival and may cause lowering of PHA accumulation at higher incubation periods (Santhanam and Sasidharan, 2010; Yamane *et al.,* 1996; Benoit *et al.,* 1990; Nam and Ryu, 1985). The interaction between PHA production and incubation period is discussed by several workers (Rawte and Mavinkurve, 2004; Ramachander *et al.,* 2010; Sangharak and Praserstan, 2008; El-Sayed *et al.,* 2009; Madison and Huisman, 1999). The initial microbial load is very crucial for production studies of

PHA (Santhanam and Sasidharan 2010; Valappil *et al.*, 2007; Vijayendra *et al.* 2007). Synthesis of PHB in *V. harveyi* was reported to be controlled by the Lux auto inducer, N-(3-hydroxybutanoyl) homoserine lactone. These findings correlate a link between luminescence and synthesis of PHAs (Sun *et al.*, 1994; Miyamoto *et al.*, 1998).

The use of submerged and solid-state fermentation (SSF) processes in PHA production from waste materials and by-products has been extensively reviewed (Castilho *et al.*, 2009). The submerged fermentation processes has been extensively studied over the last 30 years and recently the use of SSF in the production of PHAs by microbes has been proposed. Oliveira *et al.* (2004) were the first to propose the usage of SSF from agro-industrial wastes for the production of PHAs. They used agro-industrial by-products like soy cake and babassu cake for the cultivation of *C. necator* and *A. latus* strains for the production of PHAs in SSF. Utilisation of agro-industrial wastes like sunflower cake, soy bran have also been reported in the production of PHAs in *C. necator* (Castilho *et al.*,1999) and the PHAs produced in both cases was identified as PHB. Further it was seen that the production of PHB by *C. necator* was growth-associated, contrary to what occurs in submerged fermentation, where polymer production by this bacterium was not growth-associated (Lee and Chang, 1995).

Statistical approach in PHA production

A well defined statistical design of experiments is considered to be necessary for optimization of a fermentation process. The Plackett-Burman design (PBD) has been frequently used for screening of process variables that have the greatest impact on the process (Plackett and Burman, 1946). There are several reports on the use of PBD in media optimization for microbial production of PHAs. A consideration in the choice of PBD in screening of process variables is the ratio of the number of experiments to be conducted to the number of variables being studied. PBD and Taguchi method were analysed for screening of process

variables in PHB production by *Ralstonia eutropha*, and it was found that PBD is more suitable (Khosravi-Darani *et al.*, 2004). The role of PBD in the selection of important media components for PHA production from methanol by *Methylobacterium extorquens* DSMZ 1340 was envisaged (Mokhtari-Hosseini *et al.*, 2009) and found that the deficiencies of nitrogen sources (NH_4Cl and NH_4NO_3), phosphorus sources (K_2HPO_4 and Na_2HPO_4) and $MgSO_4$ in the medium increased PHB accumulation. PBD was successfully applied in optimization of PHB production in *Ralstonia eutropha* in batch culture (Khosravi-Darani *et al.*, 2003)

Statistical design of experiments by the application of factorial experimental design in maximizing PHA production by optimizing major bioprocess variables in various bacteria were studied by several workers. Statistical analysis of PHB production by *Bacillus sphaericus* NCIM 5149 under submerged fermentation condition by Response Surface Methodology (RSM) using central composite design (CCD) was done by Ramadas and his co-workers (2010). In their studies the cultural and nutritional parameters were optimized using jackfruit seed hydrolysate as the sole carbon source. Statistical analysis in optimization of PHB by *Bacillus* sp. CFR 256 with corn steep liquor as a nitrogen source using response surface methodology has been evaluated (Vijayendra *et al.*, 2007). RSM was applied successfully in medium optimization for the production of PHAs in *Rhodobacter sphaeroides* U7 cultivated in glutamate–acetate medium (Kemovangse *et al.*, 2008), in *Ralstonia eutropha* NRRL B14690 (Khanna and Srivastava, 2005), in *Ralstonia eutropha* from ice cream residue (Lee and Gilmore, 2006) and in recombinant *Escherichia coli* (Nikel *et al.*, 2005).

2.15. DETECTION, ISOLATION AND ANALYSIS OF PHAs

2.15.1. Screening for polyhydroxyalkanoates

Various phenotypic detection methods such as Sudan black B staining (Schlegel *et al.*, 1970), Nile blue A (Ostle and Holt, 1982) and Nile red

(Spiekermann *et al.*, 1999) have been widely used to screen microbes accumulating PHAs. Sudan black B is non-specific to PHA as it also stains other lipid bodies. Nile blue A and Nile red are reported to be more specific than Sudan black B for detection. In Nile Blue A and Nile red staining techniques, viable cell staining techniques can be employed and the PHA producers can be examined by the fluorescence production under UV light (Spiekermann *et al.*, 1999).

In phenotypic identification methods prior to screening for PHA-producing bacteria, it is necessary to provide appropriate nutrient limitation conditions to the bacterial cells to support PHA accumulation. This is a laborious process and hence alternative methods have been developed for the rapid detection of PHA producing bacteria where one method is based on Fourier transform infrared (FTIR) spectroscopy of intact cells (Hong *et al.*, 1999). Other techniques employed for detection depends on genotypic method involving polymerase chain reaction (PCR) technique (Sheu *et al.*, 2000; Solaiman *et al.*, 2000; Romo *et al.*, 2007).

By combining the colony PCR and semi-nested PCR techniques, a rapid, reliable and highly accurate detection method has been developed for detecting PHA producers (Sheu *et al.*, 2000) and this method is most accurate for class I PHA synthase gene specific for scl-PHAs. PCR amplification method for detecting mcl-PHA synthase gene fragments has also been described (Solaiman *et al.*, 2000). In this study degenerate primers were generated for the amplification of type II *phaC1* and *phaC2* subgenomic fragments. Romo *et al.*, (2007) proposed a molecular approach for the detection of broad range of scl and mcl-PHA accumulating Gram-negative bacteria from different genera.

2.15.2. Isolation of polyhydroxyalkanoates (PHAs)

In the PHA production process, the PHA recovery and purification steps are also reported as important components in the production cost (Nonato *et al.*, 2001; Choi and Lee, 1999; De Koning and Witholt, 1997).

2.15.2.1. Pretreatment

Before extraction of PHAs from bacterial biomass, cells are specifically pre treated so that the cell is easily disrupted. A preliminary heat treatment has an impact on the cell solidity by destabilizing the outer membrane (Kapritchkoff *et al.* 2006). In this type of pretreatment, the PHB depolymerase is denatured as evidenced from the studies in *R. eutropha* DSM545 strain (Kapritchkoff *et al.* 2006; Steinbuchel, 1996) and *Pseudomonas* (De Koning and Witholt, 1997). Another type of pretreatment step involves the alkaline pretreatment where a solution of sodium hydroxide is used (Tamer *et al.*, 1998) which results in release of proteins. In the salt pretreatment steps for PHA extraction *in A. latus* and *R. eutropha* where NaCl is used, water moves out from cells as water is attracted by high salt concentrations. This leads to dehydration and shrinkage of cells (Tamer *et al.*, 1998; Khosravi-Darani *et al.*, 2004). In certain cases salt treatment is combined with an alkaline pretreatment (Khosravi-Darani *et al.*, 2004, Tamer *et al.*, 1998). Pretreatment steps involving freezing method, releases PHA granules and cell contents which can be easily digested by SDS and NaOCl in further PHA extraction procedure (Dong and Sun, 2000).

2.15.2.2. Extraction Methods

Extraction and purification of PHA polymers from non-PHA cell mass (NPCM) presents a technical challenge due to the solid phase of both PHA granules and NPCM. Isolation and purification of bacterial PHAs have been extensively elucidated by Jacquel and co-workers (2008). According to them, two types of strategies are adopted in the downstream processing of PHA recovery ,viz. PHA solubilization and NPCM dissolution. In PHA solubilization method, the PHAs are dissolved in appropriate organic solvents and subjected for further steps, and in NPCM dissolution, NPCM is removed by chemical agents while PHA granules are left in the solid state. The resultant solid and liquid phases are

then separated by unit operations like filtration, centrifugation etc (Yu and Chen, 2006).

In 1926, Lemoigne introduced PHB extraction from lyophilised biomass with chloroform followed by precipitation with diethyl ether or acetone. In 1958, Williamson and Wilkinson reported that under optimized conditions of time and temperature all cell material, except PHB granules, dissolved in alkaline sodium hypochlorite solution.

The majority of the separation processes that have been proposed involve PHA extraction from cells with the usage of organic solvents including acetone (Jiang *et al.,* 2006), chloroform, methylene chloride or dichloroethane (Anderson and Dawes, 1990 , Baptist, 1962, Brandl *et al.,*1990, Lemoigne, 1926).

The intracellular PHA was extracted with hot chloroform and precipitated in methanol in the methodology of Lageveen *et al.* (1988). Solvent extraction of PHAs from *R. eutropha* biomass with methylene chloride has yielded a purity above 98% (Zinn *et al.,* 2001). The Austrian Company Chemie Linz GmbH also employed a solvent extraction method to recover PHB from *Alcaligenes latus* cells with methylene chloride (Hanggi, 1990).

Another type of recovery method is chemical digestion. Among these sodium hypochlorite is widely used in differential digestion method (Berger *et al.,* 1989, Ramsay *et al.,* 1990, Williamson and Wilkinson, 1958). To take advantage of both differential digestions by hypochlorite and solvent extraction, a new process was developed to recover PHB from *C. necator* by dispersions of a sodium hypochlorite solution and chloroform (Hahn et al., 1994). Aqueous enzymatic digestion methods have also been developed (Holmes and Lim, 1990). A combined method involving enzyme and sodium hypochlorite was carried out on *Burkholderia* sp. *PTU9* by using papain (Alper *et al.,* 1963).

Enzymes have been used successfully to recover and purify the PHAs by Kapritchkoff *et al.,* (2006) in *Ralstonia eutropha* DSM545. Major advantage of enzymatic recovery is its high specificity. De Koning and Witholt (1997) proposed

a combined method involving consecutive treatment with heat, alcalase and SDS assisted by EDTA for the recovery of PHA from *Pseudomonas*. Yasotha *et al.*, (2006) have incorporated the use of alcalase, SDS and EDTA in PHA extraction studies with *P. putidia* culture. Selective dissolution of non-PHA cell mass by protons was described by Yu and Chen (2006).

PHB were recovered from the dry cell mass by sodium dodecyl sulfate (SDS) treatment followed by the addition of sodium hypochlorite (Dong and Sun 2000). A simple and effective method for the recovery of poly(3-hydroxybutyrate) directly from high cell density culture broth with no pretreatment steps has been developed by Kim and workers (2003) involving direct addition of sodium dodecyl sulfate (SDS) to the culture broth, followed by shaking, heat treatment, and washing.

Another method for cell separation which is widely used for recovering intracellular proteins is mechanical disruption divided into two main categories of disruption: solid shear (e.g. bead mill disruption) and liquid shear (e.g. high pressure homogenizer) (Tamer *et al.*, 1998). Cell disruption by ultrasonication was also studied for PHA extraction from *Haloferax mediterranei* (Hwang *et al.*, 2006). The recovery of PHB by centrifugation in combination with chemical treatment for PHA extraction has also been investigated (Van-Wegen *et al.*, 1998) and Ling *et al.*, 1997).

Supercritical fluids like supercritical CO_2 have been experimented with successfully in PHA extraction procedure in *R. eutropha* (Hejazi *et al.*, 2003). Scientists have exploited the property of cell fragility in PHA extraction process. In fish peptone medium *Azotobacter vinelandii* become fragile after the accumulation of large amounts of PHA and can easily be separated by treatment with 1N aqueous NH_3 at 45^oC for 10 min (Page and Cornish, 1993). PHA extraction has also been carried out by other techniques like air classification method developed by Noda of Procter & Gamble (1998), dissolved-air flotation

(Van Hee *et al.*, 2006) and spontaneous liberation (Jung *et al.*, 2005, Resch *et al.*, 1998).

2.15.3. Analysis of PHAS

In the earlier times the most common analytical technique used for PHA estimation was gravimetric method (Lemoigne, 1926). Several methods are now available for determination of the PHA contents of microorganisms, the composition of PHAs, and the distribution of the monomer units.

Formerly, the widely used quantitative PHA estimation method was spectrophotometric method of Law and Slepecky (1961) which depends upon conversion of the polymer to crotonic acid by heating with concentrated sulfuric acid.

The gas chromatographic (GC) identification of PHA components was later proposed by Braunegg *et al.*, (1978). This method involves simultaneous extraction and methanolysis of PHA, in mild acid or alkaline conditions, to form hydroxyalkanoate methylesters which are then analysed by GC. This method is rapid (4 h), sensitive, reproducible and requires only small samples enabling concentrations as low as 10 μM to be assayed.

An alternative GC method for increased PHA recovery was proposed by carrying out propanolysis in HCl (Riis and Mai, 1988) rather than acidic methanolysis in sulphuric acid which causes less degradation. Comeau *et al.*, (1988) have described a convenient GC method for PHA analysis of activated-sludge samples. Brandl *et al.*, (1989) determined the PHA content and composition by GC after methanolysis of lyophilized cells for 140 min at 100°C to yield the methyl esters of the constituent 3-hydroxyalkanoic acids.

Findlay and White (1983) quantitated chloroform-extracted PHAs from *B. megaterium* and from environmental samples by acid ethanolysis and GC-mass spectrometric (MS) analysis of the resulting 3-hydroxyalkanoic acid ethyl esters. In *Rhizobium japonicum* bacteroids, ion exclusion high-pressure liquid

chromatography have been used for the rapid analysis of PHB following digestion with concentrated sulfuric acid (Karr *et al.*,1983)

PHA analysis can also be performed by using ionic chromatography and enzymatic methods. PHA detection by ionic chromatography is based on the conversion of monomers to alkanoic acids. The determination involves acid propanolysis followed by an alkaline hydrolysis with $Ca(OH)_2$ or acidic hydrolysis with concentrated H_2SO_4 (Hesselmann *et al.*, 1999)

The determination of PHA inside intact cells by two-dimensional fluorescence spectroscopy and flow cytometry have also been proposed (Degelau *et al.*, 1995, Gorenflo *et al.*, 1999). Cells stained with Nile Blue A showed a clear fluorescence maximum between 570 and 605 nm and when excited between 540 and 560 nm. A good correlation between fluorescence intensity and PHB concentration was obtained.

2.16. APPLICATION OF POLYHYDROXYALKANOATES

2.16.1. Industry

The usefulness of the PHAs comes from their thermoplasticity, a property found in numerous plastics including polystyrene, polycarbonate and polypropylene (Weiner, 1997). The prokaryotic PHAs form the base material of biodegradable plastics e.g. PHB with a similar structure to polypropylene, which is used in over 200 products, including pipes, packaging materials and domestic appliances (Glazer and Nikaido, 1994).

2.16.2. As packaging material

The major applications of PHAs are in the packaging and coating applications (Bucci and Tavares, 2005). The gas barrier property of P (3HB-3HV) is useful for applications in food packaging and for making plastic beverage bottles. These properties can be exploited to make coated paper and films which can be used for coated paper milk cartons (Hocking and Marchessault, 1994). It

was reported that unlike synthetic plastics, PHB does not float, but sinks to the sediment, where it can be degraded in approximately one month (Luzier, 1992) and this property can be exploited for reducing the major drawback of pollution problem caused by synthetic plastics in aquatic realms.

2.15.3. Chiral intermediates for the synthesis of valuable compounds

PHAs represent a potential source of chiral hydroxyl acid feed stock for the fine chemical industry. These compounds are mainly used as biodegradable carriers for long term dosage of drugs, medicines, hormones, insecticides and herbicides and they find application as osteosynthetic materials in the stimulation of bone growth owing to their piezoelectric nature (Reddy *et al.,* 2003, Oeding and Schlegel, 1973; Senior and Dawes, 1973).

Chiba and Nakai (1985) reported the use of 3HB for the synthesis of carbapenem antibiotics. Seebach *et al.* (1986, 1987) demonstrated that 3HA can be used as chiral building blocks for the total synthesis of macrolides. 3HB can also be used to synthesize dendrimers that possess the advantages of biodegradability, monodispersity, and large numbers of surface-functional moieties (Seebach *et al.,* 1996), which can be expected to be promising as *in vivo* drug carriers. In contrast to the introduction of new polymers, the PHA and the related derivatives can be readily integrated into existing fine chemical markets (William and Peoples, 1996).

2.16.4. Other industrial applications

Bioplastics are already used in several electronic products like mobile phones (NEC Corporation and UNITIKA Ltd. 2006). Utilization of PHA latex in film formation and paper coating has been reported by Lauzier *et al.* (1993). Biomer, a German based company produces PHB from *Alcaligenes latus* on a large scale and the polymer thus produced is used to make articles such as combs,

pens and bullets. The polymer pellets are sold commercially for use in classical transformation processes (Chen and Wu, 2005). P(3HB-3HHx) has been industrially produced from *Aeromonas hydrophila* and the polymer thus produced is used to make flushables, nonwovens, binders, flexible packaging, thermoformed articles, synthetic paper and medical devices (Chen *et al.*, 2001).

2.16.5. In nutrition

Metabolix (US) produce Metabolix PHA (blend of PHB and poly(3-hydroxyoctanoate)) that has been approved by the FDA for the production of food additives (Clarinval and Halleux, 2005).

Ketone bodies including 3HB have been considered as a means of providing energy, because of their good penetration and rapid diffusion in peripheral tissues. Tasaki *et al.*, (1999) reported that 3HB can be used as an energy substrate for injured patients. He concluded that dimers and trimers of 3HB can be converted rapidly to monomers in rat and human tissues.

2.16.6. Agricultural applications

In agriculture PHA can find several applications including seed encapsulation, encapsulation of fertilizers, crop protective (biodegradable plastic films) and as biodegradable containers for hothouse facilities. PHAs have been used as mulch films for agricultural purposes (Hocking and Marchessault, 1994). In agriculture P(3HB-3HV) can be used in the controlled release of insecticides (Holmes 1985). Another use of PHAs in agriculture is as bacterial inoculants used to enhance nitrogen fixation in plants (Dobbelaere *et al.*, 2001).

In recent years, Procter & Gamble have produced Nodax^{TM} (copolymer containing mainly 3(HB) and small quantities of mcl PHA monomers) which can be used to manufacture biodegradable agricultural film which can degrade anaerobically and hence be used as a coating for urea fertilizers to be used in rice fields or for herbicides and insecticides.

2.16.7. As bio-indicator

Foster *et al.* (2001) reported the use of PHAs as pollution bio-indicators in preliminary assessments of environmental health. He proposed a protocol based on gas chromatography to identify and measure environmental concentrations of microbial polyhydroxyalkanoates. The biota in environmental samples can be induced to produce significant concentrations of PHAs (Nickels *et al.*, 1979). In the studies of research on measuring environmental PHA concentrations in sediment, Findley and White (1983) speculated that PHAs could be used to elucidate the nutrient history of the sampling environment.

Foster *et al.* (2001), in their studies observed that environments impacted by anthropogenic activity displayed PHA concentrations 14 to 40 times higher than unpolluted sites characterised by agricultural land or native vegetation. Thus they proposed that environmental concentrations of PHAs can effectively be used as a biomarker for pollution in that area.

2.16.8. Marine application

It was reported that unlike synthetic plastics, PHB does not float, but sinks to the sediment, where it can be degraded in approximately one month (Luzier, 1992). BIOPOL®, major commercially available PHAs developed by Metabolix (USA), finds several applications in marine and related fields. The monofilaments have been used to make fishing nets and ropes. These fibres have been used to make ropes and nets for crab cages and these exhibited good strength and biodegradability in the sea. BIOPOL® coated with polyvinyl alcohol is a good matrix for growing seaweed. When reinforced with PCL, it develops anti-algal properties and can be used in nets for seafood cultivation (Asrar and Gruys, 2002).

2.16.9. Medicine

PHAs can be exploited for numerous medical applications and these have been reviewed extensively by Williams and Martin (2002). PHA has an ideal

biocompatibility as it is a product of cell metabolism and also 3HB, the product of degradation, is normally present in blood at low concentrations. The PHAs show a wide range in their degree of degradability from slow to quick degradation and these properties also aid in their usage in medicine and pharmacy (Williams and Martin, 2002).

2.16.9.1. As tissue engineering material

The application of PHA as tissue engineering material is attributed to its intrinsic properties like biocompatibility and non-toxicity (Chen and Wu, (2005). PHAs, especially PHB, P(3HB-3HV), P(4HB) and PHO are frequently used in tissue engineering. They are widely used as bone plates, osteosynthetic materials and surgical sutures (Philip *et al.*, 2007). Depending on the property requirement of different applications, PHA can be either blended, surface modified or composited with other polymers, enzymes or even inorganic materials to further adjust their mechanical properties or biocompatibility. Research shows that PHA materials can be useful in bone healing processes, where blends with hydroxyapatite (HA) can find applications in hard tissue replacement and regeneration (Chen and Wu, 2005) particularly because these composites possess mechanical strength similar to that of human bones (Galego *et al.*, 2000).The many possibilities to tailor-make PHA for medical implantations, indicates its suitability as tissue engineering materials (Chen and Wu, 2005).

2.16.9.2. As drug delivery material

Several researchers have worked on the use of PHA as agents of drug delivery (Juni and Nakano, 1987; Koosha *et al.*, 1989). Polymer implants for targeted drug delivery can be made out of PHAs (Chen and Wu, 2005; Park *et al.*, 2005) as subcutaneous implants, compressed tablets for oral administrations and microparticulate carriers for intravenous use. Williams and Martin (2001) reported the use of P(4HB) as prodrug of 4-hydroxybutyrate. These cause prolonged

release of the monomer from P(4HB) and this might potentially be beneficial in the treatment of narcolepsy, alcohol withdrawal and several other indications.

2.16.9.4. Other medical and pharmaceutical applications

Major application of PHAs is in the development of cardiovascular products like pericardial patches (Bowald and Johansson, 1990; Bowald and Johansson-Ruden, 1997; Malm *et al.*, 1992a), atrial septal defect repair (Malm *et al.*, 1992b), cardiovascular stents (Schmitz and Behrend, 1997), vascular grafts (Marois *et al.*, 2000) and development of heart valves (Sodian *et al.*, 2000).

Tepha (US based company) specializes in manufacturing pericardial patches, artery augments, cardiological stents, vascular grafts, heart valves, implants and tablets, sutures, dressings, dusting powders, prodrugs and microparticulate carriers using PHAs (Williams and Martin, 2002). PHB and its copolymers can be used to make the non-woven cover stock and the plastic film moisture barriers in nappies and sanitary towels along with some specialty paramedical film applications in hospitals (Hocking and Marchessault, 1994). Baptist and Ziegler (1965) suggested the potential use of PHB as an absorbable suture. Fine PHB powders can be used as medical dusting powders mainly for surgical gloves (Holmes, 1985). The piezoelectric property of the PHA had drawn attention toward its application for nerve repair (Aebischer *et al.*, 1988).

Amphiphilic PHA copolymers also find interesting applications in drug delivery and tissue engineering (Li *et al.*, 2003). Chemical modification reactions make it easier to insert hydrophilic segments into the hydrophobic PHAs and to produce amphiphilic copolymers. Amphiphilic graft copolymers have the potential to be used as blood-contacting devices in a broad range of biomedical applications because of their excellent blood compatibilities (Hazer and Steinbuchel, 2007).

2.17. INDUSTRIAL PRODUCTION OF PHAS

During the 1980s, the British company, Imperial Chemical Industries (ICI), developed a commercial process to produce PHB and a related copolymer known as poly-(R-3-hydroxybutyrate-co-R-3-hydroxyvalerate) (P(3HB-co-3HV). These polymers were sold under the trade name of BIOPOL® and were developed primarily as renewable and biodegradable replacements for petroleum-derived plastics (Asrar and Gruys, 2002). After the production of BIOPOL®, a number of companies have come up to engage in industrial production of PHAs.

BIOPOL® is a co-polymer of P(3HB-co-3HV) is a thermoplastic which was initially manufactured by ICI and is now produced by Metabolix (Cambridge, MA, USA). With an increase in the amount of 3HV in the copolymer, the crystallinity decreases and the polymer becomes more elastic (Philip *et al.*, 2007).

BIOPOL® can be used to coat paper and paperboards. In addition to being suitable for injection, blow moulding and film production, BIOPOL® has antistatic properties that can be exploited for electric and electronic packaging (Philip *et al.*, 2007). BIOPOL® products have found wider application in aquaculture related fields (Asrar and Gruys, 2002). BIOPOL® is used to produce shampoo bottles, motor oil bottles and disposable razors (Clarinval and Halleux, 2005). BIOPOL® has found wider application in marine and related fields (Asrar and Gruys, 2002).

Nodax™ is a recent addition to the PHA copolymer family which consists of 3HB and a comparatively small quantity of mcl-monomers (Noda *et al.*, 2005). Nodax ™ is developed by Procter and Gamble and is available as foams, fibres or nonwovens, films and latex. This polymer can be used to make flushables that can degrade in septic systems and this would include hygienic wipes and tampon applicators. They can also be used to manufacture medical surgical garments, upholstery, carpet, packaging and compostable bags (Philip *et al.*, 2007).

DegraPol is a commercially available chemically synthesised block copolyster that finds applications in bone healing methods such as autologous osteoblast or chondrocyte transplantation (Saad *et al.*, 1999).

2.18. NATIONAL STATUS OF PHA RESEARCH

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

Khardenavis *et al.* (2007) studied the biotechnological conversion of agro industrial wastes into PHB. Ramadas *et al.* (2010) reported PHB production in *B. sphaericus NCIM 5149* and optimized the bioprocess variables enhancing PHA production by central composite design. Nair *et al.* (2009) reported the accumulation of PHB in a phenol degrading *Alcaligenes* sp. d2 under phenol stressed condition. Grafting of medium chain length polyhydroxyalkanoates onto carboxymethyl chitosan (CMCH) using ceric ammonium nitrate as an initiator was done and its biodegradation studies were carried out by Bhat *et al.* (2008). These CMCH grafted mcl PHA is proposed to have applications in the field of medicine such as tissue engineering and drug-delivery systems.

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

3 Materials and Methods

3.1 SCREENING AND IDENTIFICATION OF VIBRIOS UPTO GENUS

3.1.1 Sources of vibrios

Vibrios were isolated from sediment samples collected from marine benthic environments along the South-West and East coasts of India. List of sampling stations, types of sampling sites and mode of collection is as detailed in Table 3.1 and schematic representation of sampling regions are represented in Fig. 3.1.

Table 3.1 Details of sampling stations, types of sampling sites and mode of collection

Sl no.	CODE	Sampling stations	Sampling sites	Mode of collection
1	BTTV	Thevara	Estuary	Grab
2	BTKB	Kumbalam	Estuary	Grab
3	BTED	Edakochi	Estuary	Grab
4	BTPT	Poothotta	Estuary	Grab
5	BTFC	Fort Cochin	Estuary	Grab
6	BTJT	Ern-Boat jetty	Estuary	Grab
7	BTVP	Vypeen	Estuary	Grab
8	BTOC	Ochanturuttu	Estuary	Grab
9	BTVL	Vallarpadam	Estuary	Grab
10	BTBG	Bolgatty	Estuary	Grab
11	BTSB	Tuticorin	Sandy beach	Corer
12	BTTC	Tuticorin	Saltpan	Corer
13	BTTN	Tuticorin	Coral island	Corer
14	BTTR	Thirichendoor	Beach	Corer
15	BTMV	Mangalavanam	Mangroves	Corer

Fig. 3.1 Various sampling regions along south west and east coast of India. The red dots represents various sampling stations. Map courtesy (*www. googlemap.com)* South west coast – 9.9°N 76°E; South east coast -8.76°N 78°E.

3.1.2 Collection of samples

Sediment samples were collected from marine benthic environments- estuaries, salt pans, mangroves and coastal waters. The samples were collected from the backwaters at a depth of about 8m using a grab, while benthic samples from the mangroves and salt pans were collected using a corer with 2mm diameter. Sediment samples were collected in sterile polythene bags, tied well and brought to the laboratory in an icebox.

3.1.3 Preparation of serial dilutions of the sample

Approximately10 g of sediment sample was weighed and transferred aseptically to 90 ml of physiological saline. This was followed by serial dilution using physiological saline and 50µl of the prepared dilution was used as inoculum.

3.1.4 Medium used for isolation and purification of *Vibrio* sp.

After serial dilution, samples were spread plated onto Thiosulphate Citrate Bile salt Sucrose (TCBS) agar (HiMedia, India) plates and incubated at 37°C for 24 hours. Isolated single colonies were picked, purified on nutrient agar (NA) plates, then sub-cultured and stocked in nutrient agar slants with 1% NaCl for further studies (Appendix I).

3.1.5 Identification of vibrios upto genus

All the isolates obtained from nutrient agar were repeatedly streaked on NA plates and their purity was confirmed by performing Gram staining and microscopic observation. Vibrios were identified based on their morphological and biochemical characteristics.

3.1.5.1 Morphological and biochemical characteristics

All the isolates were assigned to various genera based on their morphological and biochemical characters outlined in Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1974).

3.1.5.2 Gram staining, oxidase and MOF test

The bacterial isolates were Gram stained to determine their Gram nature.

Oxidase test detects the ability of the organism to produce cytochrome oxidase enzyme. This can be determined by the addition of Kovacs reagent-Tetramethyl-p-phenylenediamine dihydrochloride, which serves as an artificial substrate, donating electrons and thereby becoming oxidized to a blue colored oxidation product indicating the formation of indophenols (Kovacs, 1956). No color change indicates the absence of cytochrome oxidase. According to the methods recommended, the organisms were freshly grown on nutrient agar.

The oxidation/fermentation reaction with glucose (MOF test) is usually determined by inoculating the organisms into deep agar medium in test tubes. The

incorporation of a pH indicator into the medium enables detection of changes in the pH resulting from the decomposition of glucose to be observed, in addition to visible signs of growth in different parts of the medium. Cultures were stab inoculated with a straight inoculation needle. Acidic changes at or near the surface indicates that the substrate is being oxidized by aerobic bacteria, whereas the development of uniform acidity throughout the tube shows that facultative anaerobic organisms are both oxidizing and fermenting the substrate (Hugh and Leifson, 1953)

MOF medium (HiMedia, India) was used for the present work. The medium was sterilized by autoclaving at 15 lbs for 15 minutes. Added 1% glucose to the sterile basal medium and transferred 4 ml aliquots aseptically into sterile tubes and autoclaved at 10 lbs for 8 minutes. Converted to slants with a long butt. The tubes were then stab inoculated and streaked and incubated at 37°C for 24 hours. The results were recorded as follows:

O- Oxidation (yellow coloration in the butt)

F- Fermentation (yellow coloration throughout the tube)

(F)- Fermentation with gas production

Alkaline reaction - Pink coloration in the slant and no reaction in the butt.

The isolates segregated as vibrios after a presumptive screening by Gram staining, oxidase, and MOF tests, were confirmed as vibrios by plating them onto TCBS agar plates.

3.1.6 Stocking

Isolates segregated as vibrios were purified and maintained as permanent stock cultures employing two methods, viz. paraffin overlay method and glycerol stocking.

3.1.6.1 Paraffin overlay method

Nutrient agar supplemented with 1% sodium chloride in glass stocking vial was inoculated with a single colony and incubated for 18hours at 37°C. Sterile liquid paraffin was added on top and the vials were covered with sterile rubber stopper. The vials were kept in dark until further use.

3.1.6.2 Glycerol stocking

Cultures were grown in nutrient broth, supplemented with 1% sodium chloride. After 18hr growth, the broths was mixed to get a 30% glycerol (final) conc. and kept at -80°C. This was prepared by adding 300µl sterile glycerol to 700µl culture in nutrient broth (Appendix I) supplemented with 1% NaCl in sterile micro tubes.

3.1.6.3 Temporary stocking (working stock)

Cultures were grown overnight in nutrient broth supplemented with 1% sodium chloride and transferred onto NA slants with 1% NaCl kept at 37°C for 24h for temporary stocking of the cultures. These cultures were kept at 4°C in refrigerator for short term use and were subcultured at 2 week intervals.

3.2 PRELIMINARY AND SECONDARY SCREENING FOR SELECTION OF POLYHYDROXYALKANOATES (PHAS) ACCUMULATING *VIBRIO* SP.

3.2.1 Preliminary screening using plate assay

3.2.1.1 Medium

Complex nitrogen limiting medium outlined by Sun *et al.,* 1994 (Appendix I) was used for PHA screening studies. Nile blue A solution (Appendix I) was used as stain in the medium and was added to the sterilized medium to give a final concentration of 0.5 $\mu g/ml^{-1}$.

3.2.1.2 Screening for PHA accumulating vibrios

The fluorescence plate assay is a preliminary screening method for PHA production (Spiekermann *et al.*, 1999). Complex nitrogen limiting agar medium (section 3.3.1.) having glycerol as C- source and Nile blue A as stain were used for preliminary screening method for identification of PHA accumulating strains.

Test cultures were grown in NA slants with 1% NaCl. 16h cultures were patched onto Nile Blue A agar plates and incubated at 37°C for 3-4 days. Colonies were directly examined for fluorescence by exposing to UV light to detect the accumulation of lipid storage compounds including PHAs (Spiekermann *et al.*, 1999). The intensity of fluorescence produced by the cultures is directly proportional to the amount of PHAs accumulated. Strains with greater intensity of fluorescence were selected as potential PHA producers. .

3.2.2 Secondary screening by spectrophotometric analysis (using SmF)

A two-stage cultivation method was employed here for PHA production. In the first stage, the bacterial cells were grown until a pre-determined cell mass concentration was reached without nutrient limitation in seed medium. The harvested cells were then transferred to the second stage medium i.e., PHA production medium with limiting nutrients and the carbon substrates, which were utilized by the cells to make PHA.

3.2.2.1 PHA production medium preparation

PHA production medium (Sun *et al.*, 1994) detailed in Appendix I having glycerol (1%) as carbon source was used. The medium was prepared in distilled water, autoclaved at 121°C and cooled to 60°C. The glycerol and $MgSO_4$. $7H_2O$ were autoclaved separately. The cooled medium was added with previously sterilized glycerol and $MgSO_4$. $7H_2O$, and the contents mixed well before inoculation. 50 ml aliquots of complex PHA production medium in a 250 ml Erlenmeyer flask was used for the second stage of production.

3.2.2.2. Seed medium

The seed medium for PHA production studies should be a nutrient sufficient medium and hence in the present study, Nutrient medium (HiMedia, India) with 1% NaCl was used. (Appendix I).

3.2.2.3 Inoculum preparation

The primary inoculum was grown in seed medium in a 250 ml Erlenmeyer flask containing 50 ml of sterile medium (autoclaved at 121°C for 15 minutes) and inoculated from the stock culture. The cell cultures were kept in an environmental shaker (Orbitek, Scigenics India) at 150 rpm at 37±2°C overnight.

The overnight culture in nutrient broth was centrifuged in a laboratory centrifuge (Sigma, 2-16K, Germany) at 8,000 rpm at 4°C for 10 minutes and the cell pellet was washed twice with sterile physiological saline and then was resuspended in physiological saline. 1ml of this culture suspension ($O.D_{600}$ = 1) was used as primary inoculum for 50ml of production medium, kept in an environment shaker (Orbitek, Scigenics, India) at 150 rpm at 37±2°C for 48h.

3.2.2.4 PHA extraction

The PHAs were recovered from the dry cell mass by extraction method outlined by Dong and Sun (2000). For the quantitative analysis of PHAs, the polypropylene tubes previously washed thoroughly with ethanol and hot chloroform to remove plasticizers were used for extraction (Law and Spleckey, 1961).

After 48h incubation, biomass was isolated from culture by centrifugation at 8,000 rpm (Sigma, 2-16K, Germany) at 4°C for 10 minutes and washed with physiological saline. The CDW (g/l) and PHA concentration (g/l) were then determined. The cell pellet was initially subjected to a pretreatment step to release PHA.

In the pretreatment step, the biomass was treated at 100°C for 1 min in a water bath and rapidly cooled to 55°C, then stored at -20°C. 1% SDS solution was then added to this pretreated pellet, held at 55°C for 15 min to effectively solubilize lipids and protein, followed by centrifugation (8,000 rpm at 4°C for 10 minutes). The resultant cell pellet was then treated with 30 % NaOCl solution at 30°C for 1 minute to remove peptidoglycan and non-PHA biomass. PHA was then recovered by centrifugation (8,000 rpm at 4°C for 10 minutes), washed with acetone, ethanol, followed by distilled water and then dried in air (Law and Spleckey, 1961). For the extraction of PHA from cell pellet in the Oakridge tube, hot boiling chloroform was used.

3.2.2.5 Analytical methods

All the analytical procedures were performed as detailed below. All the experiments were conducted in triplicates and the experimental data was statistically analyzed using Microsoft Excel 2007.

3.2.2.5.1 Spectrophotometric determination of PHA accumulation

The PHA in the extracted sample was estimated spectrophotometrically using Shimadzu UV-Visible spectrophotometer, Japan. (Law and Spleckey, 1961).

Method - The PHA extracted as above was dissolved in hot boiling chloroform and was transferred into clean dry test tubes. The chloroform was allowed to evaporate completely and the PHA was converted into crotonic acid by treatment with conc. H_2SO_4 at 100°C for 10 minutes and the solution was cooled in ice. It was then diluted with 10% H_2SO_4 and the absorbance was determined at 210 nm (Sun *et al.*, 1994). A standard curve was plotted with maximum absorbance as a function of the concentrations of standard polyhydroxybutyrate (Sigma-Aldrich, USA) converted to crotonic acid and was quantified. The PHA concentration was expressed in g/l.

3.2.2.5.2 Cell Dry Weight (CDW) determination

The CDW was determined according to Pozo *et al.*, 2002 and expressed in g/l. Cells from 10 ml culture in nutrient medium were collected by centrifugation at 5000 rpm (Sigma, 2-16K, Germany) for 10 min at 4°C. The resultant cell pellet was washed once with sterile distilled water, and used for total cell dry weight determination after drying at 110°C in a Hot Air Oven (Labline, India) for 24- 48 h until constant weight was attained.

3.2.2.5.3 PHA content determination

PHA content (% of CDW) was calculated as percentage of PHA concentration (g/l) to cell dry weight (g/l) from CDW and PHA concentration (Chien *et al.*, 2007).

3.3 PHENOTYPIC CHARACTERIZATION OF PHA PRODUCING *VIBRIO* SP.

Phenotypic characterization of PHAs accumulating vibrios was done utilizing various biochemical tests, antibiotic susceptibility tests, exoenzyme profiling and hemolytic screening.

3.3.1 Biochemical characterization

3.3.1.1 Hi-Vibrio identification system

Biochemical characterization of PHAs accumulating vibrios were carried out using Hi-Vibrio identification systems (Himedia, Mumbai, India) which involved 12 biochemical tests (Voges Proskauers's test (VP test), arginine dihydrolase test, 1% salt tolerance test, ONPG test, citrate utilization test, ornithine, mannitol, arabinose, sucrose, glucose, salicin and cellobiose utilization tests). The results of these biochemical tests were also used to support the identification of the isolates upto species level.

3.3.1.2 Preparation of the inoculum and inoculation of the strip

The test strain was grown on nutrient agar plate and a single, well-isolated colony was inoculated into 5 ml alkaline peptone water and incubated 6-8 hours until it reached 1.0 $O.D_{600}$. Using sterile microtip 50 µl of bacterial suspension was added to the wells of strips containing the different substrates and incubated overnight at 37°C.

3.3.1.3 Reading of the strips

The VP tests, which required the addition of reagents after overnight incubation, were performed and interpreted after adding one drop of VP reagent 1 (Himedia, Mumbai, India) and VP reagent 2 (Himedia, Mumbai, India). The development of red color within 10 minutes would indicate the positive reaction. In case of other tests, reading of reactions were carried out 24h after incubation and the results were interpreted and recorded using the identification table provided in the chart, supplied by the manufacturer.

3.3.2 Screening of *Vibrio* sp. for extracellular enzyme production

Different agar plate assays were performed in order to detect the production of extracellular hydrolytic enzymes such as amylase, caseinase, lipase, cellulase, pectinase, xylanase, alginase, DNAse, gelatinase and phosphatase,

3.3.2.1 Extracellular amylase production:

Amylase activity on starch agar plates (Appendix 1) was routinely determined following the method described by Furniss *et al.* (1978) using nutrient agar plates containing 0.1% soluble starch and observing for a zone of clearance surrounding the colonies after flooding the plate with Gram's iodine.

3.3.2.2 Extracellular caseinase production

Caseinolytic activity was indicated by a zone of clearance surrounding the colony after spot inoculation on Casein agar plates (Appendix 1) containing 1.5% Casein (Kazanas, 1968).

3.3.2.3 Extracellular lipase production

Lipase production was screened on agar plates with minimal medium (Appendix 1) with 1% Tween 80 as outlined by Furniss *et al.* (1978). A positive result was observed as opacity in the medium surrounding the colonies due to crystal formation due to lipolytic activity of the culture.

3.3.2.4 Extracellular cellulase production

Cellulase producters were screened on carboxymethylcellulose (0.5%) agar plate (Appendix 1) described by Kasing *et al.* (2000). To visualize the clearing zones, the plates were flooded with an aqueous solution of congo red (1 mg/ml) for 15 min and washed with 1 M NaCl (Teather and Wood, 1982). The cellulolytic organisms will produce a clearing zone surrounding the colony due to the digestion of carboxymethylcellulose.

3.3.2.5 Extracellular pectinase production

Pectinolytic isolates were detected by using JG agar medium (Jayasankar and Graham, 1970) with 1% pectin (Appendix 1). After incubation, a 1% solution of CTAB was poured over the medium (Hankin *et al.*, 1971). The zone of clearance surrounding the colony indicated the presence of extracellular pectinase production.

3.3.2.6 Extracellular alginase production

Alginolytic activity was detected by observing for pitting around the growth (Furniss *et al.*, 1978) on plates containing marine agar with 2% sodium alginate (Appendix I).

3.3.2.7 Extracellular xylanase production

Xylanase producers were screened on oat spelt xylan medium (Appendix I) with 1% xylan, and were identified by zone of clearance surrounding the colonies (Anuradha *et al.*, 2007).

3.3.2.8 Extracellular DNase production

For the detection of DNase producers, DNA agar plate (Appendix I) containing 0.2% DNA was used (Zierdt *et al.*, 1970). After incubation, plates were flooded with 1 N HCl and the zone of clearance surrounding the colonies indicates extracellular DNase production.

3.3.2.9 Extracellular gelatinase production.

The presence of gelatinase production on plates was determined using gelatin agar plates (Furniss *et al.*, 1978) (Appendix I) containing 0.1% gelatin, where gelatinase production was indicated by a zone of clearance surrounding the colonies.

3.3.2.10 Extracellular phosphatase production.

Phosphatase producers were screened on NBRIP medium (Appendix I) with $Ca_3(PO_4)_2$ (Nautiyal, 1999). Phosphate solubilising isolates were identified by zone of clearance surrounding the colonies.

pH of all media were adjusted to 7.0 ± 0.2. Overnight cultures from NA medium with 1% NaCl were spot inoculated onto agar plates and incubated at 30°C. Results were checked at regular intervals for 2-3 days, with the exception of

pectinase, xylanase and cellulase, where the activity was determined after 7 day incubation period.

3.3.3 Antibiotic susceptibility test

Antibiotic susceptibility testing was done according to Kirby-Bauer disc diffusion method (Bauer *et al.*, 1966) as described below.

A single colony of the test strain was transferred into 3ml of sterile physiological saline. Turbidity of the cell suspension was adjusted to 0.5 Mc Farland's standard, either by adding new colonies or adding more sterile saline. With the help of a sterile cotton swab, a uniform bacterial smear was made on to Mueller-Hinton agar (HiMedia, India) plate (Appendix I). Antibiotic discs were placed on the plate, each plate holding not more than five discs. The discs were spaced to provide room for the development of the zone of inhibition. The plates were incubated at 37°C for 24 hours before examination. The result was interpreted as resistant, intermediate or sensitive based on the size of the inhibition zones around each disc as provided by the manufacturer (Himedia, India). (Performance standard for antimicrobial disc susceptibility tests, 2007).

The antibiotics used were Vancomycin (V, 30mcg/disc), Ampicillin (Am, 10mcg/ disc), Co-Trimoxazole (Co, 25mcg/disc), Carbenicillin (Cb, 100mcg/disc), Tetracyclin (T, 30mcg/disc), Trimetroprim (Tr, 5mcg/disc), Azithromycin (At, 30mcg/disc), Ciprofloxacin (Cf, 5mcg/disc) Rifampicin (R, 5mcg/disc) and Gentamycin (G, 10mcg/disc).

3.3.3.1. MAR (Multiple Antibiotic Resistance) indexing

MAR index was calculated using the formula a/b, where 'a' is the number of antibiotics to which the isolate was resistant, and 'b' is the number of antibiotics to which the isolate was exposed (Krumperman, 1983).

3.3.4. Hemolytic activity of vibrios

Hemolysis on blood agar plates is an indication of the potential for pathogenesis in bacteria. Hemolytic screening was performed on blood agar medium containing 5% human blood in blood agar base (Appendix I), to check pathogenicity of *Vibrio* sp. accumulating PHAs.

16h growth cultures in Nutrient medium were spot inoculated onto the blood agar plates and incubated at 37°C. The blood agar plates were examined at regular intervals - 16h, 24h and 48h. The formation of clearing zone/ green discoloration of the medium around the colonies indicated the hemolytic nature of isolates and they were considered as pathogenic (Isenberg 1992).

3.3.5. Phase Contrast Microscopy

A drop (100 µl) of cell culture was put on a microscopic glass slide with cover slip and visualized with a phase contrast microscopy (Nikon, Japan) for the detection of brightly refractile cytoplasmic inclusions interpreted as PHA inclusions (Lopez-Cortes *et al.*, 2010).

3.3.6. Sudan Black Staining

Sudan Black staining was performed on heat-fixed samples and prepared as 0.3 g Sudan Black B (HimEdia, India) dissolved in 75 ml (95%) ethanol, bringing it to 100 ml with distilled water. Samples were stained with Sudan Black solution for 10 min, dried with filter paper, and clarified with xylene drops, dried again with filter paper, and counterstained with 0.5% aqueous saffranine for 5s (Lopez-Cortes *et al.*, 2008). The smear on the glass slide was visualized under 100X magnification using oil immersion objective of a compound microscope (Radical, RXL-LT, India).

3.3.7. Bacterial Growth Curve of selected organism

The Nutrient medium (Appendix I) with 1% NaCl was used for the growth kinetic studies. The growth of selected organism was studied for 72h. Aliquots were sampled at regular intervals of 6h to analyze the cell growth by checking O.D. at 660nm in a spectrophotometer (Shimadzu UV-Vis Spectrophotometer, Japan). All the experiments were carried out in triplicates. From the results obtained, the growth curve of the isolate was prepared by plotting a graph of O.D. at 660nm against incubation time. From the growth curve, generation time and specific growth rate were calculated (Kratz and Myers, 1955).

3.4 16S rDNA SEQUENCE ANALYSIS FOR IDENTIFICATION OF PHA PRODUCING *VIBRIO* SP.

Selected strains of vibrios after secondary screening by spectrophotometric analysis were subjected to genotypic characterization using 16S rRNA partial gene sequencing and sequence analysis using bioinformatics tools for identification upto species level.

3.4.1 Template preparation for PCR

DNA extraction was performed employing phenol-chloroform method (Ausebel *et al.*, 1995). Transferred 2ml bacterial overnight culture in LB medium (Hi Media, Mumbai, India) into a micro centrifuge tube, spun for 10min. at 8000 rpm and decanted the supernatant completely. Resuspended the pellet in 875 ml of Tris-EDTA (TE) buffer and added 5µl Proteinase K and 100µl 10% sodium dodecyl sulphate (SDS) (Appendix I) to it. The mixture was incubated in a water bath at 37°C for 1hr. After incubation, added equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and mixed properly by inverting the tube until a white precipitate was seen and centrifuged at 8000 rpm for 10 minutes. With the help of a wide mouth-cut-tip carefully collected the upper aqueous layer into a fresh tube, taking care not to carry over any phenol while pipetting. Repeated the process

twice and added equal volume of chloroform and spun for 5min. at 8000 rpm. Collected the upper layer into a fresh tube and added 0.1 volume sodium acetate (Appendix I) and double volume isopropyl alcohol and spun at 8000 rpm for 10 min and decanted the supernatant. To the pellet, added 1ml 70% ethanol and spun. Decanted the alcohol and kept the pellet for air in a covered tray. The pellet containing the isolated DNA was then dissolved in 1 ml TE buffer 10 mM Tris (pH 7.8) and stored at -20°C for further studies.

The extracted DNA was dissolved in TE buffer and the concentration of genomic DNA was estimated spectrophotometrically. The purity of DNA was checked by reading the absorbance ratio A_{260}/A_{280}. The quantification of DNA was done using DNA/Protein pack® software of spectrophotometer (Shimadzu UV Spectrophotometer, Japan). An appropriate dilution (~80-100ng) of genomic DNA was used as template for PCR reactions.

3.4.2 Agarose Gel Electrophoresis

The agarose gel electrophoresis was carried out for the visualization of isolated DNA. 1 % (w/v) agarose in 1X TAE buffer (Appendix I) was used with 10 mg/ml EtBr solution (Appendix I). Electrophoresis was carried out at 70 V in an electrophoresis apparatus (GeNei, India) (Sambook *et al.*, 2000). The gel was then viewed on an UV transilluminator and the image was captured with the help of G: BOX Fluorescence Gel Documentation system (Syngene, USA).

3.4.3 16S rDNA sequence analysis

A PCR based method using a primer pair for 16S rDNA was used for species identification of the *Vibrio* strains (Shivaji *et al.*, 2000; Reddy *et al.*, 2000, 2002a, 2002b). A portion of the 16S rRNA gene (1.5kb) was amplified from the genomic DNA. Products after PCR amplification was purified by gene clean kit (Bangalore Genei, India) and subsequently sequenced followed by homology analysis.

Sequence	Reference
16SF 5' AGTTTGATCCTGGCTCA 3'	Shivaji *et al.*, 2000
16SR 5' ACGGCTACCTTGTTACGACTT 3'	Reddy *et al.*, 2000,2002 a, b

3.4.3.1. Polymerase Chain Reaction (PCR)

PCR was performed using the genomic DNA (~100ng/ml) as template and 16S rDNA specific primers as detailed above.

PCR Mix composition (20 µl)

10X PCR buffer	2.0µl
2.5mM dNTPs	2.0µl
Forward primer (10 µM)	1.0µl
Reverse primer (10 µM)	1.0µl
*Taq*DNA polymerase	1U (0.2 µl)
Template DNA	0.5 µl
$MgCl_2$	1.2µl

Sterile distilled water to make final volume to 20µl

PCR conditions

Annealing - 56°C -30 sec.

Extension - 72°C -2 min.

PCR was performed in a thermal cycler (Bio Rad, USA).

3.4.3.2 *In-silico* analysis of the sequence

3.4.3.2.1 DNA sequencing and analysis

Products after PCR amplification were purified by gene clean kit (Bangalore Genei, India). The products were sequenced by Sanger's Dideoxy method using ABI 3730 Excel at SciGenom Labs Pvt Ltd, Cochin, Kerala. The sequenced PCR products were analyzed online using BLAST software

(http://www.ncbi.nlm.nih.gov/blast) and the identity of the sequences were determined (Altschul *et al.*, 1980).

3.4.3.2.2 Multiple sequence alignment and phylogenetic tree construction

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

3.5 SCREENING FOR VIRULENCE GENES IN PHA PRODUCING *VIBRIO* SP.

The screening for the presence of virulence genes in vibrios were carried out using specific primers for three virulence genes using a PCR based method.

3.5.1 PCR for *toxR*

ToxR is a direct virulence factor. PCR was carried out for *toxR* using *toxR* F and *toxR* R primers (Singh *et al.*, 2001) as detailed below. Positive amplification will indicate its presence.

Primer sequence

Forward primer: 5' – CCTTCGATCCCCTAAGCAATAC – 3'

Reverse primer: 5' – AGGGTTAGCAACGATGCGTAAG – 3'

Amplicon size: 779 bp

PCR Mix composition

10X PCR buffer	2.5µl
2.5mM each dNTPs	2.0µl
Forward primer (10 picomoles)	1.0µl
Reverse primer (10 picomoles)	1.0µl
Taq DNA polymerase	1U
Template DNA	1.0µl
Sterile Distilled water to final volume of 20µl	

PCR conditions

Annealing -60°C - 1 min.

Extension - 72°C - 1.5 min.

PCR was performed in a thermal cycler (Bio Rad, USA).

3.5.2 PCR for *zot*

Zonula occludens toxin is known as ***zot***. It can be called a direct virulence factor. PCR to detect *zot* was carried out using *zot* F and *zot* R primers (Singh *et al.*, 2001) as detailed below. Positive amplification will indicate its presence.

Primer sequence

Forward primer: 5' – TCGCTTAACGATGGCGCGTTTT – 3'

Reverse primer: 5' – AACCCCGTTTCACTTCTACCCA – 3'

Amplicon size: 947 bp

PCR mix composition and conditions are similar as mentioned as for detection of *toxR* gene as under section 3.5.1

3.5.3 PCR for *ctxA*

ctxA is the most important virulence factor in *Vibrio cholera* and it codes for cholera toxin A subunit. PCR to detect *ctxA* was carried out using *ctxA* F and *ctxA* R primers (Singh *et al.*, 2001) as detailed below. Positive amplification will indicate its presence.

Primer sequence

Forward primer: 5' – CGGGCAGATTCTAGACCTCCTG – 3'

Reverse primer: 5' – TCTATCTCTGTAGCCCCTATTACG – 3'

Amplicon size: 564 bp

PCR mix composition and conditions are as mentioned for detection of *toxR* gene as under section 3.5.1

3.6. MOLECULAR CHARACTERISATION OF PHA SYNTHASE GENE IN PHA PRODUCING *VIBRIO* SP.

3.6.1 Cultivation of bacterial strains

The selected strains of *Vibrio* sp. after preliminary, secondary and hemolytic screening were cultivated in the Nutrient medium with 1% NaCl (Appendix I). 50 ml of the medium was taken in a 250 ml Erlenmeyer flask and cultivated at 35°C on a rotary shaker at 120 rpm for 12h.

3.6.2 Isolation of genomic DNA

Genomic DNA extraction was done according to Ausebel *et al.* (1995) as mentioned under section 3.4.1.

3.6.3 Nucleic acid quantification and agarose gel electrophoresis

The purity of DNA was checked by reading the absorbance ratio A_{260}/A_{280}. The quantification of DNA was done using DNA/Protein pack® software of Shimadzu UV-VIS spectrophotometer. The amount of DNA used as a template

was ~100ng/ml in 20µl of PCR mixture. The agarose gel electrophoresis was carried out according to the method mentioned in 3.4.2.

3.6.4 Detection of class I PHA synthase gene

Class I PHA synthase gene represents the gene for short chain length PHAs (scl-PHAs). The PCR reaction for class I PHA synthase gene (*phaC*) was performed using PCR based method outlined by Sheu *et al.* (2000). Three degenerate primers were used as PCR primers to detect PHA synthase genes (Sheu *et al.*, 2000). The details of primers used are represented below and positions of primers on the Class I PHA synthase gene of *R. eutropha* is as represented in Fig. 3.2.

Primer	Sequence 5'-3'	Reference
CF1	ATCAACAARTWCTACRTCYTSGACCT	Sheu *et al.*, 2000
CF2	GTSTTCRTSRTSWSCTGGCGCAACCC	Sheu *et al.*, 2000
CR4	AGGTAGTTGTYGACSMMRTAGKTCCA	Sheu *et al.*, 2000

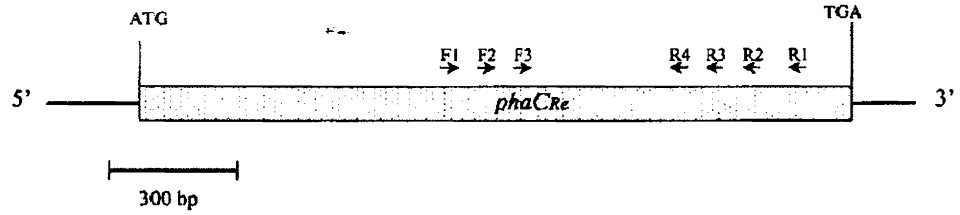


Fig. 3.2 Positions of primers on the Class I PHA synthase gene of *R.eutropha.* F1-phaCF1; F2-phaCF2; F3-phaCF3; R4-phaCR4; R3-phaCR3; R2-phaCR2; R1-phaCR1 (adopted from Sheu *et al.*, 2002)

3.6.4.1. PCR for class I PHA synthase gene

The PCR reaction for scl PHA synthase gene (*phaC*) was performed with the forward primer CF1 and the reverse primer CR4.

The PCR mixture consisted of 0.5µl of 100ng/ml template DNA (100ng/ml), 2µl of 2.5mM of dNTP, 1U units of Taq DNA polymerase (Sigma), 2 µl of 10X Taq buffer, 1 M of Betain, 0.03% of DMSO, 1 µl of acetylated BSA, 5 µl of each primers and added sterile distilled water to make final volume of 20µl. The predicted PCR product was ~ 496bp length.

The PCR mixture was pre-incubated at 94°C for 5 min, 51°C for2 min, and 72°C for 2 min. The PCR cycle consisted of 20 s of denaturation at 94°C, 45 s of annealing at 61°C (decreased by 1 sec/cycle), and 1 min of extension at 72°C. This cycle was repeated 35 times and then incubated at 72°C for 10 min for the final extension. PCR was performed in a thermal cycler (Bio Rad, USA).

3.6.4.2. Semi nested PCR for class I PHA synthase gene

Semi-nested PCR was done with the primers phaCF2 and phaCR4, with a predicted product of 406 bp (Sheu *et al.*, 2000), to further confirm the PCR results obtained previously. For positive products, 1 µl of 100-fold-diluted colony PCR products was subjected to semi-nested PCR. For negative products, 1 µl of undiluted colony PCR products was directly supplied as DNA templates for semi-nested PCR. PCR reactions were done using a PCR mixture consisting of 0.5µl of template DNA (100ng/ml), 2µl of 2.5mM of dNTP, 1U units of Taq DNA polymerase (Sigma), 2 µl of 10X Taq buffer, 1 M of Betain, 0.03% of DMSO, 1 µl of acetylated BSA, 5 µl of each primers and added sterile distilled water to a final volume of 20µl. PCR reactions for Semi nested PCR for class I PHA synthase gene were as mentioned in 3.6.4.1. PCR was performed in a thermal cycler (Bio Rad, USA). Positive amplification will indicate its presence.

3.6.5 Detection of class II PHA synthase gene

Class II PHA synthase gene represents the gene for medium chain length PHAs (mcl-PHAs). The PCR reaction for class II PHA synthase genes (*phaC1* and *phaC2*) was performed with methods outlined by Solaiman *et al.*, 2000. The details of primers used is represented below and positions of primers on the Class II PHA synthase genes of *Pseudomonas resinovorans* is as represented in Fig. 3.3

Primer	Sequence 5'-3'	Reference
I-179L	**ACAGATCAACAAGTTCTACATCTTC**	Solaiman 2000
I-179R	**GGTGTTGTCGTTGTTCCAGTAGAGG**	Solaiman 2000

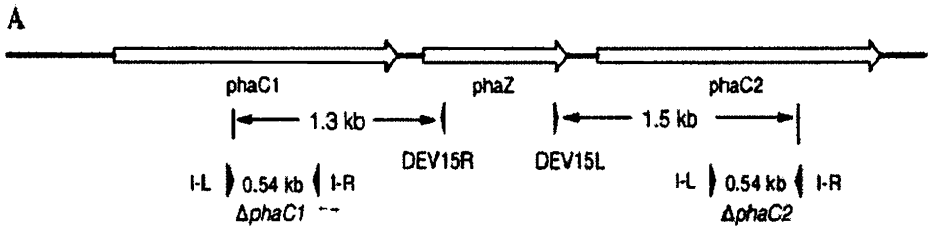


Fig. 3.3 The schematic of the *Pseudomonas resinovorans* pha locus (gb AF129396), showing the positions of the various PCR primers and the approximate sizes of the expected amplicons. The abbreviations I-L and I-R represent the binding-sites of I-179L and I-179R primers, respectively. (adopted from Solaiman, 2000)

3.6.5.1 PCR for class II PHA synthase gene (Solaiman *et al.*, 2000)

The PCR reaction for mcl PHA synthase gene was performed with the forward primer I-179L and the reverse primer I-179R the and expected PCR product was ~ 540bp.

Template DNA for mcl-PCR was prepared as detailed under section 3.4.1. PCR reactions were done using a PCR mixture consisting of 0.5µl of template DNA (100ng/ml), 2µl of 2.5mM of dNTP, 1U units of Taq DNA polymerase

(Sigma), 2 µl of 10X Taq buffer, 5 µl of each primers and add sterile distilled water to a final volume of 20µl.

The PCR mixture was pre-incubated at 94°C for 5 min, 51°C for2 min, and 72°C for 2 min. The PCR cycle consisted of 20 s of denaturation at 94°C, 45 s of annealing at 57°C and 1 min of extension at 72°C. This cycle was repeated 35 times and then incubated at 72°C for 5 min and a final incubation at 4°C (Sujatha *et al.*, 2005). PCR was performed in a thermal cycler (Bio Rad, USA). Positive amplification will indicate its presence.

3.6.6 Agarose gel electrophoresis of PCR products

The PCR-amplified DNA fragments were observed by agarose gel electrophoresis in 1% agarose gel as mentioned in section 3.4.2. Ten µl of the PCR product were electrophoresed, the gel viewed using an UV transilluminator and the image was captured by Gel Doc (Syngene, USA) as mentioned under section 3.4.2.

3.6.7 *In-silico* analysis of the sequence

Products after PCR amplification were purified by gene clean kit (Bangalore Genei) and were sequenced at SciGenom Labs Pvt Ltd, Cochin, Kerala. The sequenced PCR products were compared with those available from GenBank, by using online BLAST tools (http://www.ncbi.nlm.nih.gov/blast) (Altschul *et al.*, 1980). The sequences was translated to aminoacids using online nucleotide translation tools like Expasy (http://web.expasy.org/translate) and compared with other protein sequences using Conserved Domain Database available in NCBI (Marchler-Bauer *et al.*, 2011). Nucleic acid and protein sequences of other PHA synthase genes were obtained from NCBI database Multiple sequence alignment and phylogenetic tree construction were done as mentioned in section 3.4.3.2.2.

3.7 PHA PRODUCTION BY *VIBRIO* SP. BTKB33: OPTIMISATION OF BIOPROCESS VARIABLES BY "ONE-FACTOR-AT-A-TIME" METHOD

Various physico-chemical and bioprocess parameters affecting PHA production by *Vibrio* sp. BTKB33 under submerged fermentation were optimized towards maximum PHA production in PHA production medium. The strategy adopted for the optimization was to evaluate individually the effect of different parameters ("one-variable-at-a-time" method) on PHA production under submerged fermentation, conduct statistical optimization, and perform a time course experiment under optimized conditions.

The parameters optimized included incubation time, initial pH of medium, sodium chloride concentration, agitation, incubation temperature, media, inoculum age, inorganic nitrogen sources, organic nitrogen sources, inorganic salts, inoculum concentration and carbon sources. PHA production medium preparation, seed medium preparation, inoculum preparation and extraction of PHA were done as described under sections 3.2.2.1, 3.2.2.2, 3.2.2.3 and 3.2.2.4 respectively, unless otherwise mentioned. In each case, samples were analyzed for PHA production, CDW determination and PHA content as detailed under sections 3.2.2.5.1, 3.2.2.5.2 and 3.2.2.5.3 respectively, unless otherwise mentioned.

All the experiments were carried out in triplicates. The results are an average of triplicate experiments and standard deviation was determined using Excel 2007 (Microsoft Corporation, Redmond, USA). The graphs were plotted with the help of Sigma plot for Windows Version 11.0 (Systat Software inc., Germany).

3.7.1 Incubation time

Time course of PHA production was studied in the PHA production medium (Sun *et al.*, 1994) using shake flask cultures. Optimum incubation time for maximum PHA production was determined by incubating the inoculated media for a total of 96h and analyzing the samples at a regular interval of 6h for PHA

production, PHA content and CDW. PHA production medium preparation, seed medium preparation, inoculum preparation and extraction of PHA were performed as detailed under sections 3.2.2.1, 3.2.2.2, 3.2.2.3 and 3.2.2.4 respectively.

3.7.2 Initial pH of the medium

For the optimization of initial pH, medium used was as described earlier. The PHA production, PHA content and CDW at different initial pH (3, 4, 5, 6, 7, 8, 9 & 10) were studied and analysis carried out after 36h incubation. The pH of the medium was adjusted to different levels using either 1 N HCl or 1 N NaOH. PHA production medium preparation, seed medium preparation, inoculum preparation and extraction of PHA were performed as detailed under sections 3.2.2.1, 3.2.2.2, 3.2.2.3 and 3.2.2.4 respectively.

3.7.3 Sodium chloride concentration

Optimal sodium chloride for maximum PHA production was evaluated by using different sodium chloride concentrations (1%, 2%, 3%, 4%, 5%, 10%, 15% & 20%). PHA content and CDW at all tested sodium chloride concentrations were also measured. The analysis of the samples was done after 36h incubation and the PHA production were carried out at pH 7. PHA production medium preparation, seed medium preparation, inoculum preparation and extraction of PHA were performed as detailed under sections 3.2.2.1, 3.2.2.2, 3.2.2.3 and 3.2.2.4 respectively.

3.7.4 Agitation

Effect of agitation on PHA production, PHA content and CDW was checked by incubating the inoculated media taken in the conical flasks both in static (0 rpm) and shake flask condition on the environmental shaker (Orbitek, Scigenics, India) at different rpm (80, 100, 120 and 150 rpm). The analyses of the samples were done after 36h incubation and the PHA production were carried out

at pH 7 and in 1.5 % NaCl concentration. PHA production medium preparation, seed medium preparation, inoculum preparation and extraction of PHA were performed as detailed under sections 3.2.2.1, 3.2.2.2, 3.2.2.3 and 3.2.2.4 respectively.

3.7.5 Incubation temperature

For the optimization of different incubation temperatures, medium used was as described earlier. The PHA production, PHA content and CDW at different incubation temperature (0°C, 4°C, 25°C, 30°C, 35°C,40°C, 45°C & 50°C) were studied and analysis carried out after 36h incubation and studies were carried out at pH 7, 1.5% NaCl concentration at 120rpm. PHA production medium preparation, seed medium preparation, inoculum preparation and extraction of PHA were performed as detailed under sections 3.2.2.1, 3.2.2.2, 3.2.2.3 and 3.2.2.4 respectively.

3.7.6 Different media

Here the effect of five different media and their components on maximum PHA production were studied. In these studies Medium A, B and C represent various PHA production media outlined by Sun *et al.*, 1994, Ramadas *et al.*, 2009 and Lageveen *et al.*, 1988 (E2 minimal medium) and Media preparation are as given in the Appendix I. The medium D and E represent nitrogen sufficient media like Nutrient medium and Zobell's marine broth respectively and media preparation are given in the Appendix I. PHA production, PHA content and CDW of the samples were analyzed after 36h incubation and studies were carried out at pH 7, 1.5 % NaCl concentration at 120rpm at incubation temperature of 35°C. Seed medium preparation, inoculum preparation and extraction of PHA were performed as detailed under sections 3.2.2.2, 3.2.2.3 and 3.2.2.4 respectively.

3.7.7 Age of inoculum

Inoculum of various age ranging from 6h -24h were added to PHA production medium for the optimization of age of inoculum for maximum PHA production, PHA content and CDW were carried out. The samples were analyzed after 36h incubation and studies were carried out in at pH 7, 1.5 % NaCl concentration at 120rpm at incubation temperature of 35°C. PHA production medium preparation, seed medium preparation, inoculum preparation and extraction of PHA were performed as detailed under sections 3.2.2.1, 3.2.2.2, 3.2.2.3 and 3.2.2.4 respectively.

3.7.8 Effect of inorganic nitrogen Sources

Effect of inorganic nitrogen source on PHA production, PHA content and CDW was studied by the addition of ammonium sulphate, ammonium oxalate, ammonium nitrate, ammonium hydrogen phosphate, ammonium chloride, ammonium ferrous sulphate, sodium nitrate, potassium nitrate and ammonium acetate individually at 0.05 % (w/v) level, replacing the inorganic nitrogen source of PHA production medium described earlier in the section 3.2.2.1. Medium without any inorganic nitrogen source was used as control. The PHA production, PHA content and CDW of the samples were analyzed after 36 h incubation and the studies were carried out at pH 7, at 120rpm at 35°C temperature in PHA production medium having 1.5 % NaCl concentration. Seed medium preparation, inoculum preparation and extraction of PHA were performed as detailed under sections 3.2.2.2, 3.2.2.3 and 3.2.2.4 respectively.

3.7.9 Effect of organic nitrogen sources

Effect of complex organic nitrogen source on PHA production, PHA content and CDW was studied using yeast extract, beef extract, malt extract, casein, peptone, soyabean and urea, added individually at 0.05 % (w/v) , replacing the organic nitrogen source of PHA production medium described earlier in the

section 3.2.2.1. Medium without any organic nitrogen source was used as control. The PHA production, PHA content and CDW of the samples were analyzed after 36 h incubation and the studies were carried out at pH 7, at 120rpm at 35°C temperature in PHA production medium having 1.5 % NaCl concentration and ammonium chloride as inorganic N-source. Seed medium preparation, inoculum preparation and extraction of PHA were performed as detailed under sections 3.2.2.2, 3.2.2.3 and 3.2.2.4 respectively.

3.7.10 Effect of inorganic salts

Effect of inorganic salts on PHA production, PHA content and CDW was studied by the addition of $MgSO_4$, $CaCl_2$, $ZnSO_4$, KCl, $FeSO_4$, $MgSO_4$, $MgCl_2$ and $CoCl_2$, used individually at 0.02 % (w/v) level, replacing the inorganic salts source of PHA production medium described earlier in the section 3.2.2.1, Medium without any inorganic salts was used as control. The samples were analyzed after 36 h incubation for PHA production, PHA content and CDW and the studies were carried out at pH 7, at 120 rpm at 35°C temperature in PHA production medium having 1.5 % NaCl concentration, yeast extract as organic N source and ammonium chloride as inorganic N source. Seed medium preparation, inoculum preparation and extraction of PHA were performed as detailed under sections 3.2.2.2, 3.2.2.3 and 3.2.2.4 respectively.

3.7.11 Inoculum concentration

Optimal inoculum concentration that support maximum PHA production in *Vibrio* sp. BTKB33 was evaluated using different concentration of initial inoculum (1%, 2%, 3%, 4%, 5%, 10%, 15% & 20) prepared as mentioned in section 3.3.2.3. The samples taken after 36 h incubation were analyzed for PHA production, PHA content and CDW and the studies were carried out at pH 7, at 120rpm at 35°C temperature in PHA production medium described earlier in section 3.2.2.1 having 1.5 % NaCl concentration, yeast extract as organic N

source, $MgSO_4$ as inorganic salt and Ammonium chloride as inorganic N source. Seed medium preparation, inoculum preparation and extraction of PHA were performed as detailed under sections 3.2.2.2, 3.2.2.3 and 3.2.2.4 respectively.

3.7.12 Effect of carbon source

Effect of various carbon source on PHA production, PHA content and CDW was studied by the addition of glucose, glycerol, lactose, mannitol, sodium acetate, starch & sucrose, used individually at a level of 2 % (w/v), replacing the carbon source of PHA production medium described earlier in the section 3.2.2.1. Medium without any carbon source was used as control. The samples taken after 36 h incubation were analyzed for PHA production, PHA content and CDW and the studies were carried out at pH 7, at 120rpm at 35°C temperature in PHA production medium described earlier in section 3.3.2.3 having 1.5 % NaCl concentration, yeast extract as organic N source, $MgSO_4$ as inorganic salt and Ammonium chloride as inorganic N source. Seed medium preparation, inoculum preparation and extraction of PHA were performed as detailed under sections 3.2.2.2, 3.2.2.3 and 3.2.2.4 respectively.

3.8 PHA PRODUCTION BY *VIBRIO* SP.BTKB33 UNDER SUBMERGED FERMENTATION: OPTIMISATION OF BIOPROCESS VARIABLES BY STATISTICAL APPROACH

Optimization of bioprocess variables for maximal PHA production by *Vibrio* sp. BTKB33 through statistical approach was carried out employing Plackett-Burman (PB) Design and Response Surface Methodology (RSM). Initially, process variables that significantly influence PHA production were selected by Plackett-Burman (PB) Design, and in the second stage Response Surface Methodology (RSM) was adopted towards selection of optimal variables and to understand the interrelationship among significant variables on PHA

production and PHA content. The statistical software package used was Design expert 8.0 (Stat Ease Inc., Minneapolis, USA).

3.8.1 Plackett-Burman design (PB Design)

After studying the effect of different parameters by "one-variable-at-at-time" method, eleven factors were selected for further optimization using statistical approach. The effect of eleven factors *viz.*; incubation period, pH, temperature, glucose, ammonium chloride, yeast extract, KH_2PO_4, $MgSO_4$, tryptone, inoculum and sodium chloride concentration on PHA production was evaluated with Plackett-Burman design (Haaland, 1989). The parameters were varied over two levels and minimum and maximum ranges selected for the parameters are given in Table 3.2

Table 3.2 The minima and maxima of the parameters selected for optimization of PHA production in *Vibrio* sp. BTKB33 by Plackett-Burman design

Sl no.	Factors	Level	
		Minimum (-)	Maximum (+)
1	Incubation period (h)	12	36
2	pH	6	9
3	Temperature (°C)	30	40
4	Glucose (%)	2	4
5	Ammonium Chloride (%)	0.05	0.1
6	KH_2PO_4 (%)	0.05	0.1
7	$MgSO_4$ (%)	0.01	0.02
8	Yeast Extract (%)	0.05	0.1
9	Tryptone (%)	0.1	0.5
10	Inoculum concentration (%)	1	10
11	Sodium chloride (%)	1	10

The statistical software package Design-Expert® 8.0 (Stat Ease Inc., Minneapolis, USA) was used to generate a set of 12 experimental designs. The production was setup by inoculating the media with respective inoculum

percentages as suggested by the model and incubated for specified incubation period (12-36h), at specified temperature (30-40°C), at 120 rpm. For each experiment, the PHA production was calculated in terms of g/l. Regression analysis of the experimental data obtained was conducted using statistical software. The experiments were done in triplicates.

Based on results obtained from Plackett-Burman design, the fitted first order model is:

$$Y = \beta o + \sum_{i=1}^{k} \beta i \, Xi$$

Y- Predicted response, βo, βi - Constant coefficients, Xi-Coded independent variables or factors.

This model does not describe interaction among factors and it is used to screen and evaluate the important factors that influence the response. Effect of each variable on production was determined by calculating their respective E-values (Gupta *et al.,* 2004)

$$E = \frac{\text{(Total response at high level)} - \text{(Total response at low level)}}{\text{Number of Trails}}$$

3.8.1.2 Validation of PB Design

In order to validate the PB Design, a random set of experiments were setup according to conditions predicted by the model. The responses obtained from the trials conducted as above following the PB design model, was used to estimate the coefficients of polynomial models using standard regression techniques. The estimate of Y was used to generate an optimal combination of factors that can support maximal PHA production using predictive models from PB Design. The software Design Expert® 8.0 (Stat Ease Inc., Minneapolis, USA)

was used to fit the PB Design model to the experimental data. All the experiments were carried out independently in triplicates.

3.8.2 Optimization of bioprocess variables for PHA production and PHA content in *Vibrio* sp. BTKB33 using RSM under submerged fermentation

The important parameters affecting PHA production and PHA content in *Vibrio* sp. BTKB33 were optimized using a response type Box-Behnken (Box and Behnken, 1960) model experimental design. Box- Behnken Design model is a second order design that allows estimation of quadratic effects, and is based on combining a two-level factorial design with an incomplete block design. The design was used for creating the quadratic response model.

The variables selected by the PB Design were applied to RSM to evaluate the individualized influence of each variable and understanding the interrelationship among significant variables on PHA production and PHA content.

Based on the results of the one variable at a time experiments and PB design, the effect of three factors *viz.* NaCl concentration, temperature and inoculum concentration were studied on PHA production and PHA content using response surface methodology. Other components of the medium were: pH 7.5; glucose concentration 3% (w/v); ammonium chloride 0.08% (w/v); yeast extract 0.08% (w/v); KH_2PO_4 0.08% (w/v); $MgSO_4$ 0.02% (w/v); tryptone 0.5% (w/v) and inoculum concentration 5.5% (v/v).

Each factor in the design was studied at three different levels. All the variables were taken at a central coded value, considered as zero. A design model with 17 runs in 1 block of 17 cases was used as exhibited in Table 3.3 and each independent variable was tested at three levels. The levels were coded in standardized units with values -1, 0 and +1 representing lower, middle and higher values respectively.

Table 3.3. Box-Behnken design for 3 variables at 3 levels and 17 runs for the optimization of PHA production and PHA content in *Vibrio* sp. BTKB33 under submerged fermentation

RUN	Incubation Period (h)	NaCl concentration (%)	Temperature (^{O}C)
1	0	0	0
2	0	0	0
3	-1	-1	0
4	+1	0	-1
5	0	+1	+1
6	+1	+1	0
7	0	0	0
8	-1	0	+1
9	0	0	0
10	+1	0	+1
11	0	+1	-1
12	+1	-1	0
13	-1	0	-1
14	-1	+1	0
15	0	0	0
16	0	-1	-1
17	0	-1	+1

Design Expert® 8.0 (Stat Ease Inc., Minneapolis, USA) was used to analyze the experimental data. The average maximum PHA production (g/l) and PHA content (g/l) was taken as the dependent variable or response (Y). Regression analysis was performed on the data obtained. The results of the Box-Behnken design were then used to fit a quadratic equation by multiple regression procedure. This resulted in an empirical model that related the response measured to the independent variables of the experiment.

The following quadratic model was chosen to represent the relationship fitted for the three variables.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j$$

In this model, Y represents the dependent variable – PHA production/PHA content; X_1, X_2 and X_3 represents the independent variables selected. The design of experiments in terms of actual factors are given in Table 3.4.

Table 3.4. Box-Behnken design for optimization of PHA production and PHA content in *Vibrio* sp. BTKB33 under submerged fermentation

RUN	Incubation Period (h)	NaCl concentration (%)	Temperature (°C)
1	24.00	5.50	35.00
2	24.00	5.50	35.00
3	12.00	1.00	35.00
4	36.00	5.50	30.00
5	24.00	10.00	40.00
6	36.00	10.00	35.00
7	24.00	5.50	35.00
8	12.00	5.50	40.00
9	24.00	5.50	35.00
10	36.00	5.50	40.00
11	24.00	10.00	30.00
12	36.00	1.00	35.00
13	12.00	5.50	30.00
14	12.00	10.00	35.00
15	24.00	5.50	35.00
16	24.00	1.00	30.00
17	24.00	1.00	40.00

The levels tested were incubation period: 12, 24 and 36h; incubation temperature: 30, 35 and 40°C and NaCl concentration: 1, 5.5 and 10 % (w/v). Analysis of Variance (ANOVA) were performed and 3-dimensional response surface curves were plotted by Design Expert software to study the interaction among various physico-chemical parameters. The experiments were carried out in triplicate.

3.8.2.2 Validation of the Box-Behnken design model

In order to validate the response surface model, a random set of experiments were setup according to conditions predicted by the model. The responses obtained from the trials conducted as above following the Box-Behnken design model for three variables, was used to estimate the coefficients of polynomial models using standard regression techniques. The estimate of Y was used to generate an optimal combination of factors that can support maximal PHA production using predictive models from response surface methodology. The software Design Expert® 8.0 (Stat Ease Inc., Minneapolis, USA) was used to fit the response surface-Box-Behnken model to the experimental data. All the experiments were carried out independently in triplicates.

3.8.3 Time course study under optimal condition

Time course experiment was conducted with the optimized conditions determined after statistical optimization of various variables.

All the experiments were conducted in triplicates and the PHA production studies were carried out under submerged condition detailed earlier and from each samples taken at interval of 6h, PHA concentration (g/l), CDW (g/l) and PHA content (% of CDW) were calculated as described earlier under optimized conditions.

3.9. EXTRACTION, PURIFICATION AND CHARACTERISATION OF POLYHYDROXYALKANOATES

3.9.1 Culture conditions and PHA extraction

The PHA from *Vibrio* sp. strain BTKB33 were extracted,purified and characterized. The preparation of PHA production medium, seed medium, and inoculum preparation were done as mentioned under section 3.2.2.1, 3.2.2.2 and 3.2.2.3. For the characterization studies PHAs were extracted from the biomass

according to the method outlined by Dong and Sun (2000) as mentioned under section 3.2.2.4

3.9.2 PHA purification

PHA purification was done according to López-Cuellar *et al.* (2011). The PHAs after extraction were subjected to a boil in chloroform for 10 min and filtered through Whatman filter paper no.1. The filtrate was allowed to precipitate in ice-cold hexane (10 fold excess). The precipitated PHAs were redissolved in chloroform, and the process repeated twice. The residual solvent was eliminated by evaporation.

3.9.3 Fourier-Transform Infra red Spectroscopy (FT-IR Spectroscopy)

Fourier-Transform infra red spectroscopy is a technique that provides information about the chemical bonding or molecular structure of materials whether organic or inorganic. The bonds and group of bonds vibrate at characteristic frequencies. A molecule that is exposed to infra red rays absorbs infrared energy at frequencies which are characteristic to that molecule. During FT-IR analysis a spot on specimen is subjected to a modulated IR beam. The specimen's transmittance and reflectance of infrared rays at different frequencies is translated to an IR absorption plot consisting of reverse peaks. The resulting FT-IR spectral pattern is then analyzed and matched with known signatures of identified materials.

The parameters used in FT- IR analysis were: spectral range 4000 - 400 cm^{-1}, resolution 4 cm^{-1}.The extracted purified PHAs sample was subjected to FT-IR spectroscopic analysis (Thermo Nicolet, Avatar 370), equipped with KBr beam splitter with DTGS (Deuterated triglycine sulphate) detector (7800-350 cm^{-1}), at Sophisticated Test and Instrumentation Centre, Cochin University of Science and Technology, Cochin, Kerala.

3.9.4 Nuclear Magnetic Resonance (NMR) analysis

Nuclear Magnetic Resonance is a property that magnetic nuclei have in a magnetic field and applied electromagnetic pulse, which cause the nuclei to absorb energy from the EM pulse and radiate this energy back out. A PHA solution was prepared by using $CDCl_3$ and the 400MHz ^{13}C NMR spectrum of PHA was obtained by using a Bruker Avance III, spectroscope at Sophisticated Test and Instrumentation centre, Cochin University of Science and Technology, Cochin, Kerala.

3.9.5 Differential Scanning Calorimetric (DSC) analysis

DSC measures the amount of heat energy absorbed or released by a sample, as it is heated, cooled or held at a constant temperature. The sample was analyzed using a Mettler Toledo 822c instrument. The PHA samples were heated at a rate of 10°C min^{-1} from 4 to 200°C. DSC analysis was carried out at Sophisticated Test and Instrumentation Centre, Cochin University of Science and Technology, Cochin, Kerala.

3.9.6 Thermo gravimetric analysis (TGA)

The thermal properties of the extracted purified PHA samples were examined by TGA instrument (Perkin Elmer, Diamond, USA). The sample was scanned from 40- 930°C with a heating rate of 10°C min^{-1} at Sophisticated Test and Instrumentation Centre, Cochin University of Science and Technology, Cochin, Kerala.

4 Results

4.1 Screening and identification of vibrios isolated from marine benthic environments upto Genus

Vibrios were isolated from sediment samples collected from marine benthic environments from locations along the South-West and East coasts of India. 828 isolates that were Gram negative rods or cocci, oxidase positive, fermentative on MOF media, and showing yellow/green coloured colonies on TCBS (Thiosulfate Citrate Bile salt Sucrose) agar, were segregated as *Vibrio* sp. employing the above routine biochemical tests.

4.2 Preliminary screening for polyhydroxyalkanoates (PHAs) accumulating *Vibrio* sp.

All 828 isolates were screened for PHA accumulation by the fluorescent Nile blue sulphate staining method. From this preliminary screening, 513 (62%) isolates that emitted pink fluorescence on exposure of plates to UV light, were grouped as PHA accumulating *Vibrio* sp. (Fig. 4.1).

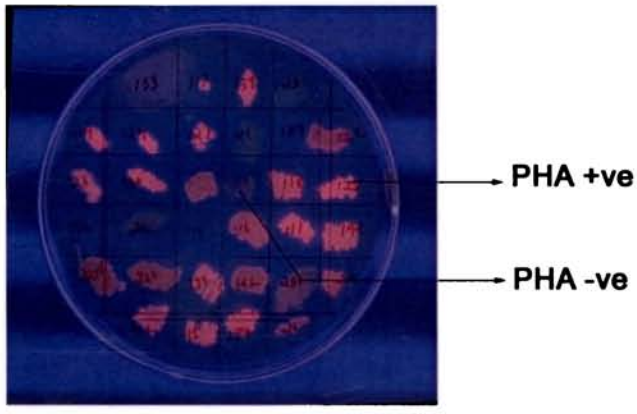


Fig. 4.1 Nile blue A staining method for the detection of PHAs accumulating strains of *Vibrio* sp. isolated from marine sediment samples. The PHAs accumulating strains emitted pink fluorescence under UV light.

The intensity of fluorescence emitted by the culture is directly proportional to the the amount of PHA accumulated by them. 80 isolates out of the 513, were selected for further studies based on the intensity of fluorescence in the preliminary plate assay method. These were then subjected to phenotypic characterization - biochemical tests, exoenzyme profiling, antibiotic sensitivity testing and secondary screening for PHA accumulation by spectrophotometric analysis.

4.3 Phenotypic characterization of potential PHA accumulating vibrios

4.3.1 Biochemical tests

Table 4.1 details the results of the biochemical tests for the 80 *Vibrio* sp. All the tested isolates were viable in 1% NaCl concentration. Only *Vibrio* sp. strain BTTR45, BTTR36, BTTN26, BTTC4, BTTC10 and BTMV9 tested positive for Voges-Proskauer test. All the tested isolates except strains BTTV7, BTKB27, BTTV40, BTKB18, BTTV33 and BTMV2, did not utilize ONPG. All the tested isolates were citrate positive except BTTV29, BTTV40 and BTTV33. Salicin utilization was shown only by strains BTKB27 and BTMV10. Only two strains BTTV10 and BTMV10 showed the capability to utilize cellobiose as a sole source of carbon. Highly diverse biochemical reactions were observed in the other biochemical tests. These biochemical results also aided in the species level identification.

It was noted that only 6 of the 80 isolates tested were non hemolytic (Table 4.1-Appendix II) - *Vibrio* sp. strain BTPT3, BTTN7, BTKB33, BTTC26, BTTN33 and BTMV10.

Table 4.1 The results of biochemical tests of the PHA producing *Vibrio* sp. from marine sediments (N= 80). (Hi-*Vibrio* identification kit, HiMedia, India)

Sl No.	CODE	VP	Arg	NaCl	ONPG	Citrate	Orni	Mann	Arab	Suc	Glu	Sal	Cell	Hem
1	BTKB32	-	+	+	-	+	+	+	+	-	+	-	-	+
2	BTKB33	-	+	+	-	+	-	-	-	-	+	-	-	-
3	BTTV19	-	+	+	-	+	+	-	-	-	+	-	-	+
4	BTTV31	-	+	+	-	+	+	-	-	-	+	-	-	+
5	BTTN22	-	+	+	-	+	-	+	+	+	+	-	-	-
6	BTSB13	-	-	+	-	+	-	+	-	+	+	-	-	+
7	BTTN4	-	+	+	-	+	-	+	+	+	+	-	-	+
8	BTTN16	-	+	+	-	+	-	+	+	+	+	-	-	+
9	BTTC7	-	+	+	-	+	-	+	+	+	+	-	-	+
10	BTTC26	-	+	+	-	+	-	+	+	+	+	-	-	-
11	BTTN3	-	+	+	-	+	-	+	+	+	+	-	-	+
12	BTTN25	-	+	+	-	+	-	+	+	+	+	-	-	+
13	BTPT3	-	+	+	-	+	+	+	+	+	+	-	-	-
14	BTTV25	-	-	+	-	+	-	+	+	+	+	-	-	+
15	BTPT5	-	+	+	-	+	+	-	+	+	+	-	-	+
16	BTTV26	-	+	+	-	+	+	+	+	-	+	-	-	+
17	BTTV30	-	-	+	-	+	-	+	+	-	+	-	-	+
18	BTTV6	-	+	+	-	+	+	+	+	-	+	-	-	+
19	BTTV22	-	-	+	-	+	-	-	-	+	+	-	-	+

Sl No.	CODE	VP	Arg	NaCl	ONPG	Citrat	Orni	Mann	Arab	Suc	Glu	Sal	Cell	Hem
20	BTTV14	-	-	+	-	+	+	+	+	-	+	-	-	+
21	BTTV21	-	-	+	-	+	-	-	-	+	+	-	-	+
22	BTPT1	-	-	+	-	+	-	+	-	+	+	-	-	+
23	BTPT2	-	+	+	-	+	-	+	-	+	+	-	-	+
24	BTTV24	-	-	+	-	+	-	+	-	+	+	-	-	+
25	BTTV4	-	+	+	-	+	-	-	+	-	+	-	-	+
26	BTTV29	-	-	+	-	-	-	-	+	+	+	-	-	+
27	BTTV10	-	+	+	-	+	-	+	+	+	+	-	+	+
28	BTTV7	-	+	+	+	+	+	+	+	+	+	-	-	+
29	BTPT4	-	-	+	-	+	+	+	+	+	+	-	-	+
30	BTED48	-	+	+	-	+	-	+	+	-	+	-	-	+
31	BTED54	-	+	+	-	+	-	-	+	-	+	-	-	+
32	BTKB25	-	-	+	-	+	-	-	+	+	+	-	-	+
33	BTED17	-	+	+	-	+	+	-	+	-	-	-	-	+
34	BTKB27	-	+	+	+	+	+	-	+	-	-	+	-	+
35	BTKB15	-	+	+	-	+	-	+	+	+	+	-	-	+
36	BTED51	-	+	+	-	+	-	+	-	-	+	-	-	+
37	BTED56	-	+	+	-	+	-	+	+	-	+	-	-	+
38	BTKB16	-	-	+	-	+	-	+	+	+	+	-	-	+
39	BTKB21	-	-	+	-	+	-	+	+	-	+	-	-	+
40	BTKB2	-	-	+	-	+	-	+	+	+	+	-	-	+
41	BTED50	-	+	+	-	+	-	-	+	-	+	-	-	+

Sl No.	CODE	VP	Arg	NaCl	ONPG	Citrat	Orni	Mann	Arab	Suc	Glu	Sal	Cell	Hem
42	BTKB4	-	+	+	-	+	-	-	+	-	+	-	-	+
43	BTKB14	-	-	+	-	+	-	-	+	-	+	-	-	+
44	BTKB5	-	+	+	-	+	-	-	+	-	+	-	-	+
45	BTED47	-	-	+	-	+	-	+	-	+	+	-	-	+
46	BTED59	-	+	+	-	+	-	+	+	-	+	-	-	+
47	BTTV40	-	+	+	+	-	+	+	+	-	+	-	-	+
48	BTKB3	-	-	+	-	+	-	+	+	+	+	-	-	+
49	BTKB31	-	+	+	-	-	-	-	-	+	+	-	-	+
50	BTED49	-	+	+	-	+	-	+	-	-	+	-	-	+
51	BTKB18	-	-	+	+	+	-	+	+	+	+	-	-	+
52	BTED58	-	-	+	-	+	-	-	-	+	+	-	-	+
53	BTKB20	-	+	+	-	+	-	-	+	+	+	-	-	+
54	BTKB23	-	-	+	-	+	-	-	+	+	+	-	-	+
55	BTED46	-	+	+	-	+	-	-	+	-	+	-	-	+
56	BTTV33	-	+	+	+	-	+	-	-	-	+	-	-	+
57	BTTR45	+	-	+	-	+	+	+	-	+	+	-	-	+
58	BTTR36	+	+	+	-	+	+	-	-	+	+	-	-	+
59	BTTN26	+	-	+	-	+	-	+	+	+	+	-	-	+
60	BTTC4	+	-	+	-	+	-	+	+	+	+	-	-	+
61	BTTC10	+	+	+	-	+	-	+	+	+	+	-	-	+
62	BTTC12	-	-	+	-	+	-	+	+	+	+	-	-	+
63	BTTC1	-	-	+	-	+	-	+	+	+	+	-	-	+

Sl No.	CODE	VP	Arg	NaCl	ONPG	Citrat	Orni	Mann	Arab	Suc	Glu	Sal	Cell	Hem
64	BTTN7	-	-	+	-	+	-	+	+	+	+	-	-	-
65	BTTN18	-	-	+	-	+	-	+	+	+	+	-	-	+
66	BTSB12	-	-	+	-	+	-	-	-	+	+	-	-	+
67	BTTR46	-	-	+	-	+	+	-	-	+	+	-	-	+
68	BTTR50	-	-	+	-	+	-	+	-	+	+	-	-	+
69	BTTR39	-	-	+	-	+	-	+	-	+	+	-	-	+
70	BTTR40	-	-	+	-	+	-	+	-	+	+	-	-	+
71	BTTC27	-	-	+	-	+	-	+	-	+	+	-	-	+
72	BTSB4	-	-	+	-	+	-	+	-	+	+	-	-	+
73	BTMV2	-	+	+	+	+	-	+	+	+	+	-	-	+
74	BTMV3	-	-	+	-	+	-	+	+	+	+	-	-	+
75	BTMV5	-	+	+	-	+	-	-	+	-	+	-	-	+
76	BTMV8	-	-	+	-	+	-	+	+	+	+	-	-	+
77	BTMV9	+	-	+	-	+	-	+	+	+	+	-	-	+
78	BTMV10	-	+	+	-	+	+	+	+	+	+	+	+	-
79	BTMV15	-	-	+	-	+	-	-	-	-	+	-	-	+
80	BTMV7	-	-	+	-	+	-	-	+	-	+	-	-	+

+ = positive; - = negative; VP = Voges-Proskauer test; Arg = Arginine dihydrolase test; NaCl = 1% salt tolerance test; ONPG = ONPG test; Citrat = Citrate test; Orni = ornithine utilization test; Mann = mannitol utilization test; Arab = arabinose utilization test; Suc = sucrose utilization test; Glu = glucose utilization test; Sal = salicin utilization test; Cell = cellobiose utilisation test; Hem = Hemolytic test.

4.3.2 Extracellular enzyme profile

The PHA accumulators were screened for 10 different hydrolytic enzymes by plate assay. Fig. 4.2. shows the amylolytic, lipolytic and caseinolytic activity of PHA accumulating vibrios.

A B C

Fig. 4.2 The exoenzyme activity of strains of PHA accumulating *Vibrio* sp. by plate assay method. (A) amylolytic (B) lipolytic (C) caseinolytic activity

Extracellular enzyme profile of selected PHA accumulating *Vibrio* sp. isolated from marine benthic samples is graphically represented in Fig. 4.3. The exoenzyme profiling showed that PHAs accumulating *Vibrio* sp. isolated from marine sediments were produced enzymes like lipase (93.75%), amylase (88.75%), DNase (88.75%), gelatinase (87.5%) and caseinase (65%) (Table 4.2: Appendix II). A few isolates also produced extracellular pectinase and cellulase.

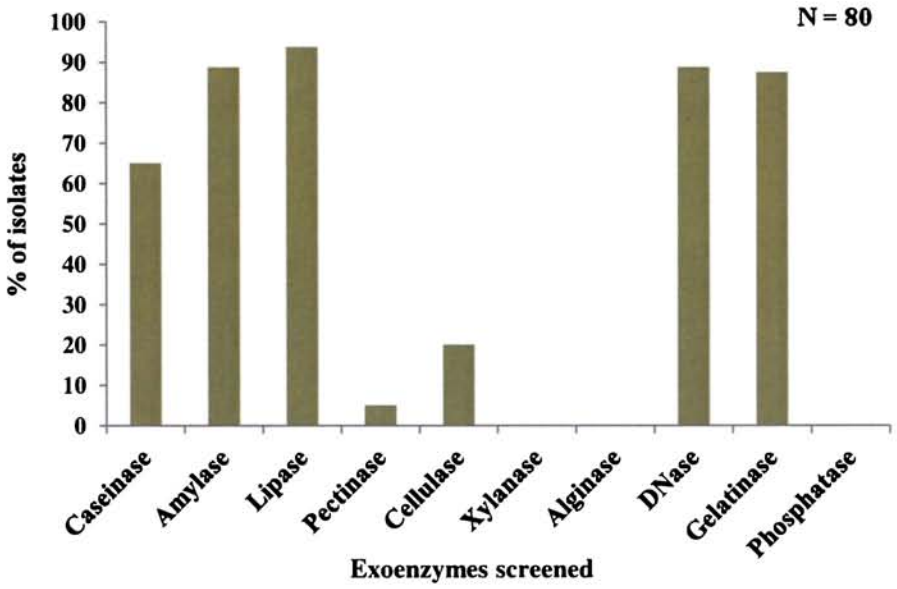


Fig. 4.3 Extracellular enzyme profile of PHA accumulating *Vibrio* sp. isolated from marine benthic samples (N=80).

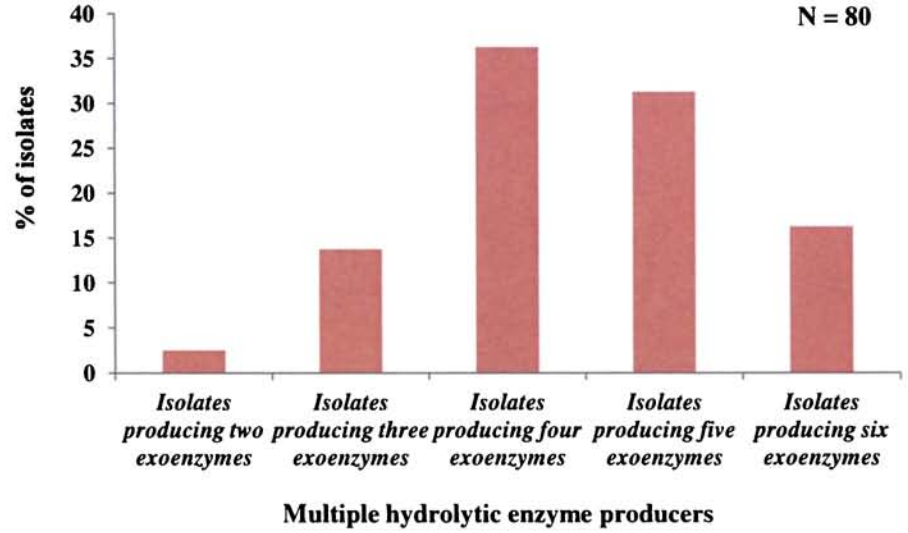


Fig. 4.4 Percentage of PHA producing *Vibrio* sp. with multiple hydrolytic enzyme activity (N=80).

It was interesting that all the selected isolates (N=80) of PHA producing *Vibrio* sp. had multiple exoenzyme producing capability i.e. ability to produce more than two hydrolytic enzymes (Fig. 4.4). In the current study, 29 isolates (36%) produced four exoenzymes out of the ten tested while 13 isolates (16%) were found to produce six hydrolytic enzymes. 25 isolates (31%) produced five exoenzymes, 11 isolates (13.75) had three exoenzyme producing capability and two isolates with two exoenzyme capability were obtained.

4.3.3 Antibiotic susceptibility test

Antibiotic resistance pattern can be used as a strain marker. The results of antibiotic susceptibility tests of PHA producing *Vibrio* sp. (N=80) are presented in Table 4.3-Appendix II.

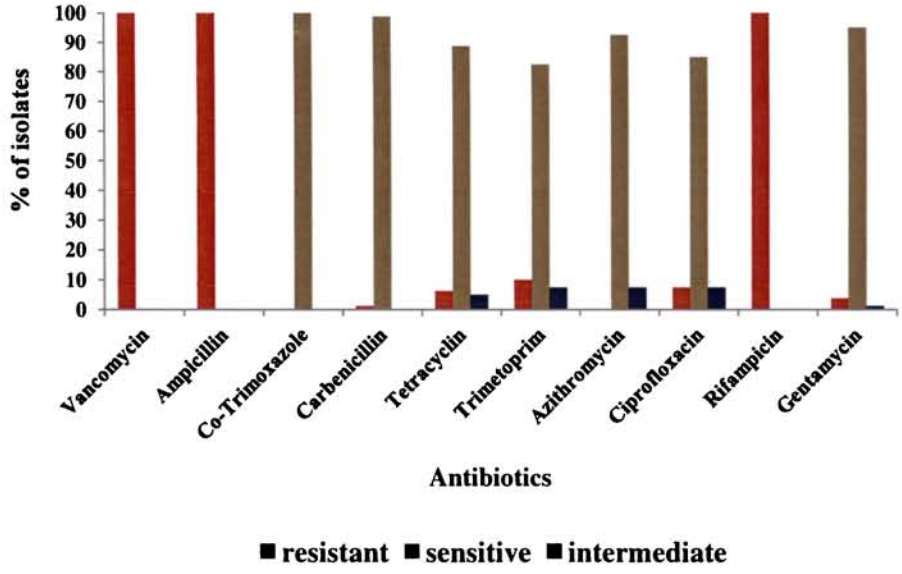


Fig. 4.5 The antibiotic susceptibility profile of PHA accumulating *Vibrio* sp. (N=80) (expressed in percentage).

The percentage resistance/ sensitivity of all the selected PHA accumulated *Vibrio* strains against ten commonly administered antibiotics is as represented in

Fig. 4.5. It is clear from the Fig. 4.5 (and Table 4.3) that all the tested isolates (N=80) were resistant to vancomycin, ampicillin and rifampicin. However, Co-trimoxazole sensitivity was observed in all the strains (N=80) tested. Most of the isolates were sensitive towards carbenicillin (99%), trimetoprim (83%), tetracycline (89%), azithromycin (93%), ciprofloxacin (85%) and gentamycin (95%). Some of the tested PHA accumulating vibrios showed intermediate resistance against tetracycline, trimetoprim, azithromycin and ciprofloxacin.

4.3.3.1 Multiple antibiotic resistance (MAR) indexing

MAR Index was calculated from the results of antibiotic susceptibility tests, and the MAR Index pattern observed in PHA accumulating *Vibrio* sp. is represented in Fig. 4.6. In the present study, a MAR Index ≥ 0.3 was obtained for all the tested isolates (N=80). Higher MAR indices (≥ 0.2) are indicative of the stressful environments encountered by microbes in the marine environments. MAR Index of 83.75% of the tested isolates was 0.3, while 11.25% isolates showed a MAR index of 0.4.

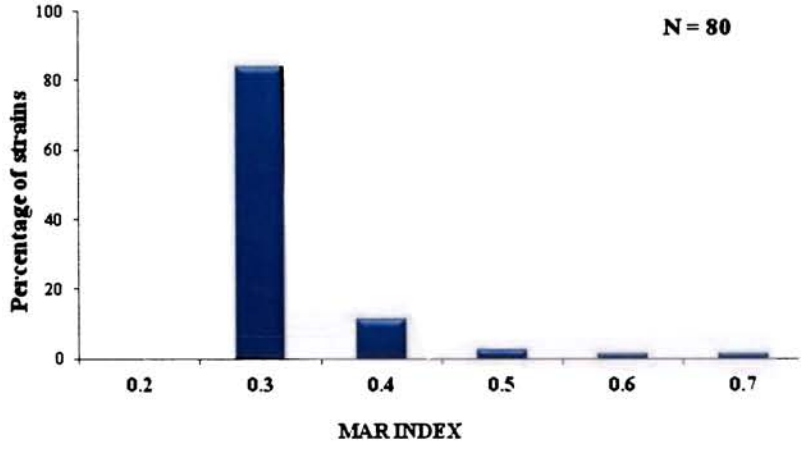


Fig. 4.6 The MAR Index pattern of PHA accumulated *Vibrio* sp. isolated from marine benthic environments (N=80).

4.4 Secondary screening for polyhydroxyalkanoates (PHAs) accumulating *Vibrio* sp.

The secondary screening for PHA accumulating *Vibrio* sp. involved spectrophotometry, where the isolates (N=80) were quantitatively analyzed for PHA accumulation (g/l) (Law and Spleckey, 1961). Their cell dry weight (CDW) was determined gravimetrically and PHA content (% of CDW) was calculated from the values of PHA accumulation and CDW measurements. 24 strains which accumulated more than 0.04 g/l of PHAs were segregated after the secondary screening, and represented in Table 4.4. These strains were selected as potential PHA producers for further genotypic characterization studies. Among these strains, *Vibrio* sp. strain BTKB33 showed maximum PHA production of 0.21 g/l and PHA content of 19.3% (% of CDW). The other strain showing PHA production of 0.2 g/l was *Vibrio* sp. strain BTTC26.

4.5. 16S rDNA sequence analysis

The 24 strains of *Vibrio* sp. producing ≥0.04 g/l of PHA were identified up to species level by partial 16S rDNA sequence analysis. After 16S rDNA sequencing, the sequences were submitted to GenBank database. Using BLAST (Altschul *et al.,* 1990), the sequences were searched against the GenBank database. The GenBank accession numbers and the species identity of these 24 strains are as detailed in Table 4.5. The strains of PHA accumulating *Vibrio* sp. were identified as *V. parahaemolyticus* (N=7), *V. alginolyticus* (N=7), *V. azureus* (N=6), and *V. harveyi* strains (N=4).

Table 4.4 Secondary screening of PHA accumulating *Vibrio* sp. from marine sediment samples (N=24) in terms of PHA accumulation (g/l), CDW (g/l) and PHA content (%).

Sl. no.	CODE	PHAs concentration[a] (g/l)	Cell dry weight[b] (g/l)	PHA content %
1	BTKB32	0.13 ± 0.01	1.07 ± 0.15	12.46
2	BTKB33	0.21 ± 0.07	1.07 ± 0.12	19.30
3	BTTN22	0.07 ± 0.01	1.43 ± 0.21	4.88
4	BTTC26	0.20 ± 0.03	1.87 ± 0.31	10.74
5	BTPT3	0.05 ± 0.03	2.10 ± 0.71	2.30
6	BTTV25	0.12 ± 0.05	2.20 ± 0.66	5.67
7	BTTV30	0.10 ± 0.04	2.37 ± 0.15	4.21
8	BTTV21	0.09 ± 0.01	1.67 ± 0.15	5.43
9	BTPT1	0.08 ± 0.01	2.63 ± 0.25	3.20
10	BTPT2	0.07 ± 0.02	2.43 ± 0.35	2.81
11	BTTV4	0.09 ± 0.03	2.37 ± 0.38	3.90
12	BTPT4	0.17 ± 0.01	1.70 ± 0.10	9.94
13	BTED48	0.17 ± 0.02	2.00 ± 0.56	8.54
14	BTED17	0.08 ± 0.02	1.43 ± 0.15	5.45
15	BTED47	0.17 ± 0.01	2.53 ± 0.40	6.78
16	BTTC10	0.17 ± 0.01	2.07 ± 0.74	8.11
17	BTTN7	0.08 ± 0.02	2.07 ± 0.61	3.94
18	BTTN18	0.10 ± 0.02	1.83 ± 0.78	5.19
19	BTSB12	0.10 ± 0.01	3.03 ± 0.60	3.18
20	BTTR39	0.16 ± 0.04	2.87 ± 0.64	5.68
21	BTTC27	0.14 ± 0.01	3.03 ± 0.40	4.54
22	BTSB4	0.16 ± 0.02	2.23 ± 0.35	7.08
23	BTMV10	0.16 ± 0.03	2.73 ± 0.32	5.75
24	BTMV15	0.11 ± 0.01	1.60 ± 0.20	6.91

[a]Calculated from spectrophotometric analysis

[b]Calculated from Gravimetric analysis

Table 4.5 Identities of PHAs accumulating strains of *Vibrio* sp. (N=24) derived by 16S rDNA sequence analysis.

Sl no	Strain no	Species identified with > 99% similarity.	GenBank accession number
1	BTKB32	*V. parahaemolyticus*	HM346670
2	BTKB33	*V. azureus*	HM346671
3	BTTN22	*V.parahaemolyticus*	HM346666
4	BTTC26	*V.azureus*	HM346664
5	BTPT3	*V.azureus*	HM346661
6	BTTV25	*V.harveyi*	HM346668
7	BTTV30	*V.alginolyticus*	HM346669
8	BTTV21	*V.parahaemolyticus*	HM346667
9	BTPT1	*V.alginolyticus*	HM030800
10	BTPT2	*V.azureus*	HM346660
11	BTTV4	*V.parahaemolyticus*	GU904007
12	BTPT4	*V.azureus*	HM346662
13	BTED48	*V.alginolyticus*	HM346657
14	BTED17	*V.parahaemolyticus*	HM346655
15	BTED47	*V.alginolyticus*	HM346656
16	BTTC10	*V.alginolyticus*	GU904005
17	BTTN7	*V.azureus*	HM346665
18	BTTN18	*V.parahaemolyticus*	GU904004
19	BTSB12	*V.parahaemolyticus*	HM346663
20	BTTR39	*V.alginolyticus*	HM030802
21	BTTC27	*V.alginolyticus*	GU904006
22	BTSB4	*V.azureus*	HM030801
23	BTMV10	*V.harveyi*	HM346658
24	BTMV15	*V.harveyi*	HM346659

4.5.1. Phylogenetic tree construction

Phylogenetic trees can tell a great deal about the interrelationship of strains in a sample. They are useful and reliable tools of molecular taxonomy. The relative position of an unknown strain in a well constructed phylogenetic tree can give a lot of information about the probable affinities of the strains and also its evolutionary progenitors.

Phylogenetic relationship based on partial 16S rDNA sequences of selected PHA accumulating strains of *Vibrio* sp. isolated from marine sediments and related taxa is represented in Fig. 4.7.

From the phylogram of PHA accumulating *Vibrio* sp. and their related taxa, it was clear that the strains were quite diverse, but grouped together as a large clade. From the tree it was observed that they grouped separately from some of the related species. Within the group, the stains BTTN22 and BTTV4 grouped together and claded separately from the rest of PHA accumulating vibrios. The strains BTED47 (*V.alginolyticus*) and BTKB33 (*V.azureus)* also showed some diversity within the large clade and stood separated from the rest of the group.

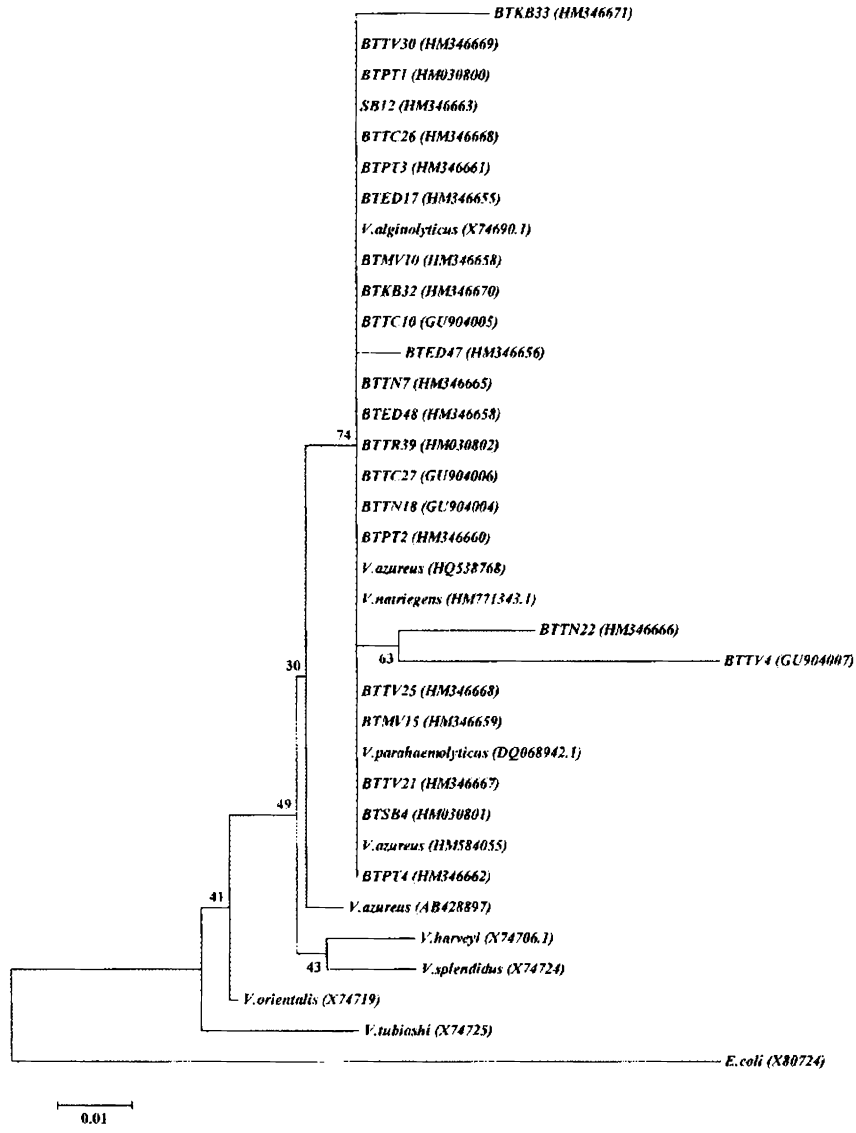


Fig. 4.7 Phylogenetic relationship based on partial 16S rDNA sequences of selected PHA accumulating strains of *Vibrio* sp. (N=24) isolated from marine sediments and related taxa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved 35 nucleotide sequences and *E.coli* (accession number X80724) used as outgroup. Accession numbers are given in parentheses.

4.5.1.1 Intraspecies variation of PHA accumulating vibrios

In order to understand the intra species variation within the PHA accumulating strains, phylogenetic tree were constructed with partial 16S rDNA sequences and are represented below.

4.5.1.1.1 Intra species variation among PHA accumulating strains of *V. alginolyticus* isolates.

Intra species variation for the seven *Vibrio alginolyticus* strains is as depicted in Fig. 4.8. The dendrogram mainly grouped into two, showing the diversity among the 7 *V.alginolyticus* strains obtained in this study. This intraspecies diversity obtained based on 16S rDNA sequence analysis is also observed in the variations in the biochemical reactions, enzyme profile and antibiotic susceptibility of these strains identified as *V.alginolyticus*. Strains BTED47, BTTC10 and BTTR39 claded together. The strains BTTV30, BTED48, BTTC27 and BTPT1 grouped together to form a major clade.

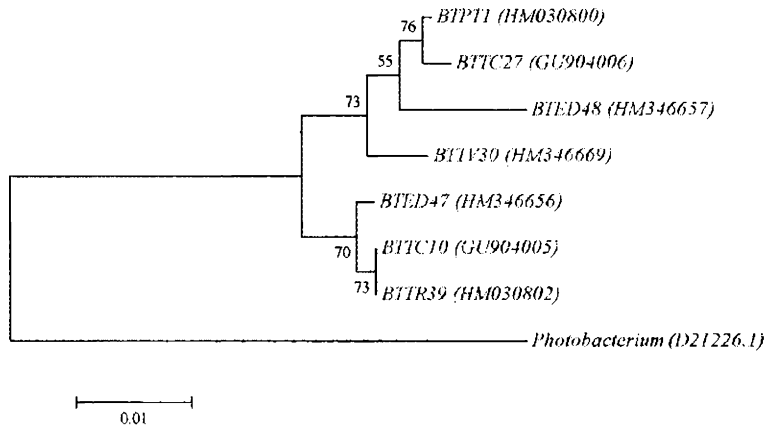


Fig. 4.8 Intra species variation among PHA accumulating strains of *Vibrio alginolyticus* isolates (N=7). The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar = 10 % sequence divergence.

4.5.1.1.2 Intra species variation among PHA accumulating strains of *V. parahemolyticus* isolates.

Intra species variation for the five *V.parahemolyticus* strains having the ability to accumulate PHAs is depicted in Fig. 4.9. All the strains together formed a major clade and showed divergence from the outgroup. But within the clade, the strains BTTN22 and BTTV4 grouped together and separate from the rest. This intraspecies diversity obtained based on 16S rDNA sequence analysis in *V.parahemolyticus* obtained in this study is also seen in the diverse patterns of their phenotypic characteristics.

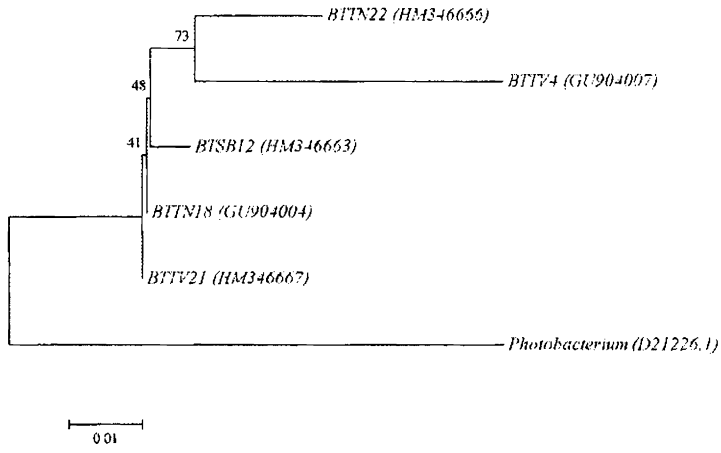


Fig. 4.9 Intra species variation among PHA accumulating strains of *Vibrio parahemolyticus* isolates (N=5). The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar = 10 % sequence divergence.

4.5.1.1.3 Intra species variation among PHA accumulating strains of *V. azureus* strains

Intra species variation for the seven *V.azureus* strains having the ability to accumulate PHAs is depicted in Fig. 4.10. In the present analysis, all the *V.azureus* strains claded together in a single group and showed higher similarity

unlike the intraspecies variation shown by the other *Vibrio* strains in this study. Within this group the strain BTKB33 claded separately from other *V. azureus* strains. The variation again only point to the diversity within *V. azureus* strains isolated from different marine benthic locations. Variation is also seen in their biochemical characteristics.

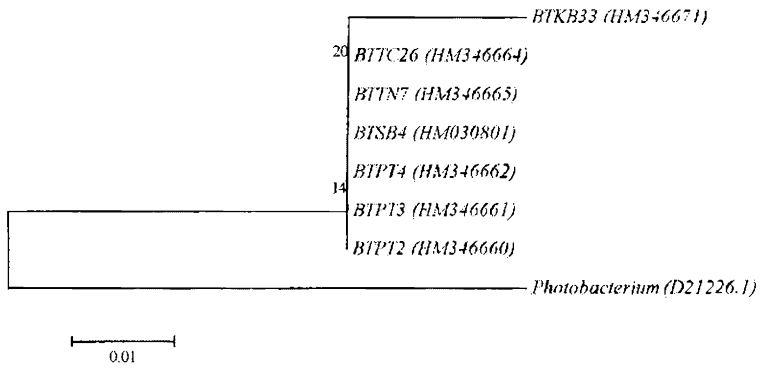


Fig. 4.10 Intra species variation among PHA accumulating strains of *Vibrio azureus* isolates (N=7). The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar = 10 % sequence divergence.

4.6 Molecular characterization of PHA synthase gene

4.6.1 Semi-nested PCR detection of Class I PHA synthase gene

The PCR based method outlined by Sheu *et al.*, 2000 for detection of Class I PHA synthase gene (scl-PHA synthase gene), gave desired amplicons of ~496bp for strain BTKB33, BTTC26, BTMV10, BTTN18 and BTTV4 and 406bp amplicon for the seminested PCR analysis of PCR products. The agarose gel electrophoresis of seminested PCR products is represented in Fig. 4.11. This helped to prove that the strains BTKB33, BTTC26, BTMV10, BTTN18 and BTTV4 harbored the Class I PHA synthase gene type. This also proved that these 5 strains of vibrios produced single chain length (scl) PHAs.

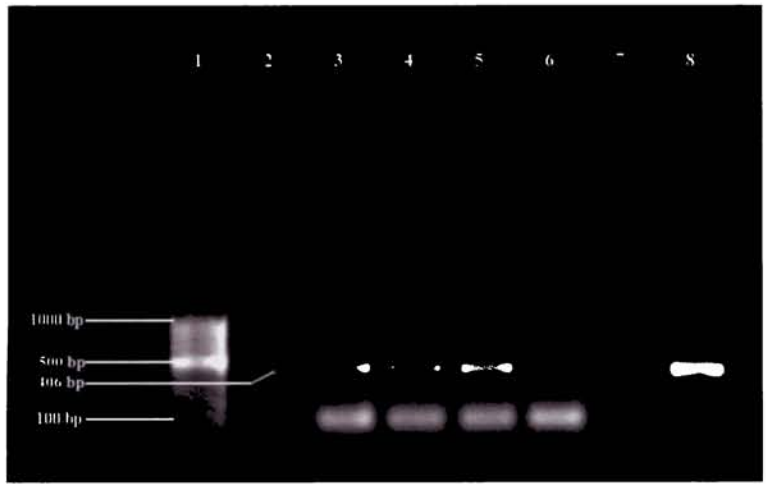


Fig. 4.11 Agarose electrophoresis for seminested PCR product of Class I PHA synthase genes (Sheu *et al.*, 2006). Lane 1 has 1kb ladder; lane 2 *Vibrio* sp. strain BTKB33; lane 3 *Vibrio* sp. strain BTTC26; lane 4 *Vibrio* sp. strain BTMV10; lane 5 has positive control for class I PHA synthase gene (seminested PCR product of *Alcaligenes eutrophus*); lane 6 has negative control for class I PHA synthase gene; lane 7 *Vibrio* sp. strain BTTN18; lane 8 *Vibrio* sp. strain BTTV4.

4.6.2 PCR detection of Class II PHA synthase gene

In the second PCR protocol used for the detection of Class II PHA synthase genes (mcl-PHA synthase gene) outlined by Solaiman *et al.*, 2000, two degenerate primers were used. None of the tested strains of PHA accumulating *Vibrio* sp. including BTKB33 gave the desired PCR product. The agarose gel electrophoresis of mcl-PHA synthase gene PCR products is as represented in Fig. 4.12.

Fig. 4.12 **Agarose electrophoresis for PCR product of Class II PHA synthase genes** (Solaiman *et al.*, 2006). Lane 1 *Vibrio* sp. strain BTKB33; lane 2 *Vibrio* sp. strain BTTN18; lane 3 *Vibrio* sp. strain BTTC26; lane 4 *Vibrio* sp. strain BTMV10; lane 5 *Vibrio* sp. strain BTTV4; lane 6 has 1kb ladder; lane 7 has positive control for class II PHA synthase gene (PCR product of *Alcaligenes eutrophus*); lane 8 has negative control

It is clear that the *Vibrio* sp. strains BTKB33, BTTC26, BTMV10, BTTN18 and BTTV4 did not harbor the Class II PHA synthase gene. It may be inferred therefore that none of the 5 strains screened were mcl PHA producers and may well accumulate only single chain length (scl) PHA. The selected production organism in this study, *Vibrio* sp. strain BTKB33 harbored Class I PHA synthase gene and may therefore accumulate scl-PHA.

4.7 The phenotypic and genotypic characteristics of production strain BTKB33

Vibrio sp. BTKB33 was selected as the production organism for further studies based on the preliminary screening employing fluorescent plate assay and followed by the secondary screening by spectrophotometric method from 513 PHA accumulating vibrios. *Vibrio* sp. strain BTKB33 showed maximum PHA production of 0.21 g/l and PHA content of 19.3% (% of CDW) under un-optimised conditions. The selected strain was considered for optimization studies

of bioprocess variables for enhancing PHA production and for characterization of the biopolymer in them.

4.7.1 Phenotypic characteristics of *Vibrio* sp. strain BTKB33

Vibrio sp. strain BTKB33 was identified using phenotypic and genotypic approaches as described previously under section 4.3 and 4.5. The phenotypic characteristics of the strain BTKB33 are represented in Table 4.6. Strain BTKB33 also produced biotechnologically important hydrolytic exoenzymes like caseinase, amylase, gelatinase and DNase. This isolate showed MAR index of 0.4, with resistance towards vancomycin, rifampicin, tetracycline and ampicillin. *Vibrio* sp strain BTKB33 non-hemolytic on blood agar.

Table 4.6 Phenotypic characteristics of *Vibrio* sp. strain BTKB33

Traits	Results	Traits	Results
Colony colour on TCBS	Green	Pectinase	-
Oxidase test	+	Cellulase	-
MOF test	Fermentative	Xylanase	-
Voges Proskaur's test	-	Alginase	-
Arginine	+	DNase	+
Salt tolerance	+	Gelatinase	+
ONPG	-	Phosphatase	-
Citrate	+	Vancomycin	R
Ornithine	-	Ampicillin	R
Mannitol	-	Co-Trimoxazole	S
Arabinose	-	Carbenicillin	S
Sucrose	-	Tetracyclin	R
Glucose	+	Trimetroprim	S
Salicin	-	Azithromycin	S
Cellobiose	-	Ciprofloxacin	S
Haemolytic screening	-	Rifampicin	R
Caseinase	+	Gentamycin	S
Amylase	+	MAR Index	0.4
Lipase	-		

+ = Positive; - = Negative; R = Resistant; S = Sensitive

4.7.2 Genotypic characteristics of *Vibrio* sp. strain BTKB33

The 16SrDNA sequence analysis and biochemical tests helped to identify strain BTKB33. On comparing the partial sequences of 16S rDNA with those in NCBI database, the strain BTKB33 showed maximum identity (99% identity) with *V. azureus* strain VPMP45 (Accession no. 663915.1). The phenotypic characterisation results also confirmed the species level identification of strain BTKB33 as *V. azureus* (Yoshizawa *et al.*, 2009). The nucleotide sequence was deposited in the GenBank database under accession number HM346671. The phylogeny based on partial 16S rDNA sequence of *Vibrio* sp. BTKB33 and related *Vibrio* sp. is shown in Fig. 4.13. In the tree, the strain BTKB33 grouped with the *V.azureus* strains to form a major clade. Within the clade, the strain grouped with *V. azureus* strain VPMP45 and showed divergence from the rest. This is the first report of PHA accumulation in *V.azureus*.

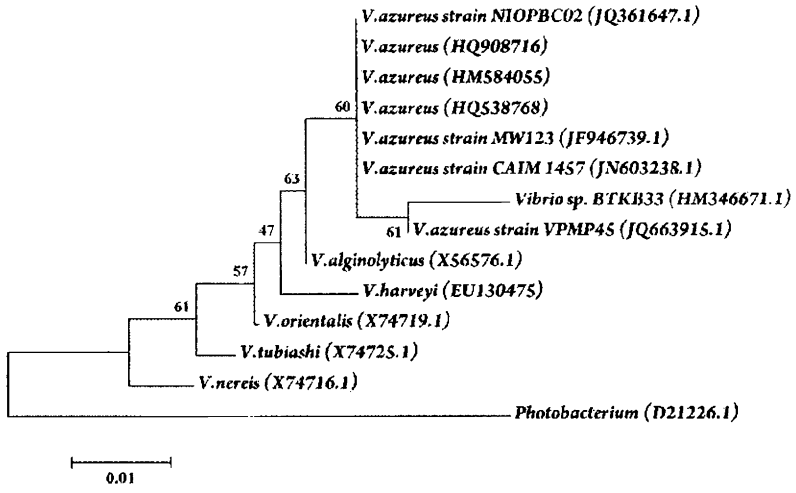


Fig. 4.13 Phylogenetic relationship based on partial 16SrDNA sequences of *Vibrio* sp. strain BTKB33 with related taxa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The *Photobacterium* (accession number D21226.1) used as outgroup. Accession numbers are given in parentheses.

4.7.3 Screening for virulence genes in *Vibrio* sp. strain BTKB33

Vibrio sp. strain BTKB33 was screened for a few common virulence genes seen in vibrios like *ctxB*, *toxR* and *zot* genes using PCR method in order to check its pathogenicity. Fig. 4.14 represents the results of the PCR-based screening method for various virulence gene in *Vibrio* sp. strain BTKB33. From the results it was clear that *Vibrio* sp. strain BTKB33 does not harboring any of the virulent genes tested. The positive control strain used in this study was *Vibrio cholerae* CO366 *Eltor* strain which harbored all the virulence genes tested. The absence of major virulence genes tested, in strain BTKB33, supports its usage as an industrial organism in industrial production of PHAs.

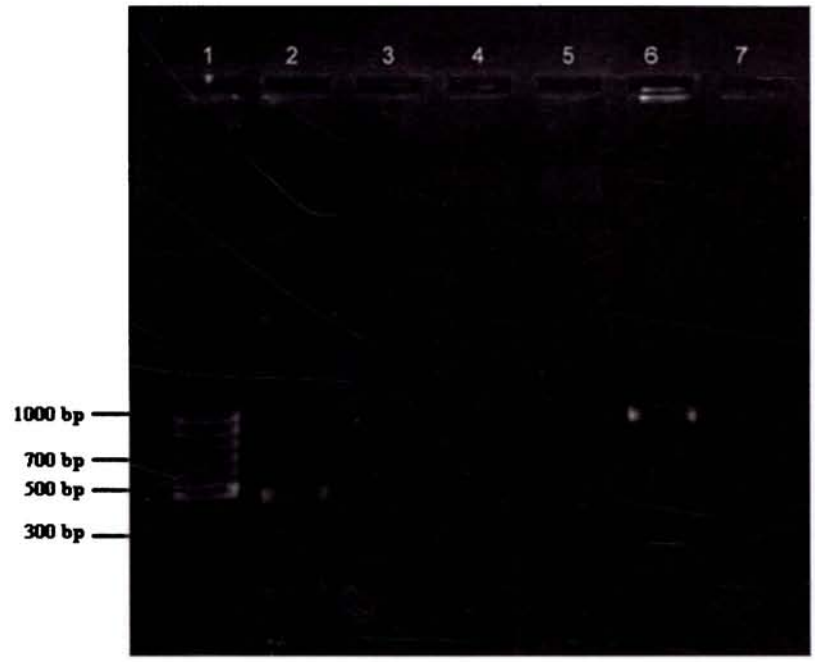


Fig. 4.14 Screening for virulence gene in *Vibrio* sp. strain BTKB33 using PCR. Lane 1 has 500bp DNA ladder; lane 2 has positive control for *ctxA* gene (*Vibrio cholerae* CO366 Eltor strain ; lane 3 has PCR product of *Vibrio* sp. strain BTKB33for *ctxA* gene; lane 4 has positive control for *toxR* gene (*Vibrio cholerae* CO366 Eltor strain ; lane 5 has PCR product of *Vibrio* sp. strain BTKB33for *toxR* gene; lane 6 has positive control for *zot* gene (*Vibrio cholerae* CO366 Eltor strain ; lane 7 has PCR product of *Vibrio* sp. strain BTKB33for *zot* gene.

4.7.4 Growth curve of *Vibrio* sp. strain BTKB33

A growth curve was prepared by measuring cell growth by checking the O.D. of cell culture at 660nm and plotting against the time of incubation and is represented in Fig. 4.15, and it was clear that the organism had a very short generation time. The log phase (exponential phase) started by 3rd hour of incubation and lasted upto 15 hours. The generation time was calculated as 8.61 minutes and the specific growth rate as 0.001465. The short generation time is one of the most important criterion in its selection as a production organism.

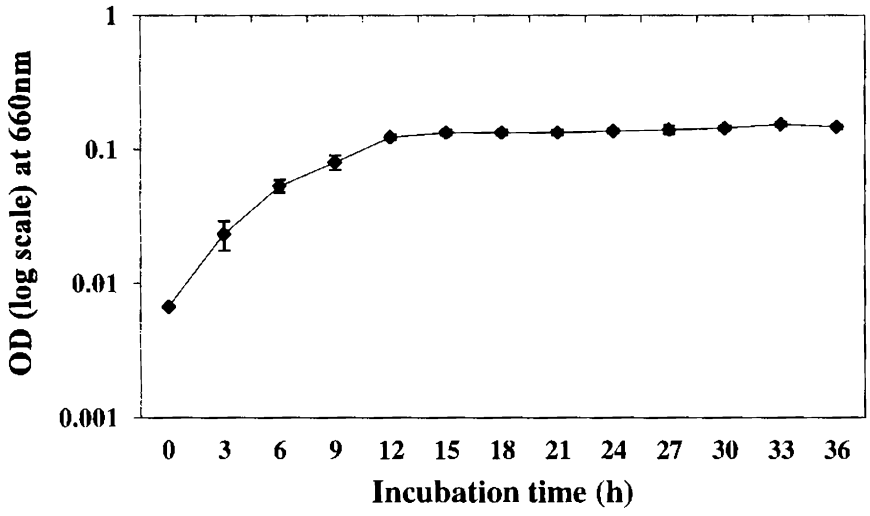


Fig. 4.15 Growth curve of production organism- *Vibrio* sp. strain BTKB33.
Data points are arithmetic means of triplicates, while error bars denote the standard deviation of the mean.

4.7.5 Phase contrast microscopy

Phase contrast micrograph of *Vibrio* sp. BTKB33 represented in Fig. 4.16, highlighted the brightly refractile cytoplasmatic inclusions.This is indicative of PHAs accumulation in this organism (Lopez-Cortez *et al.*, 2008).

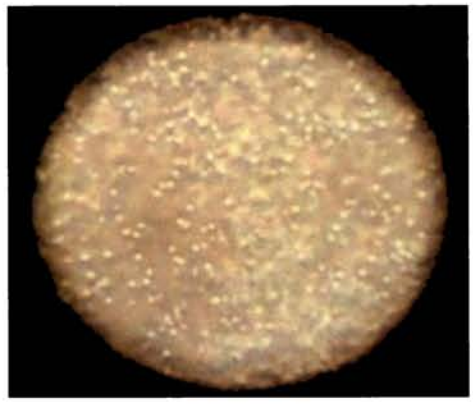


Fig. 4.16 Phase contrast microscopic image of the brightly refractile cytoplasmic inclusions in *Vibrio* sp. strain BTKB33.

4.7.6 Sudan Black staining

The micrograph of Sudan Black stained cells of *Vibrio* sp. strain BTKB33 under bright field compound microscope (100X magnification) is represented in Fig. 4.17. Bacteria with lipophilic inclusions like PHAs are stained by Sudan Black stain and the cell wall by saffranine. The lipophilic staining by Sudan Black was used to identify PHA under bright field compound microscope.

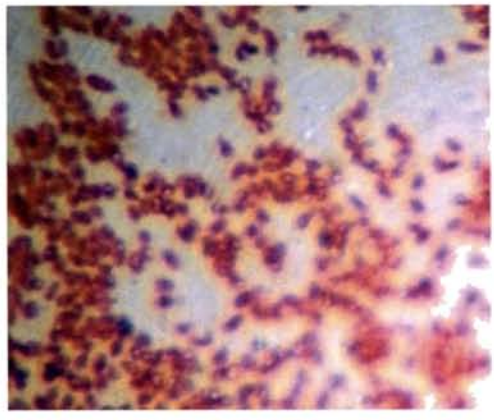


Fig. 4.17. Micrograph of Sudan Black stained cells of *Vibrio* sp. strain BTKB33 under bright field compound microscope (100X magnification)

4.7.7 *In-silico* analysis of the partial sequence *of phaC* gene

The seminested PCR for *Vibrio* sp. strain BTKB33 gave a ~406 bp product, which on sequencing gave a partial sequence of length 231 bp (Table 4.7). This sequence was compared with those in the GenBank database using BLAST (Altschul *et al.*, 1990) and it was found to have 98 % identity with the *phaC* gene of *V.parahemolyticus* strain K5030 (Accession no. ACKB01000050.1).

This BLAST analysis confirmed that the partial gene sequence obtained was indeed that encoding Class I PHA synthase and the sequence was submitted in GenBank under accession number JQ781052 as *Vibrio* sp. strain BTKB33 PHA synthase Class I family gene, partial sequence.

In addition, the partial sequence was translated into its amino acid sequence (Table 4.8.) and aligned using PSI-BLAST algorithm. An ORF that coded for 74 residues was obtained, with maximum E value of 6e-43 and 93% identity with the amino acid sequence of the *phaC* gene of *Vibrio alginolyticus* strain 40B (Accession no. NZ ACZB01000148.1). The lineage report for the partial PHA synthase gene of *Vibrio* sp. BTKB33 is presented in Table 4.9

The deduced amino acid sequence alignment of partial polyhydroxyalkanoic acid synthase, class I from *Vibrio* sp. strain BTKB33 is represented in Fig. 4.18. The aminoacid sequence of the *Vibrio* sp. in the alignment showed similarity with each other, when compared by the multiple sequence alignment. These results showed the similarity of *phaC* gene product within the genus *Vibrio* and their divergence from that in other bacteria, included in the study. The deduced amino acid sequence of PHA synthase showed some intra-generic variation within genus *Vibrio*. On the analysis of the multiple sequence alignment, it was observed that the translated *phaC* gene of strain BTKB33 had serine instead of alanine in the 6^{th} position. Likewise, there was cysteine instead of tyrosine, in the 22^{nd} and leucine instead of alanine in the 34^{th} position of PHA synthase of *Vibrio* sp. BTKB33. Red arrow marks these positions in the Fig. 4.18.

A dendrogram was constructed to study the relationship of amino acid sequence of Class I *phaC* gene of *Vibrio* sp. BTKB33 with other related bacteria and is represented in Fig 4.19. From the tree based on the amino acid sequences of *phaC* genes, it was observed that all the *Vibrio* strains claded separately from the rest of the bacteria included in the study. Within the clade, *Vibrio* sp. BTKB33 showed divergence from other *Vibrio* sp. included in this study. This divergence may be due to the amino acid variations revealed in the multiple sequence alignment as described above. In the present analysis, amino acid sequence of *phaC* gene of *Paracoccus denitrificans* was used as an outgroup.

Conserved Domain Database is a protein annotation resource that consists of a collection of well-annotated multiple sequence alignment models for ancient domains and full-length proteins. CD-Search is NCBI's interface to searching the Conserved Domain Database with protein query sequences. It uses RPS-BLAST, a variant of PSI-BLAST, to quickly scan a set of pre-calculated position-specific scoring matrices (PSSMs) with a protein query. The protein query sequence of *phaC* gene of strain BTKB33 was then compared using the putative conserved domains search service (RPS-BLAST) to find conserved domains. It was observed that the protein sequence showed maximum resemblance with conserved domain model TIGR01838, which represented the class I subfamily of PHA synthase, with an E value of 2.18e-37. The conserved domain model TIGR01838 represents the class I subfamily of poly(R)-hydroxyalkanoate synthases, which polymerizes hydroxyacyl-CoAs with three to five carbons in the hydroxyacyl backbone into aliphatic esters termed poly(R)-hydroxyalkanoic acids.

This result is highly relevant, considering that this sequence (JQ781052) of *phaC* is the first deposit in GenBank from the *Vibrio azureus* species of genus *Vibrio* as the review of the description of the *V.azureus* (Yoshizawa *et al.*, 2009) has not included PHA production by *V.azureus*. The outcome of this study allowed us to determine that the partial *phaC* gene sequence of *Vibrio* sp. strain

BTKB33 was that of a PHA synthase class I, specifically that of a poly-β-hydroxybutyrate polymerase.

Table 4.7 The 231 bp sequence obtained for Class I PHA synthase gene of *Vibrio* sp. BTKB33 (GenBank Accession number JQ781052)

5'AGTGAACGACCATCCATGTAACCTTTTGCGTTGTTCTGCGTCTCGATA
GCGCTAATGATTGTGTCGTTAATGTACGCGCCGACTTCGCCCGGTTGAG
AGAAATCCAAAAGCGTGGTGAAGAACGTCGCCAATTTGATGCGCTTCT
TCATACGTTTTGCTGCATAGCACGCAACAGTGCTCGCTAAAACCGTAC
CACCAATGCAATAGCCTGCCGAGTTGATTTGCTCTTGC 3'

Table 4.8 The deduced aminoacid sequence of Class I PHA synthase gene of *Vibrio* sp. BTKB33. Nucleic acid to protein translation was done using http://web.expasy.org/translate. (76 amino acids)

QEQINSAGYCIGGTVLASTVACYAAKRMKKRIKLATFFTTLLDFSQPGEV
GAYINDTIISAIETQNNAKGYMDGRS

Table 4.9 The lineage report for the partial PHA synthase gene of *Vibrio* sp. BTKB33

Lineage Report
root
. Bacteria [bacteria]
. . *Proteobacteria* [proteobacteria]
. . . *Gammaproteobacteria* [g-proteobacteria]
. . . . *Vibrionales* [g-proteobacteria]
. . . . . *Vibrionaceae* [g-proteobacteria]
. . . . . *Vibrio* [g-proteobacteria]
. . . . . *Vibrio harveyi* group [g-proteobacteria]
. . . . . *Vibrio alginolyticus* [g-proteobacteria]
. . . . . *Vibrio alginolyticus* 40B ---------- 152 2 hits [g-proteobacteria] polyhydroxyalkanoic
. . . . . *Vibrio alginolyticus* 12G01 ..... 152 2 hits [g-proteobacteria] polyhydroxyalkanoic acid
. . . . . *Vibrio harveyi* 1DA3 ---------- 155 2 hits [g-proteobacteria] polyhydroxyalkanoic
. . . . . *Vibrio parahaemolyticus* AQ3810 ..... 155 2 hits [g-proteobacteria] polyhydroxyalkanoic
. . . . . *Vibrio parahaemolyticus* AQ4037 ..... 155 2 hits [g-proteobacteria] poly(R)-
. . . . . *Vibrio parahaemolyticus* RIMD 2210633 ..... 154 2 hits [g-proteobacteria]
. . . . . *Vibrio parahaemolyticus* K5030 ..... 154 2 hits [g-proteobacteria] polyhydroxyalkanoic
. . . . . *Vibrio parahaemolyticus* AN-5034 ..... 154 2 hits [g-proteobacteria] polyhydroxyalkanoic
. . . . . *Vibrio parahaemolyticus* Peru-466 ..... 154 2 hits [g-proteobacteria] polyhydroxyalkanoic
. . . . . *Vibrio parahaemolyticus* 10329 ..... 154 1 hit [g-proteobacteria] polyhydroxyalkanoic
. . . . . *Vibrio harveyi* ATCC BAA-1116 ..... 154 2 hits [g-proteobacteria] hypothetical protein
. . . . . *Vibrio parahaemolyticus* ..... 153 1 hit [g-proteobacteria] polyhydroxyalkanoic acid
. . . . . *Vibrio rotiferianus* DAT722 ..... 153 1 hit [g-proteobacteria] polyhydroxyalkanoic acid
. . . . . *Vibrio harveyi* HY01 ..... 153 2 hits [g-proteobacteria] poly-beta-hydroxybutyrate
. . . . . *Vibrio parahaemolyticus* 16 ..... 142 2 hits [g-proteobacteria] poly(3-hydroxyalkanoate)
. . . . . *Vibrio* sp. Ex25 ---------- 152 4 hits [g-proteobacteria] poly(R)-

..... *Vibrio* sp. AND4 ..... 152 2 hits [g-proteobacteria] polyhydroxyalkanoic acid
..... *Vibrio coralliilyticus* ATCC BAA-450 ..... 151 2 hits [g-proteobacteria]
..... *Vibrio furnissii* CIP 102972 ..... 150 2 hits [g-proteobacteria] polyhydroxyalkanoic acid
..... *Vibrio furnissii* NCTC 11218 ..... 150 2 hits [g-proteobacteria] polyhydroxyalkanoic
..... *Vibrio brasiliensis* LMG 20546 ..... 149 2 hits [g-proteobacteria] polyhydroxyalkanoic
..... *Vibrio* sp. EJY3 ..... 149 2 hits [g-proteobacteria] unnamed protein product
..... *Vibrio* sp. RC586 ..... 149 2 hits [g-proteobacteria] polyhydroxyalkanoic acid
..... *Vibrio mimicus* VM223 ..... 149 2 hits [g-proteobacteria] polyhydroxyalkanoic acid

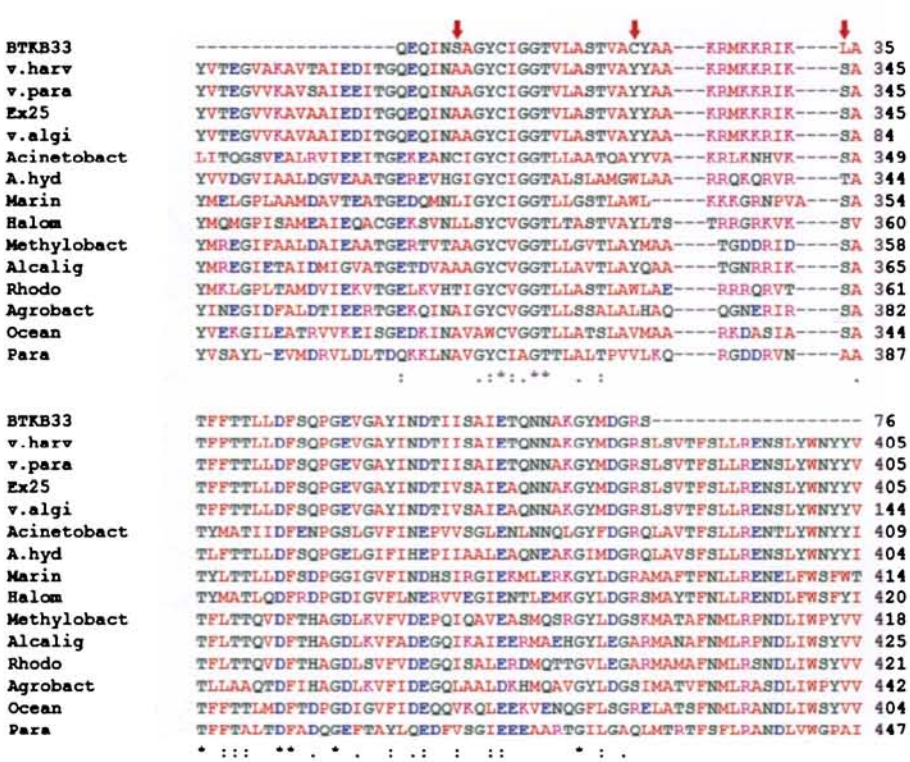


Fig. 4.18 Amino acid sequence alignment of partial poly-(R)-hydroxyalkanoic acid synthase (phaC), class I from *Vibrio* sp. strain BTKB33.

The protein sequences was aligned using Clustal W programme (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

BTKB33 = *Vibrio* sp. strain BTKB33; v . harv = *V.harveyi*; v.para = *V. parahemolyticus*; Ex25= *Vibrio* sp. Ex 25; v.algi = *V. alginolyticus* ; Acinetobact = Acinetobacter sp.; A. hyd = *Aeromonas hydrophila*; Marin = *Marinobacter manganoxydans*; Halom = *Halomonas* sp.; Methylobact = *Methylobacterium nodulans*; Alcalig = *Alcaligenes eutrophus*; Rhodo = *Rhodopseudomonas palustris*; Agrobact = *Agrobacterium tumefacians*; Ocean = *Oceanospirullum* sp.; Para = *Paracoccus denitrificans*.

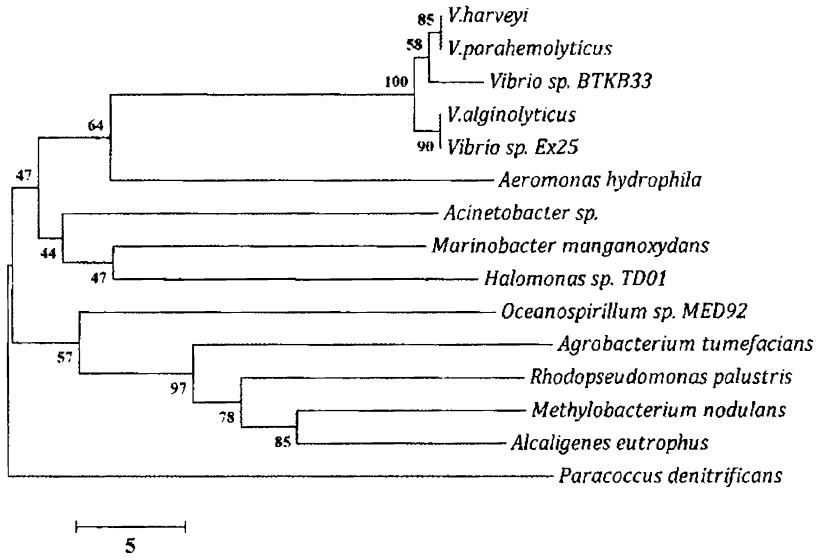


Fig. 4.19 Phylogenetic tree showing diversity of aminoacid sequences of *phaC gene* of *Vibrio* sp. BTKB33 with other bacteria. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987) The bootstrap consensus tree inferred from 1000 replicates (Felsenstein J, 1985) is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein J, 1985). The evolutionary distances were computed using the method (Nei and Kumar, 2000) and are in the units of the number of amino acid differences per sequence. The analysis involved 15 amino acid sequences. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2007).

4.8 Submerged fermentation (SmF) for the production of polyhydroxyalkanoates by *Vibrio* sp. strain BTKB33

4.8.1 Optimization of bioprocess variables for PHA production by *Vibrio* sp. strain BTKB33 by "one-factor-at-a-time" method.

4.8.1.1 Effect of incubation time

The results of the studies on optimization of incubation period for PHA accumulation in *Vibrio* sp. strain BTKB33 is presented in Fig 4.20. It was observed that PHA accumulation increased with increase in incubation period upto 36h, while the PHA production gradually decreased. The maximum PHA accumulation of 0.21 g/l was obtained at 36h of incubation. The CDW

measurement was also found to increase with increase in incubation period upto 42h and then decreased. The optimum incubation time for obtaining maximum CDW was found to be at 42h incubation (0.9 g/l). The PHA content was maximum at 12h incubation (29.5 % of CDW) and drastically decreased with further increase in incubation time.

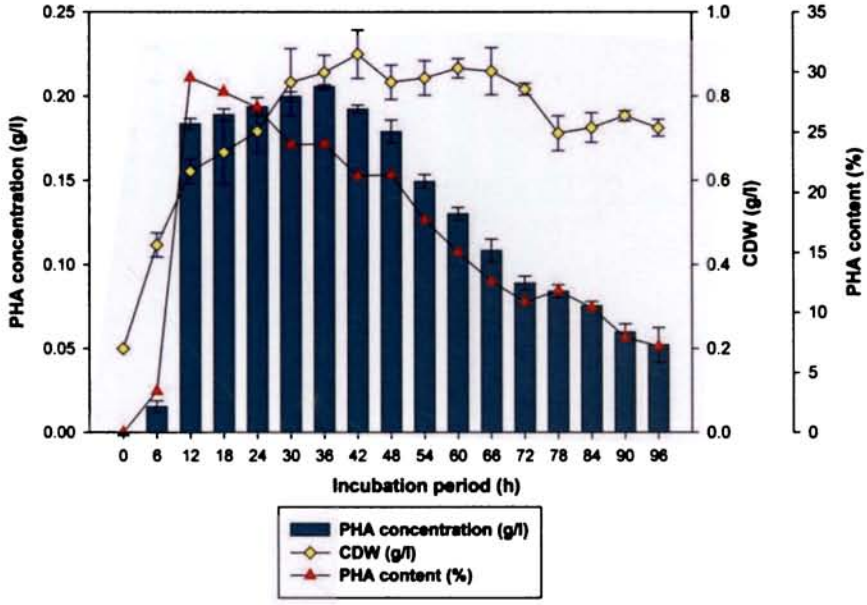


Fig. 4.20 Optimization of incubation period for PHA accumulation, PHA content and CDW by *Vibrio* sp. strain BTKB33 under submerged fermentation. Data points are arithmetic means of triplicates, while error bars denote the standard deviation of the mean.

4.8.1.2 Effect of initial pH

Studies conducted for the optimization of initial pH of the medium on PHA production in *Vibrio* sp. strain BTKB33 is indicated in Fig. 4.21. The bacterium could produce PHA over a broad pH range of 6 to 9. The maximum PHA production was at pH 7 (0.22 g/l). Little PHA or no production was observed at pH lower than 6 and above pH 9. It was clear from the results that the organism favored alkaline pH for PHA production. Like the PHA production, the bacterium showed maximum cell dry weight measurement and PHA content at pH 7 (1.13 g/l

and 19.3 % of CDW respectively). The effect of initial pH of the medium on PHA production, CDW, and PHA content of the bacterium all showed a similar pattern.

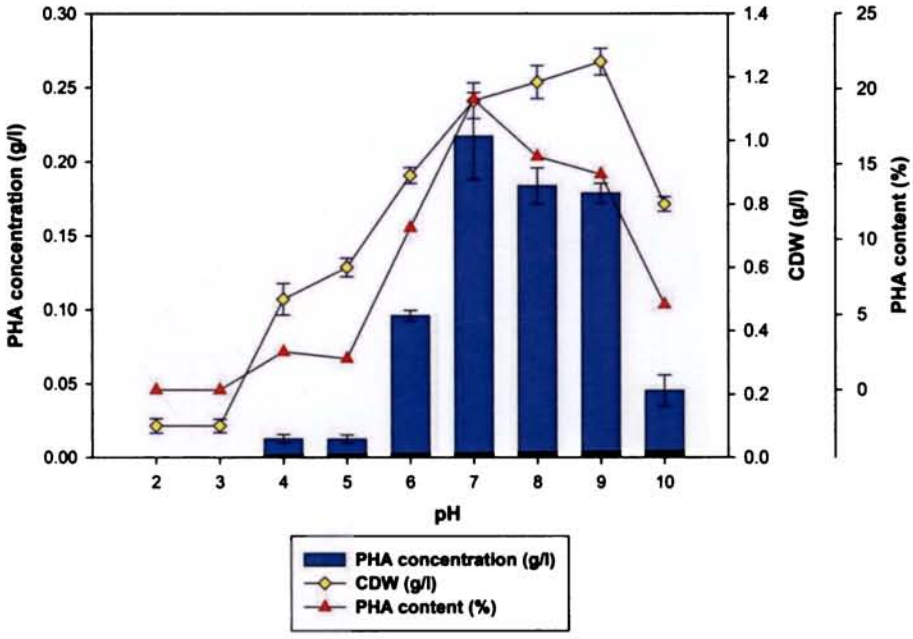


Fig. 4.21 Optimization of initial pH for PHA accumulation, PHA content and CDW by *Vibrio* sp. strain BTKB33. Data points are arithmetic means of triplicates, while error bars denote the standard deviation of the mean.

4.8.1.3 Effect of sodium chloride concentration

The PHA concentration (g/l) and PHA content (% of CDW) increased upto 1.5 % of NaCl concentration in the medium and was 0.23 g/l and 18% of CDW respectively (Fig. 4.22). The cell dry weight measurement was highest at 2 % NaCl concentration (1.3 g/l). Increase in NaCl concentration above 1.5% resulted in lower accumulation of PHA in the bacterium and PHA production decreased with further increase in NaCl concentration. However, PHA content of the bacterium showed almost similar values upto 5 % NaCl concentration and thereafter decreased drastically with further increase in NaCl concentration.

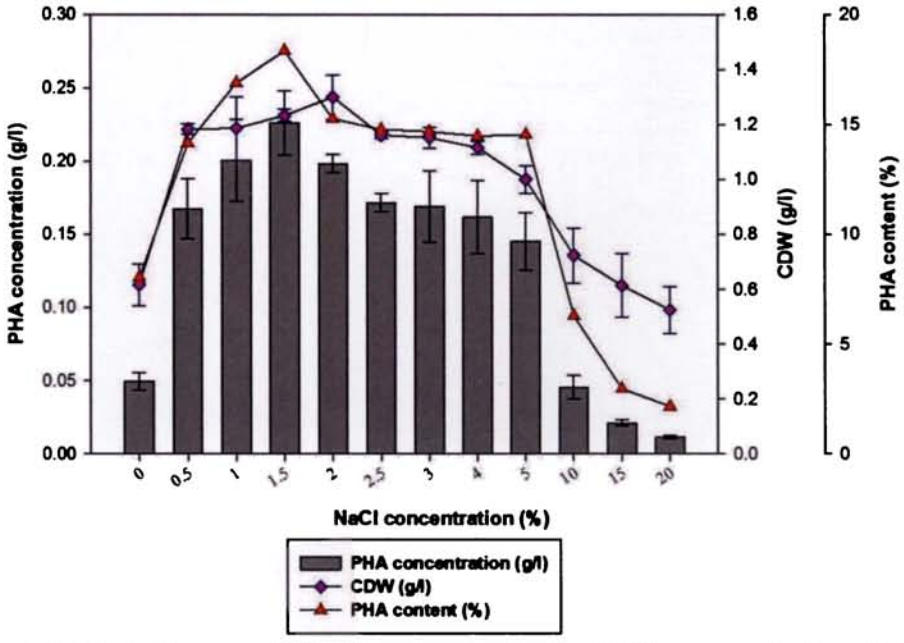


Fig. 4.22 Optimization of NaCl concentration for PHA accumulation, PHA content and cell dry weight by *Vibrio* sp. strain BTKB33. Data points are arithmetic means of triplicates, while error bars denote the standard deviation of the mean.

4.8.1.4 Effect of agitation

It was evident from the results presented in Fig. 4.23., that agitation is a very important factor for enhanced production of PHA in *Vibrio* sp. strain BTKB33, since there was only a little PHA production in static culture condition. It was evident that maximum PHA production and PHA content was at 120 rpm (0.24 g/l and 18 % of CDW respectively). The PHA production by the bacterium was almost similar in all the other agitation rates except at stationary phase. However, PHA content of the bacterium did not vary much with variation in agitation rate. The CDW measurement increased with increase in agitation speed and maximum CDW measurement was 1.47 g/l at 150 rpm.

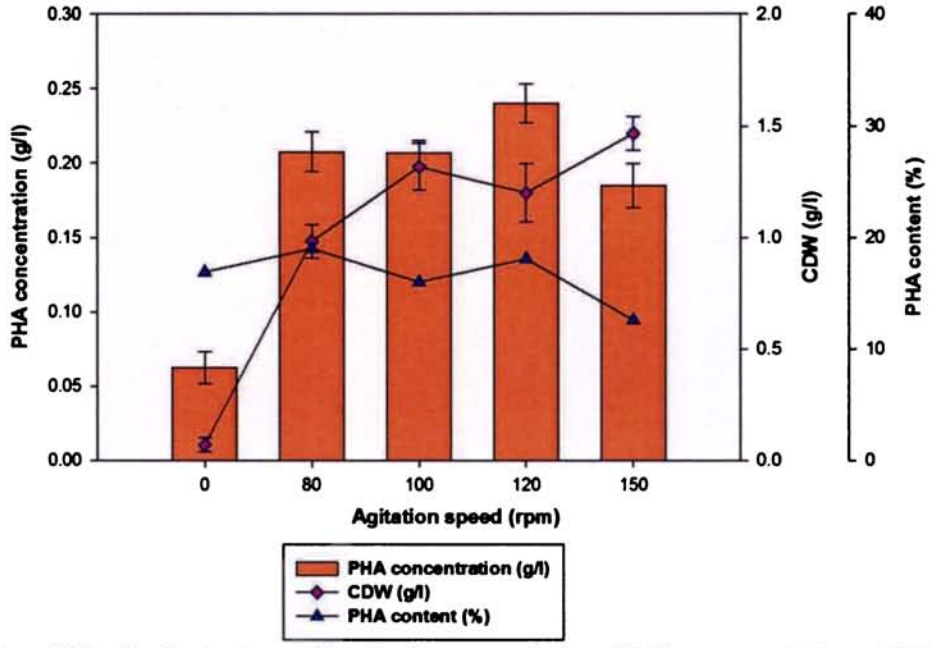


Fig. 4.23. Optimization of agitation speed for PHA accumulation, PHA content and cell dry weight by *Vibrio* sp. strain BTKB33. Data points are arithmetic means of triplicates, while error bars denote the standard deviation of the mean.

4.8.1.5 Effect of temperature

Fig 4.24 shows the results of the effect of different incubation temperature on PHA production in *Vibrio* sp. strain BTKB33. The optimum PHA production was observed at temperature ranging between 30-35°C. Maximum PHA production was found to be at 35°C (0.25 g/l). The PHA production decreased drastically with increase in incubation temperature above 35°C. Below 30°C temperature, PHA production was lower. The cell dry weight and PHA content of the strain was also maximum at 35°C (1.39 g/l and 17.7 % of CDW respectively) and pattern observed was similar to that of PHA accumulation by the bacterium.

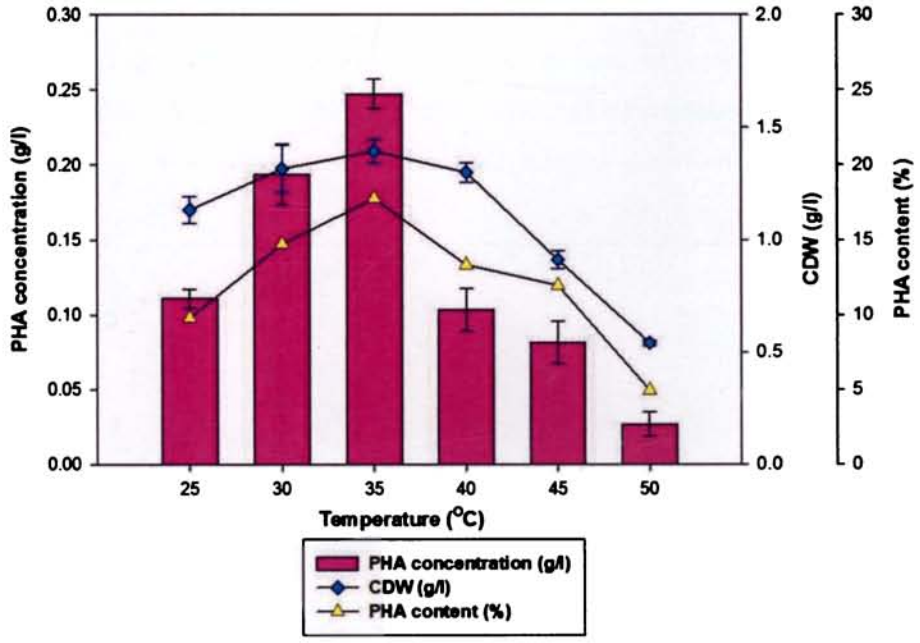


Fig. 4.24 Optimization of incubation temperature for PHA accumulation, PHA content and cell dry weight by *Vibrio* sp. strain BTKB33. Data points are arithmetic means of triplicates, while error bars denote the standard deviation of the mean.

4.8.1.6 Optimization of medium

The outcome of the optimization studies for the selection of a suitable medium for PHA accumulation is indicated in Fig. 4.25. In the above studies Medium A, B and C represented various Nitrogen limiting medium outlined by Sun *et al.* (1994), Ramadas *et al.* (2009) and Lageveen *et al.* (1988) (E2 minimal medium). The medium D and E represented Nutrient medium and Zobell marine medium respectively. From the studies, it was clear that the medium outlined by Sun *et al.*, 1994 favoured maximum PHA accumulation compared to others. Usage of Nitrogen sufficient media like Nutrient medium and Zobell's Marine medium resulted in lower PHA accumulation compared to various nitrogen minimal media tested. In case of PHA content per CDW determination of the bacterium, similar pattern of results was observed. Nutrient medium favored

maximum cell growth than any other medium. It was obvious that the bacterium required a N- limiting medium for PHA production and medium A representing PHA production medium outlined by Sun *et al.* (1994) was found to be optimum for PHA production *Vibrio* sp. BTKB33.

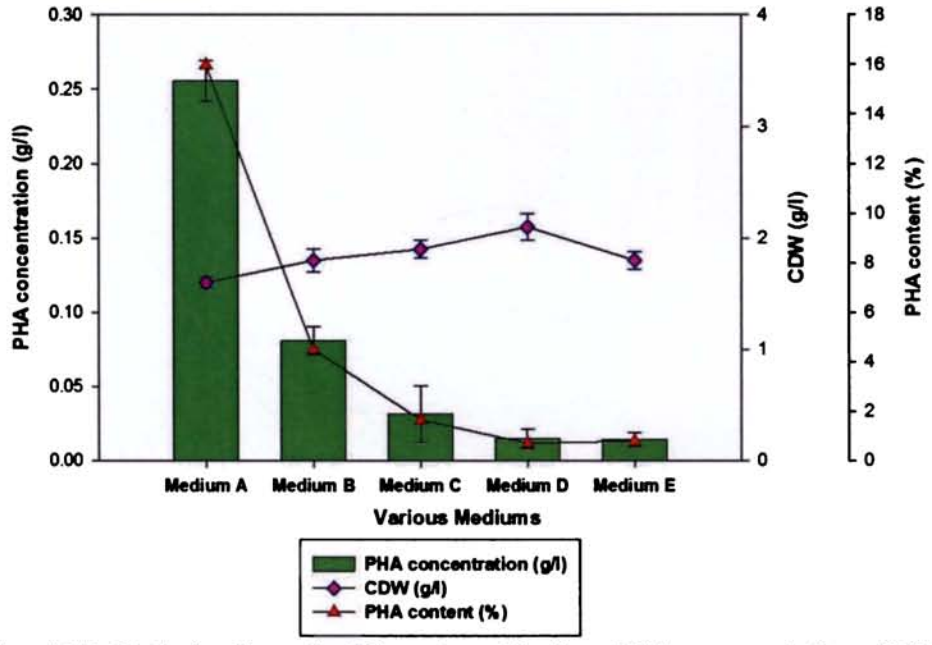


Fig. 4.25 Optimization of different media for PHA accumulation, PHA content and cell dry weight by *Vibrio* sp. strain BTKB33. Data points are arithmetic means of triplicates, while error bars denote the standard deviation of the mean.

Medium A = PHA production medium (Sun *et al.*, 1994);
Medium B = PHA production medium (Ramadas *et al.*, 2009);
Medium C = E2 minimal medium (Lageveen *et al.*, 1988);
Medium D = Nutrient Broth (HiMedia, India) and
Medium E = Zobells Marine Broth (HiMedia, India).

4.8.1.7 Effect of age of inoculum

The results of the studies conducted for the optimization of age of primary inoculum is as represented in Fig. 4.26. It can be inferred that production of PHA was maximum in PHA production medium with 12h old seed inoculum (0.31 g/l), with a steady decline with further increase in age of seed. The PHA content and

cell dry weight measurement were also maximum using 12h inoculum (20% of CDW and 1.5 g/l). The 6h and 9h old cultures gave lower PHA production. CDW was not much influenced by the age of inoculum.

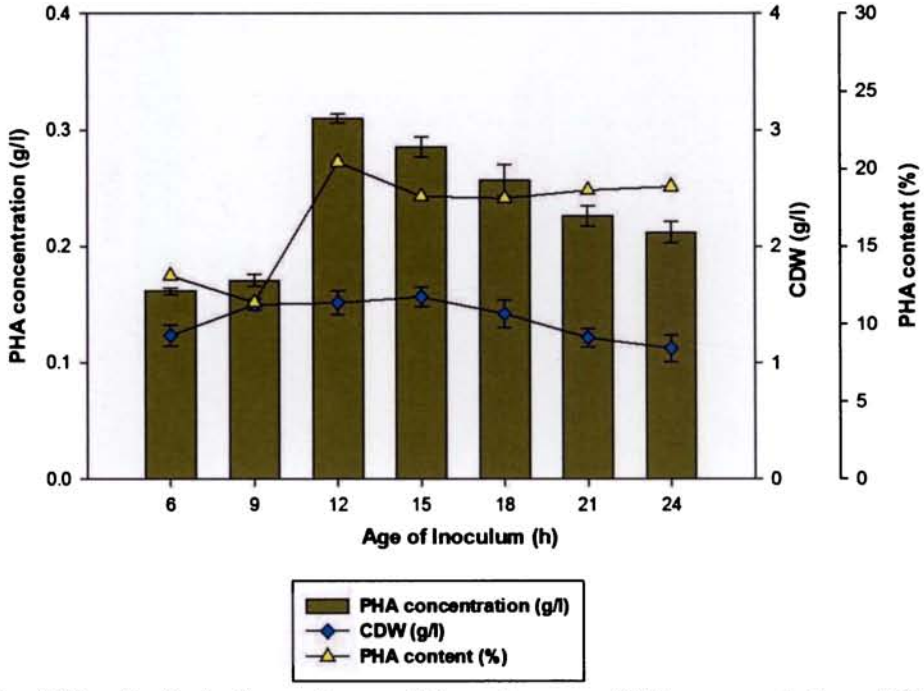


Fig. 4.26 Optimization of age of inoculum on PHA accumulation, PHA content and cell dry weight by *Vibrio* sp. strain BTKB33. Data points are arithmetic means of triplicates, while error bars denote the standard deviation of the mean.

4.8.1.8 Effect of inorganic nitrogen source

Different inorganic nitrogen sources were tested for their effect on the PHA production, CWD and PHA content and all enhanced PHA production, PHA content and cell dry weight compared to the control, which was devoid of any inorganic nitrogen source (Fig 4.27). PHA production by ammonium chloride (0.32 g/l)> ammonium hydrogen phosphate (0.29 g/l)>ammonium oxalate (0.22 g/l)> ammonium acetate (0.21 g/l)> potassium nitrate (0.16 g/l)> ammonium nitrate (0.13 g/l)>ammonium ferrous sulphate (0.1 g/l)> ammonium sulphate (0.09 g/l) > sodium nitrate (0.08 g/l). PHA content was found to be maximum in the

presence of ammonium chloride (24 % of CDW) as inorganic nitrogen source. But Cell dry weight was maximum in the presence of sodium nitrate (2.85 g/l) as inorganic nitrogen source.

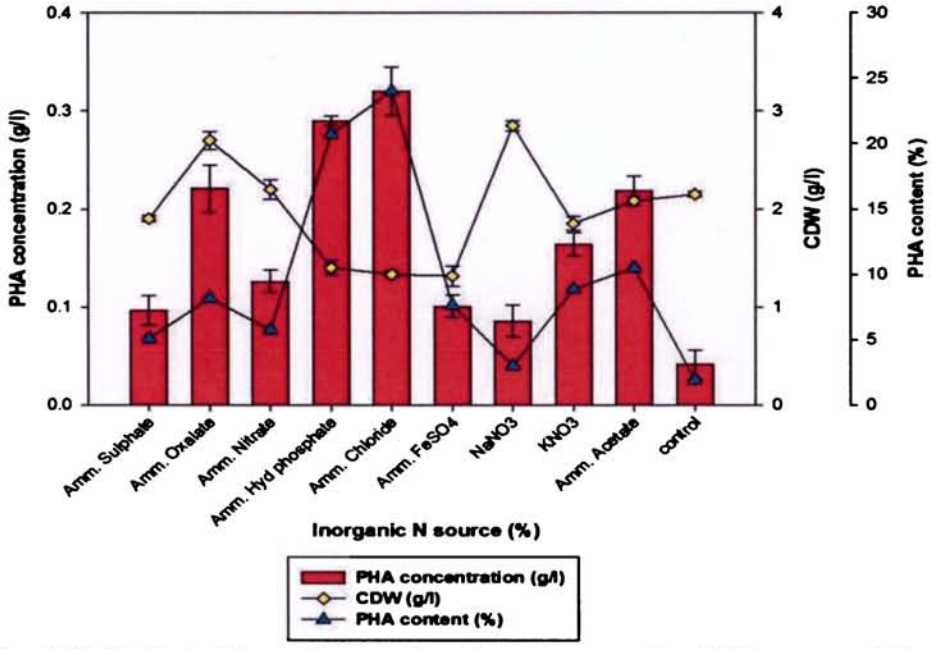


Fig. 4.27 Optimization of inorganic nitrogen source for PHA accumulation, PHA content and cell dry weight by *Vibrio* sp. strain BTKB33. Data points are arithmetic means of triplicates, while error bars denote the standard deviation of the mean.

4.8.1.9 Effect of organic nitrogen sources

It is evident from the results presented in Fig. 4.28., that all the organic nitrogen sources tested had a positive effect on PHA production compared to control, which was devoid of any organic nitrogen source. Maximum PHA production was supported by yeast extract (0.33 g/l) followed by soyabean (0.22 g/l), urea (0.21 g/l), casein (0.18 g/l), peptone (0.16 g/l), beef extract (0.12 g/l) and malt extract (0.1 g/l). Use of yeast extract as organic nitrogen sources also maximised PHA content per CDW (25.9 % of CDW). It was followed with soyabean (12.9 % of CDW), casein (12.7 % of CDW), urea (10 % of CDW), beef extract (9.79 % of CDW), peptone (8.88 % of CDW) and malt extract (9.06 % of CDW). Urea enhanced CDW to a maximum (2.1 g/l) followed by peptone (1.8

g/l), soyabean (1.7 g/l), casein (1.4 g/l), yeast extract (1.3 g/l), beef extract (1.2 g/l) and malt extract (1.2 g/l).

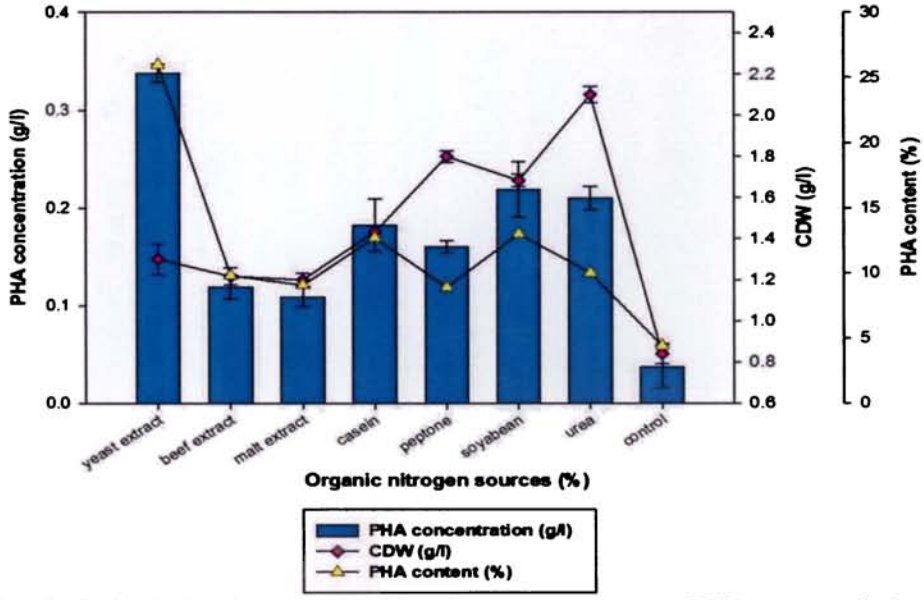


Fig. 4.28 Optimization of organic nitrogen source on PHA accumulation, PHA content and cell dry weight by *Vibrio* sp. strain BTKB33. Data points are arithmetic means of triplicates, while error bars denote the standard deviation of the mean.

4.8.1.10 Effect of inorganic salts

From the data obtained from the optimization of inorganic salts for PHA production in *Vibrio* sp. BTKB33, it was evident that presence of inorganic salts like $MgSO_4$, $MgCl_2$, $ZnSO_4$, $CaCl_2$ and KCl, in the medium had a positive effect on PHA production compared to control, which was devoid of any inorganic salts (Fig. 4.29.). Inorganic salts like $FeSO_4$ and $CoCl_2$ had a negative effect on PHA production compared to control. Maximum PHA production was supported by $MgSO_4$ (0.36 g/l) followed by $MgCl_2$ (0.17 g/l), $ZnSO_4$ (0.16 g/l), $CaCl_2$ (0.15 g/l) and KCl (0.15 g/l). Maximum PHA content was supported by $MgSO_4$ (22.5% of CDW) followed by $MgCl_2$ (9% of CDW), KCl (7.5% of CDW), $ZnSO_4$ (7% of CDW) and $CaCl_2$ (5% of CDW). Maximum CDW was supported by $CaCl_2$ (2.8

g/l) followed by $ZnSO_4$ (2.3 g/l), KCl (2 g/l), $MgCl_2$ (1.7 g/l) and $MgSO_4$ (1.6 g/l).

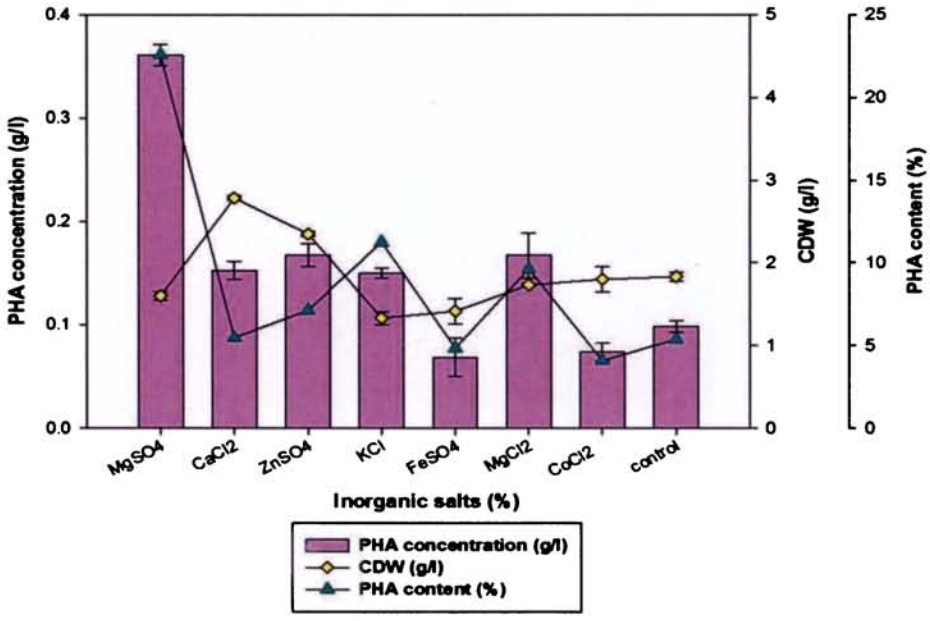


Fig. 4.29 Optimization of various inorganic salts for PHA accumulation, PHA content and cell dry weight by *Vibrio* sp. strain BTKB33. Data points are arithmetic means of triplicates, while error bars denote the standard deviation of the mean.

4.8.1.11 Effect of inoculum concentration

From the studies on the effect of concentration of primary inoculum on PHA production in *Vibrio* sp. strain BTKB33 (Fig. 4.30.), it was obvious that a 2.5 % primary inoculum concentration gave maximum PHA production (0.37 g/l) and PHA content (22% of CDW). The results also showed that the PHA production increased with increase in concentration of primary inoculum upto 2.5 % and gradually decreased with further increase in inoculum concentration. However, the cell dry weight increases with increase in primary inoculum concentration and 10 % primary inoculum gives maximum cell dry weight for the bacterium (2.2 g/l).

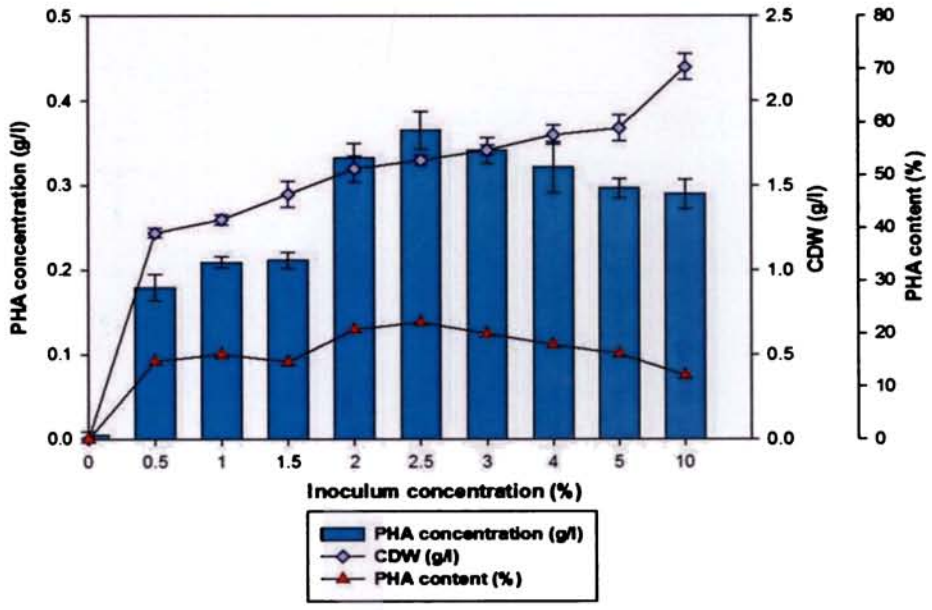


Fig. 4.30 Optimization of inoculum concentration for PHA accumulation, PHA content and cell dry weight by *Vibrio* sp. strain BTKB33. Data points are arithmetic means of triplicates, while error bars denote the standard deviation of the mean.

4.8.1.12 Effect of various carbon sources

The effect of different carbon sources on PHA production in *Vibrio* sp. strain BTKB33 is as shown in (Fig 4.31). It was found that the bacterium grown in production medium with glucose (2%) as sole carbon source showed maximum PHA production (0.48 g/l) and PHA content (42.7 % of CDW), although the cell dry weight was higher in medium using glycerol(1.38 g/l). It was clearly seen that all the carbon sources tested in the present study favored production of PHA in *Vibrio* sp. strain BTKB33 compared to control, which was devoid of any carbon sources. The PHA production in the presence of other carbon sources were 0.39 g/l, 0.25 g/l , 0.24 g/l, 0.21 g/l, 0.18 g/l, 0.16 g/l and 0.15 g/l for glycerol, sucrose, lactose, pectin, mannitol, starch and sodium acetate respectively.

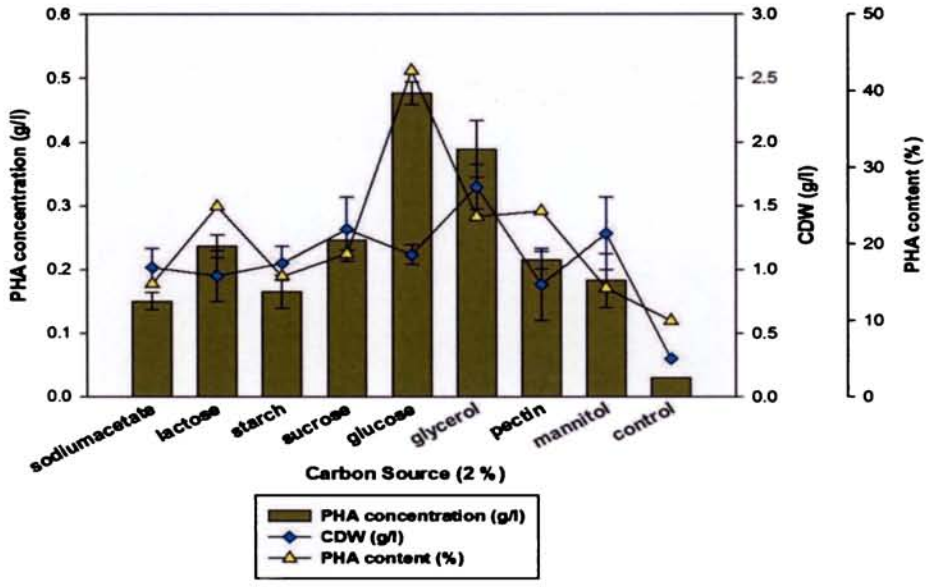


Fig. 4.31 Optimization of various carbon sources for PHA accumulation, PHA content and cell dry weight by *Vibrio* sp. strain BTKB33. Data points are arithmetic means of triplicates, while error bars denote the standard deviation of the mean.

From the "one-factor-at-a-time-method" for optimization of bioprocess variables in PHA production by *Vibrio* sp. BTKB33 under submerged fermentation condition, a 2.28 fold increase in PHA production in *Vibrio* sp. strain BTKB33 was obtained. The optimized factors are - pH 7; C source - glucose (2% w/v); Inorganic N source - Ammonium Chloride (0.05% w/v); Organic N source - yeast extract (0.05% w/v); Inorganic salt – $MgSO_4$ (0.02% w/v); Inoculum concentration - 2.5% (v/v); NaCl 1.5 % (w/v); Temperature 35°C; Incubation period 36 h; Inoculum age - 12h; Medium- PHA minimal medium (Sun *et al.*, 1999) and Agitation - 120 rpm. All these factors were used for statistical evaluation of factors for PHA production.

4.8.2 Optimization of bioprocess variables for PHA production by *Vibrio* sp. strain BTKB33 by "statistical approach"

Medium that could support maximum PHA production by *Vibrio* sp. strain BTKB33 was optimized employing statistical approach. Initially process variables were optimized using Plackett-Burman design and in the second stage, Response surface methodology was adopted towards selection of optimal variables and understanding the probable interaction among the significant variables. Submerged fermentation (SmF) was utilized for optimization of suitable variables that support maximal PHA production in *Vibrio* sp. strain BTKB33.

4.8.2.1 Plackett-Burman Design (P-B Design)

Data obtained from the "one-factor-at-a-time" optimization of various factors affecting PHA production in *Vibrio* sp. BTKB33 by Plackett-Burman design were analyzed by Design Expert software 8.0 and a first order model was fitted to the data obtained from the experiment. The experimental results of PHA production by Plackett-Burman design are as represented in table 4.10. The experimental results of the PB design were used for navigating the Paretto chart and are represented in Fig. 4.32. From the results of the studies with PB design, it was noted that incubation period, pH, tryptone, inoculum concentration, $MgSO_4$ and Glucose effected an enhancement in PHA production, whereas yeast extract, process temperature, ammonium chloride, KH_2PO_4 and sodium chloride concentration recorded a negative effect on PHA production when there was an increase in the value of variable. Further it was noted that among the 11 variables which were selected based on one factor per trial (data showed earlier), only three factors, namely sodium chloride concentration, temperature and incubation period were identified as the most significant variables that influenced the PHA concentration (g/l). The experimental results presented in table 4.10 were statistically analyzed by the software Design Expert 8.0. The ANOVA for the experiments with Plackett-Burman design for the PHA production by *Vibrio* sp. BTKB33 is represented in table 4.11.

Table 4.10 The matrix of the Plackett-Burman design experiments and the observed experimental data for PHA production in *Vibrio* sp. BTKB33

Sl. No.	RUN	Incubation period (h)	pH	Temperature (^{O} C)	Glucose (% w/v)	Ammonium chloride (% w/v)	Yeast extract (% w/v)	KH_2PO_4 (% w/v)	$MgSO_4$ (% w/v)	Tryptone (% w/v)	Inoculum concentration (% v/v)	NaCl (% w/v)	PHA concentration (g/l)
1	1	36.00	6	30.00	2.00	0.10	0.10	0.10	0.01	0.50	10.00	1.00	0.316
2	2	36.00	9	30.00	4.00	0.05	0.05	0.05	0.02	0.50	10.00	1.00	0.582
3	3	12.00	6	30.00	4.00	0.10	0.10	0.05	0.02	0.50	1.00	10.00	0.018
4	4	36.00	6	40.00	4.00	0.05	0.10	0.05	0.01	0.10	10.00	10.00	0.035
5	5	12.00	9	30.00	2.00	0.05	0.10	0.10	0.02	0.10	10.00	10.00	0.059
6	6	12.00	9	40.00	2.00	0.10	0.05	0.05	0.01	0.50	10.00	10.00	0.012
7	7	36.00	9	40.00	2.00	0.10	0.10	0.05	0.02	0.10	1.00	1.00	0.285
8	8	36.00	6	40.00	2.00	0.05	0.05	0.10	0.02	0.50	1.00	10.00	0.026
9	9	12.00	6	30.00	2.00	0.05	0.05	0.05	0.01	0.10	1.00	1.00	0.259
10	10	12.00	6	40.00	4.00	0.10	0.05	0.10	0.02	0.10	10.00	1.00	0.192
11	11	12.00	9	40.00	4.00	0.05	0.10	0.10	0.01	0.50	1.00	1.00	0.249
12	12	36.00	9	30.00	4.00	0.10	0.05	0.10	0.01	0.10	1.00	10.00	0.028

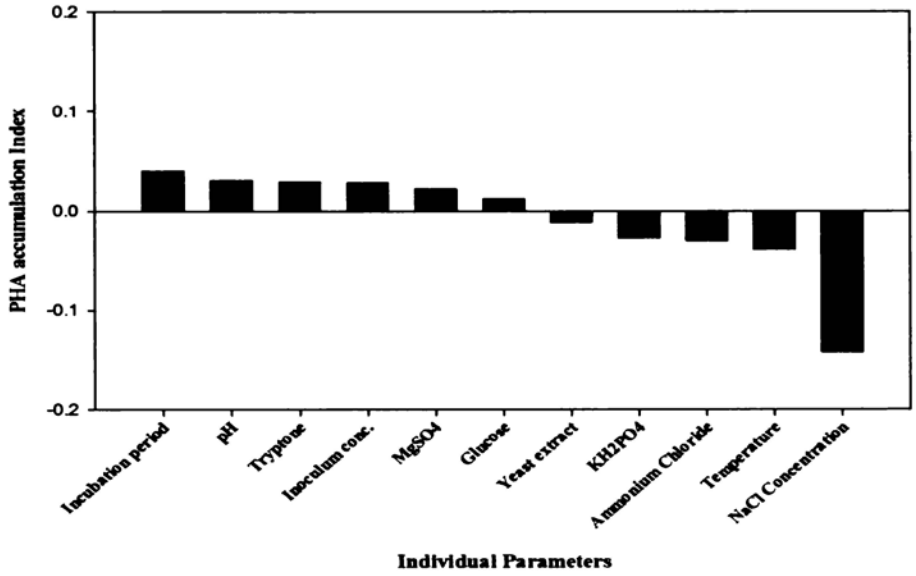


Fig 4.32 Pareto chart showing the effect of individual factors on PHA production by *Vibrio* sp. BTKB33

Table 4.11 ANOVA for the experiments with Plackett-Burman design for the PHA production by *Vibrio sp.* BTKB33

Term	PHA yield
F Value	12.67
Prob>F	0.0021
Mean	0.17
R-Squared	0.8262
Adjusted R-Squared	0.7610
Coefficient of Variance	49.86
Predicted R-Squared	0.6089
Adequate Precision	8.923

Application of the PB method enables selection of variables which have the largest influence on the desired product yield. In the present study, this fact could be experienced in the rapid recognition of the three most important variables from among the eleven that were originally studied. In the present PB design for maximum PHA production in *Vibrio* sp. BTKB33, the significant variables selected for further RSM studies were NaCl concentration, temperature and incubation period.

The statistical significance of the model was evaluated using ANOVA, and the results were used to fit a first-order polynomial equation; the model equation for PHA production (Y) could be written as

PHA concentration (Y) g/l = + 0.17
+ 0.040 * Incubation period
- 0.039 * Temperature
- 0.14*NaClconcentration

The model coefficients estimated by multiple linear regressions and ANOVA showed that the model was significant with F value of 12.67. Values of "Prob > F" less than 0.0500 indicates the significance of this model. The coefficient of determination R^2 was calculated as 0.8262, which indicated that approximately 82.62 % of the variability in the dependent variable (response) could be explained by the model. The adjusted R^2, which is more suited for comparing models with different numbers of variables was 0.7610. The "Pred R-Squared" of 0.6089 is in reasonable agreement with the "Adj R-Squared" of 0.7610. The coefficient of variance was found to be 49.86, and the adequate precision that measures the signal-to-noise ratio was 8.923. A ratio greater than 4 is desirable as it indicates an adequate signal. Thus this model could be used to navigate the design space.

Validation of the PB design was carried out in shake flasks under conditions predicted by the model and it was noted that the experimental values were very close to the predicted values and hence the model was successfully validated (Fig. 4.33).

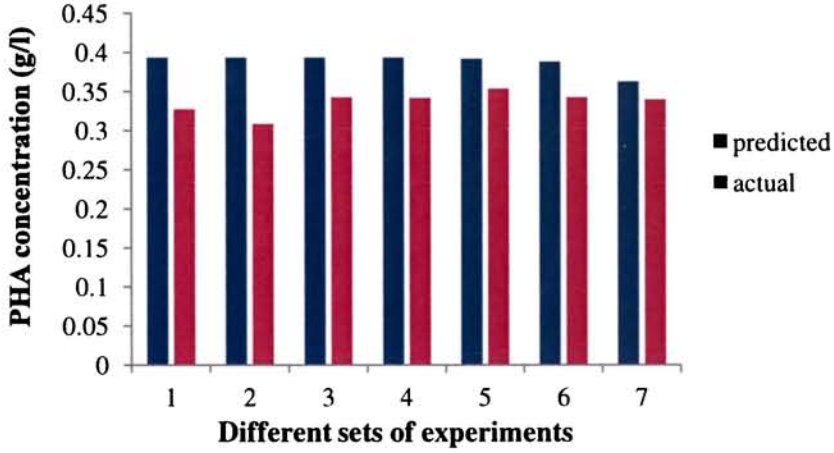


Fig. 4.33 Validation of Plackett-Burman Design

4.8.2.2 Response surface methodology (RSM) by Box-Behnken design

Response surface methodology using Box-Behnken design was adopted for the selection of optimal levels of the significant variables. Sodium chloride concentration (X_1), temperature (X_2), and incubation period (X_3) were identified as significant variables based on Plackett-Burman design experiment.

The design evaluated these three independent variables, each one at three levels for PHA production (g/l) and PHA content (% of CDW). The present RSM studies focus on two responses viz., PHA accumulation (g/l) and PHA content (%).

The quadratic model chosen to represent the relationship fitted for the three variables was

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=1}^{3} \beta_{ij} X_i X_j$$

Where Y is the PHA production (g/l) and PHA content (% of CDW), and X_1, X_2 and X_3 represented the three independent variables for incubation

temperature, sodium chloride concentration and incubation period and β_0 is the intercept term. The results obtained for the Box-Behnken design experiments were analyzed by ANOVA, which yielded the following regression equation for the level of (1) PHA production:

PHA concentration, Y (g/l) = +0.26 +0.074 * Incubation period - 0.19 * NaCl - 0.083 * Temperature - 0.028 * Incubation period^2 - 0.024 * NaCl^2 - 0.028 * Temperature^2 + 4.165E-004 * Incubation period * NaCl - 0.11 * Incubation period * Temperature + 5.900E-003 * NaCl*Temperature

And (2) for the level of PHA content per CDW:

PHA content, Y (% of CDW) = + 28.08 + 4.19 * Incubation period - 18.47 * NaCl - 10.55 * Temperature - 2.17 * Incubation period^2 - 3.52 * NaCl^2 + 0.18 * Temperature^2 - 2.99 * Incubation period * NaCl - 7.07 * Incubation period * Temperature - 0.36 * NaCl * Temperature

The design matrix (Box-Behnken design) and the corresponding experimental data obtained are shown in table 4.12. Experimental data obtained for the effect of three selected physicochemical factors on PHA production and PHA content by *Vibrio* sp. BTKB33 showed strong dependence on the presence and levels of selected factors since it was noted that PHA production varied between 0.02 and 0.62 g/l, while PHA content varied between 1.73 and 60.27 (% of CDW) under the studied experimental conditions.

Table 4.12 Optimization of medium composition and physical parameters for the PHA production and PHA content per CDW by *Vibrio* sp. BTKB33 using Box-Behnken design experiment

RUN	Incubation period (h)	NaCl conc. (%)	Temperature (°C)	PHA conc. (g/l)	PHA content (% of CDW)
1	24.00	5.50	35.00	0.27	26.38
2	24.00	5.50	35.00	0.20	28.66
3	12.00	1.00	35.00	0.40	36.42
4	36.00	5.50	30.00	0.62	60.27
5	24.00	10.00	40.00	0.02	1.73
6	36.00	10.00	35.00	0.03	2.37
7	24.00	5.50	35.00	0.27	36.57
8	12.00	5.50	40.00	0.02	6.05
9	24.00	5.50	35.00	0.29	23.87
10	36.00	5.50	40.00	0.08	9.03
11	24.00	10.00	30.00	0.03	7.57
12	36.00	1.00	35.00	0.40	42.02
13	12.00	5.50	30.00	0.11	29.29
14	12.00	10.00	35.00	0.02	8.71
15	24.00	5.50	35.00	0.29	26.53
16	24.00	1.00	30.00	0.42	47.03
17	24.00	1.00	40.00	0.38	42.64

Table 4.13 ANOVA for the response surface experiments conducted using Box Behnken design for PHA production by *Vibrio* sp. BTKB33

Term	PHA yield
F Value	3.87
Prob>F	0.0441
Mean	0.23
R-Squared	0.8327
Adjusted R-Squared	0.6175
Adequate Precision	6.276
Co efficient of Variance	49.73

The ANOVA analysis of PHA production yielded values of Prob>F less than 0.05, suggesting that the model terms were significant, with a P value of 0.0001 and an F value of 3.87 (Table 4.13). An estimated value of 0.8327 for the coefficient of determination R^2 ensured a satisfactory adjustment of the quadratic model to the experimental data, indicating that approximately 83.27 % of the variability in the dependent variable (response) could be explained by the model. It was observed that all the selected parameters were significant, and varied levels of interactions were recorded for the variables in their cumulative effect on PHA production. The coefficient of variance was found to be 49.73, and the adequate precision that measures the signal-to-noise ratio was 6.276. The ratio greater than 4 was desirable as it indicated an adequate signal. Thus, this model could be used to navigate the design space.

Table 4. 14 ANOVA for the response surface experiments conducted using Box- Behnken design for PHA content by *Vibrio sp.* BTKB33

Term	PHA yield
F Value	4.10
Prob>F	0.0382
Mean	25.49
R-Squared	0.8404
Adjusted R-Squared	0.6353
Adequate Precision	7.201
Co efficient of Variance	41.23

The ANOVA analysis of PHA content yielded values of Prob>F less than 0.05, suggesting that the model terms were significant, with a P value of 0.0001 and an F value of 4.10 (Table 4.14). An estimated value of 0.8404 for the coefficient of determination R^2 ensured a satisfactory adjustment of the quadratic model to the experimental data, indicating that approximately 84.04 % of the variability in the dependent variable (response) could be explained by the model. It was observed that all the selected parameters were significant, and varied levels of interactions were recorded for the variables in their cumulative effect on PHA content. The coefficient of variance was found to be 41.23, and the adequate

precision that measures the signal-to-noise ratio was 7.201. The ratio greater than 4 was desirable as it indicated an adequate signal. Thus, this model could be used to navigate the design space.

4.8.2.2.1 Analysis of factors influencing PHA production

Three dimensional response surface curves were plotted to study the interaction among various physicochemical factors, and to determine the optimum concentration of each individual variable for PHA production in *Vibrio* sp. BTKB33 under submerged fermentation conditions.

The pairwise interaction among the factors in terms of PHA production in the optimized set was assessed by examining the response surfaces. Three dimensional response surfaces were generated holding three factors constant at a time and plotting the response obtained for varying levels of the other two.

4.8.2.2.1.1 Interaction between incubation period and temperature on PHA production

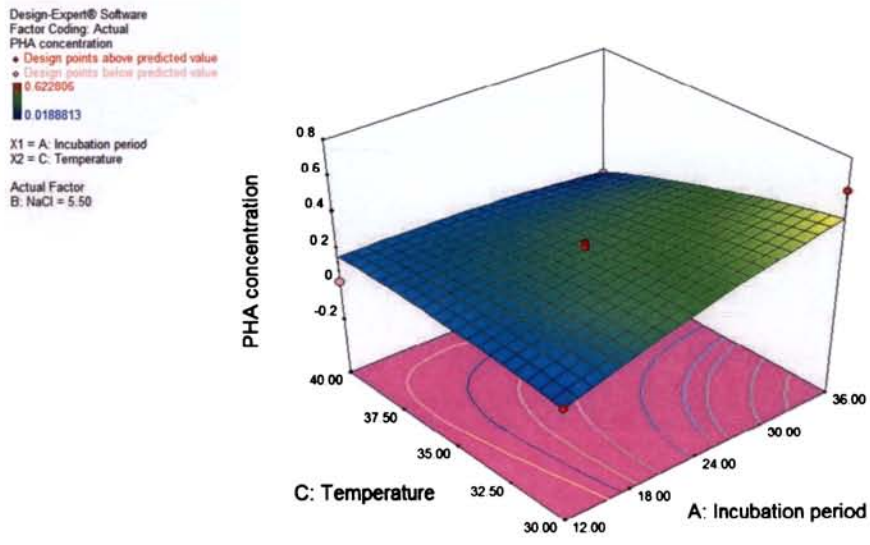


Fig. 4.34 Effect of incubation period and temperature on PHA production in *Vibrio* sp. BTKB33

When the concentration of NaCl was held at its optimum level (5%), the PHA production increased along with increase in incubation period (Fig. 4.34.). The PHA production is found to increase slightly at higher temperature, when the concentration of NaCl was held at its optimum level. The response surface curves clearly indicate a positive interaction between temperature and incubation time in their effect on PHA production in *Vibrio* sp. BTKB33.

4.8.2.2.1.2 Interaction between NaCl concentration and temperature on PHA production

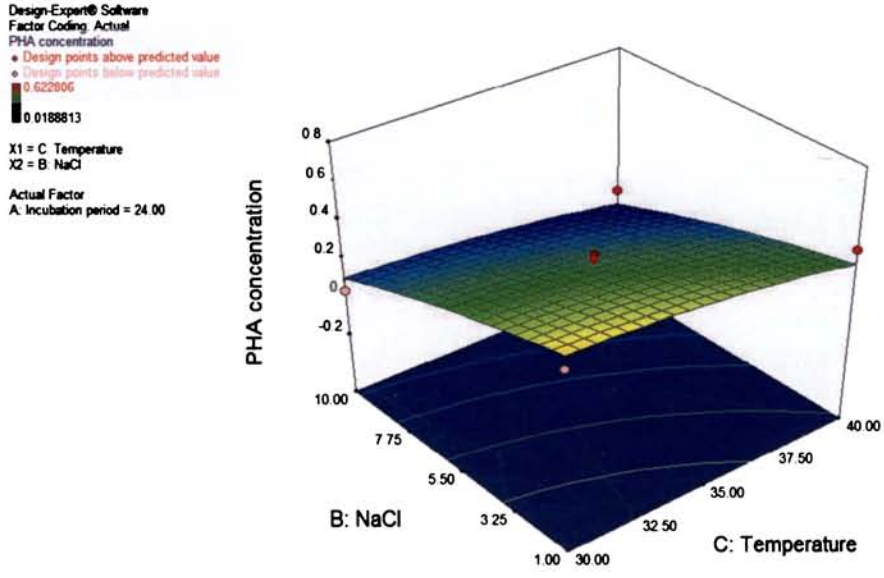


Fig. 4.35 Effect of temperature and NaCl concentration on PHA production in *Vibrio* sp. BTKB33

When the incubation period was held at its optimum level (24h), the PHA production was found to decrease with increase in NaCl concentration (Fig. 4.35.). The PHA concentration remained unchanged in response to increase or decrease in incubation temperature suggesting a neutral interaction between temperature and NaCl concentration.

4.8.2.2.1.3 Interaction between NaCl concentration and incubation period on PHA production

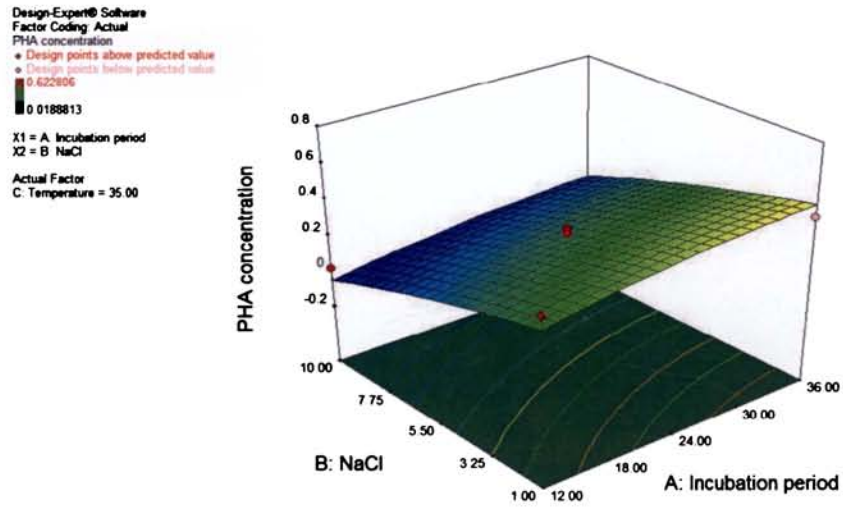


Fig. 4.36 Effect NaCl concentration and incubation period on PHA production in *Vibrio* sp. BTKB33

When the temperature was held at its optimum level (35° C), the PHA production decreased with increase in NaCl concentration (Fig.4.36) and increased with increase in incubation period. This suggests a positive interaction between incubation period and NaCl concentration affecting PHA production.

4.8.2.2.2 Analysis of factors influencing PHA content

Three dimensional response surface curves were plotted to study the interaction among various physicochemical factors and to determine the optimum level of each individual variable for PHA content (% of CDW) in *Vibrio* sp. BTKB33 under submerged fermentation conditions.

The pairwise interaction among the factors in terms of PHA content in the optimized set was assessed by examining the response surfaces. Three dimensional response surfaces were generated holding three factors constant at a time and plotting the response obtained for varying levels of the other two.

4.8.2.2.2.1 Interaction between NaCl concentration and temperature on PHA content (%)

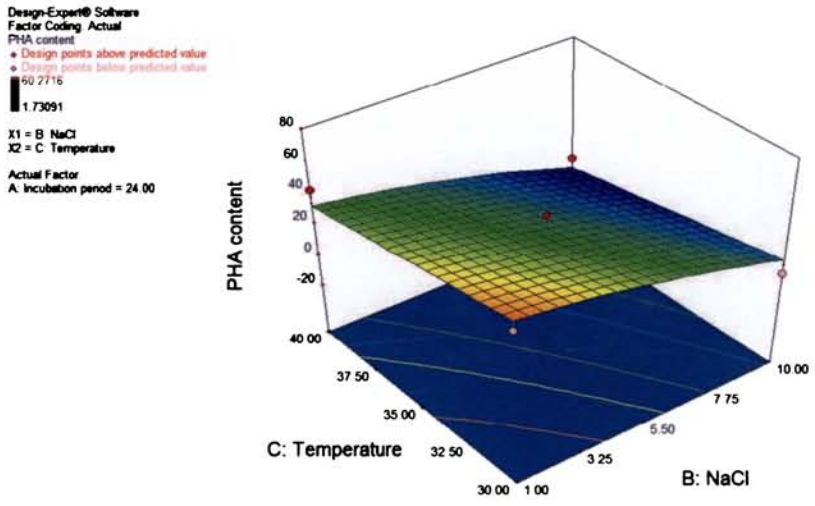


Fig. 4.37 Effect of temperature and NaCl concentration on PHA content in *Vibrio* sp. BTKB33

When the incubation period was held at its optimum level (24h), the PHA content (%) decreased with increase in NaCl concentration (Fig. 4.37) and also decreased with increase in temperature suggesting a positive interaction between temperature and NaCl concentration affecting PHA content in *Vibrio* sp. BTKB33.

4.8.2.2.2.2 Interaction between of incubation period and temperature on PHA content (%)

When the concentration of NaCl was held at its optimum level (5%), the PHA content increased along with increase in incubation period (Fig. 4.38.), while it remained unchanged in response to increase or decrease in temperature suggesting a neutral interaction between temperature and incubation period on PHA content in *Vibrio* sp. BTKB33.

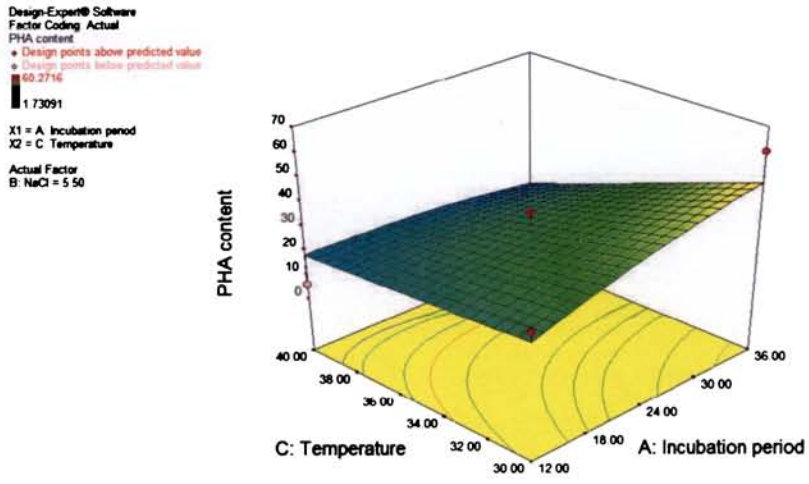


Fig. 4.38 Effect of incubation period and temperature on PHA content in *Vibrio* sp. BTKB33

4.8.2.2.2.3 Interaction between NaCl concentration and incubation period on PHA content (%)

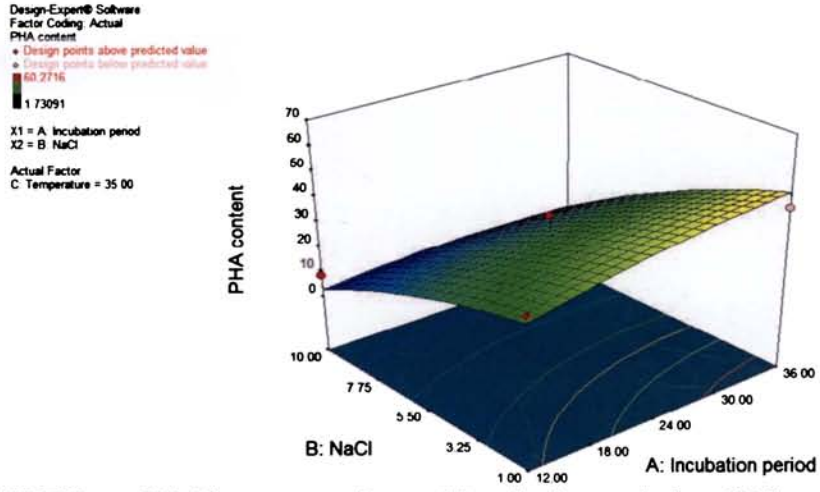


Fig. 4.39 Effect of NaCl concentration and incubation period on PHA content in *Vibrio* sp. BTKB33

When the temperature was held at its optimum level (35^{o}C), the PHA content in *Vibrio* sp. BTKB33 decreased with increase in NaCl concentration (Fig. 4.39.) while it increased with increase in incubation period suggesting a positive

interaction between incubation period and NaCl concentration on PHA content in *Vibrio* sp. BTKB33.

4.8.2.3 Validation of response surface model

Validation of the deduced response surface model based on previous experiments was carried out in shake flask under conditions predicted by the model. The experimental values were found to be very close to predicted values, and hence the model was successfully validated (Table 4.15). Validation of the RSM model for PHA production by *Vibrio* sp. BTKB33 is represented in Fig. 4. 40. and validation of the RSM model for PHA content by *Vibrio* sp. BTKB33 is represented in Fig. 4.41.

The model predicted maximum PHA production upto 0.64 g/l and PHA content upto 64.56 %; this could be achieved using 1.26% w/v NaCl concentration, at 30.02°C after 35.97 h of incubation. An overall 2.96-fold increase in PHA accumulation and 3.11 fold increase in PHA content was achieved after validation of RSM in shake flasks. The NaCl concentration range that supported maximum PHA accumulation and PHA content was 1.02-1.62%, and the incubation period was 33.9-36h.

Optimized conditions of major bioprocess variables of the medium for the PHA production and PHA content in *Vibrio* sp. BTKB33 after statistical analysis is represented in Table 4.16 and these conditions were used for the time course studies.

Table 4.15 Predicted and experimental values of PHA production and PHA content obtained for validation of the RSM model

Sl. No.	Incubation period (h)	NaCl concentration (%)	Temperature (°C)	PHA content		PHA concentration	
				Predicted	Experimental	Predicted	Experimental
1	35.97	1.26	30.02	64.560	54.927	0.639	0.604
2	35.34	1.06	30.17	64.151	55.304	0.634	0.602
3	34.94	1.02	30.09	64.212	58.362	0.634	0.612
4	35.66	1.2	30.32	63.441	55.258	0.629	0.613
5	35.92	1.24	30.17	64.048	51.935	0.635	0.597
6	35.23	1.11	30.06	64.275	55.508	0.635	0.610
7	35.98	1.23	30.29	63.717	59.971	0.632	0.619
8	35.19	1.08	30.14	64.049	53.797	0.633	0.602

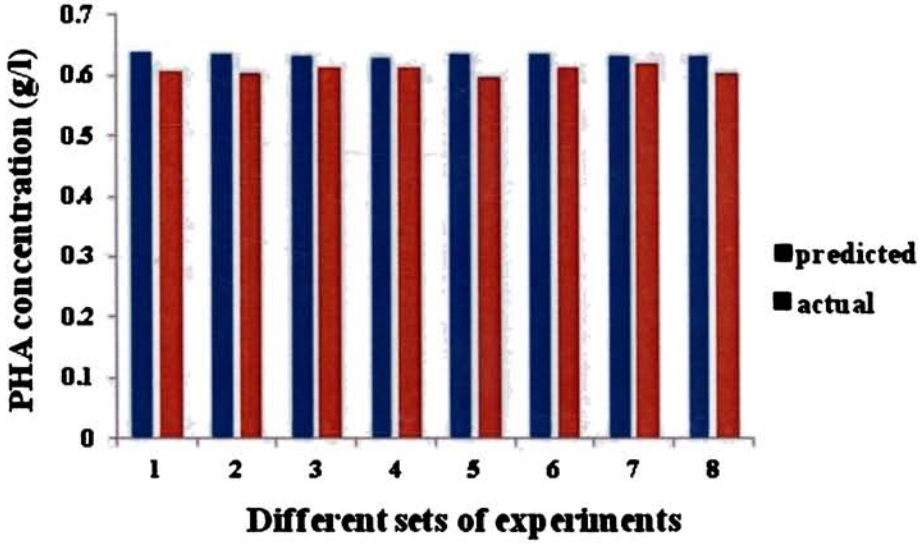


Fig. 4.40 Predicted and experimental values of PHA production by *Vibrio* sp. BTKB33 obtained for validation of the RSM model

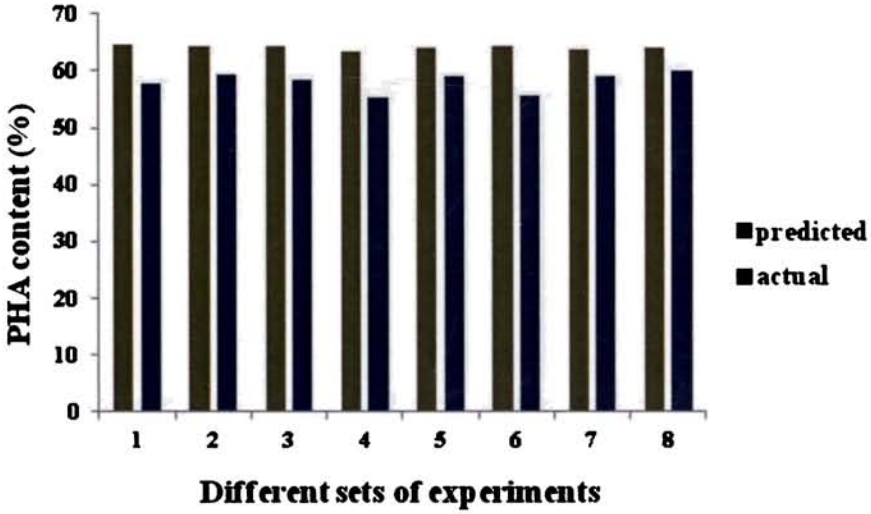


Fig. 4.41 Predicted and experimental values of PHA content by *Vibrio* sp. BTKB33 obtained for validation of the RSM model

Table 4.16 Optimized condition selected after statistical optimization studies for time course study for maximal PHA production in *Vibrio* sp. BTKB33

Bioprocess variables	Conditions optimized
Incubation period	36 h
pH	7.5
Agitation	120 rpm
Inoculum age	12 h
NaCl conc.	1.26 % (w/v)
Inoculum conc.	5.5 % (v/v)
Yeast extract	0.08 % (w/v)
$MgSO_4$	0.01 % (w/v)
Temperature	30.02°C
KH_2PO_4	0.08 % (w/v)
NH_4Cl	0.08 % (w/v)
Glucose	3 % (w/v)
Tryptone	0.3 % (w/v)

4.8.3 Time course study under optimal conditions

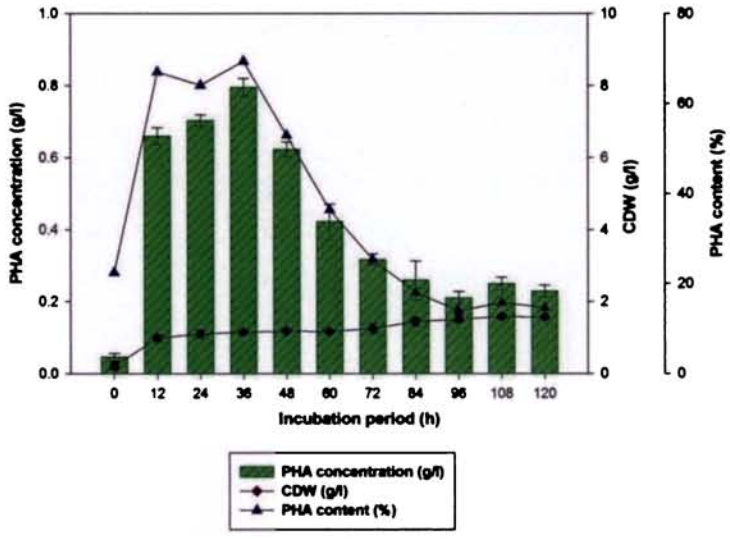


Fig. 4.42 Time course study for PHA production and PHA content by *Vibrio* sp. BTKB33 under optimized conditions

The RSM model obtained gave 0.62 g/l of PHA and 60% PHA content after 36 h of incubation, whereas after time course experiment, PHA concentration was 0.79 g/l and PHA content 69 % of CDW after 36 h of incubation (Fig. 4.42). The time course study was conducted over a period of 120 h under optimized condition. The production of PHA and PHA content per CDW attained under optimized condition was nearly 4 times higher than that obtained under the unoptimized conditions (PHA concentration of 0.79 g/l and PHA content of 69 % of CDW). The PHA production and PHA content increased with increase in incubation period upto 36h and suddenly declined with further increase in incubation period.

The results of optimization studies for PHA production and PHA content in *Vibrio* sp. BTKB33 under SmF conditions is represented in Table 4.17. Under unoptimised condition, PHA concentration and PHA content were 0.21 g/l and 19.3 % of CDW respectively. After "one-factor-at-a-time" optimization studies,

PHA concentration and PHA content increased to 0.48 g/l and 42% of CDW respectively. After statistical optimization of the variables, PHA concentration and PHA content further increased to 0.62 g/l and 60 % of CDW respectively. After final time course study under optimized condition for PHA production by *Vibrio* sp. BTKB33, PHA concentration and PHA content increased to 0.79 g/l and 69 % of CDW respectively. A total of 3.76 fold increase in PHA concentration and 3.58 fold increase in PHA content was observed after final time course study in *Vibrio* sp. BTKB33.

The optimized conditions for maximum PHA production obtained in the present study were as follows: NaCl 1.26% (w/v); temperature 30.02^{0}C; incubation period 36 h; pH 7.5; glucose concentration 3% (w/v); ammonium chloride 0.08% (w/v); yeast extract 0.08% (w/v); KH_2PO_4 0.08% (w/v); $MgSO_4$ 0.01% (w/v); tryptone 0.3% (w/v); inoculum concentration 5.5% (v/v); inoculum age 12 h and agitation 120 rpm.

Table 4.17 The results of optimization studies for PHA production and PHA content in *Vibrio* sp. BTKB33 under SmF conditions

Conditions	PHA conc. (g/l)	Increase in yield	PHA content (%)	Increase in yield
Under unoptimised condition	0.21	NA	19.3 %	NA
After one-factor-at-a-time optimization	0.48	2.29	42 %	2.22
After statistical optimization using PB design and RSM (Box Behnken Design) employing Design Expert. 8	0.62	2.96	60 %	3.11
After final time course study under optimized conditions	0.79	3.76	69 %	3.58

4.9 Characterization of polyhydroxyalkanoates

4.9.1 Fourier transform infrared spectroscopy (FTIR)

The functional groups of the extracted PHA was confirmed as C=O groups by the presence of bands at 1736.90 cm^{-1} in FT-IR spectroscopy of extracted PHAs from *Vibrio* sp. BTKB33 as represented in Fig. 4.43 and this result confirmed the presence of PHAs in this strain.

The typical spectra of methyl and methylene groups in the purified PHAs could be observed in the regions between 3415.71 cm^{-1}-2861.70 cm^{-1}. The bands obtained at 2926.79 cm^{-1} indicated the presence of methyl group in them and the smaller band at this position inferred the presence of PHB in them.

In the FTIR spectrum, stretching between 3415.71 and 1452.04 cm^{-1} indicated C-H stretching and bending. . The presence of bands at 1395.39 cm^{-1} indicated presence of methyl group

The presence of bands at 1290.08 cm^{-1} is characteristic for PHB . The bands at 1233.23 and 1052.71 cm^{-1} may be contributed by the presence of polyhydroxybutyrate (PHB) in them. The FTIR spectrum of the purified PHAs extracted from *Vibrio* sp. BTKB33 was compared with that of commercial PHB (sigma) as represented in Fig. 4.44 and also with previous results available in literature.

It was inferred that there was maximum resemblance between spectra of the *Vibrio* sp. BTKB33 and that of polyhydroxybutyrate (PHB). The FTIR analysis result are in agreement with previous results which indicated the presence of Class I PHA synthase gene responsible for scl-PHAs.

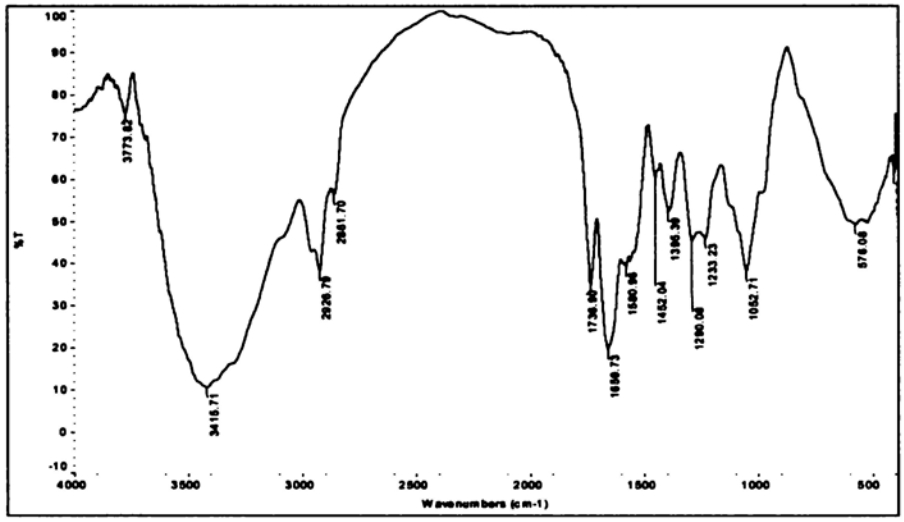


Fig. 4.43 FTIR spectrum of PHA produced by *Vibrio* sp. BTKB33 in PHA minimal medium containing glucose as carbon source

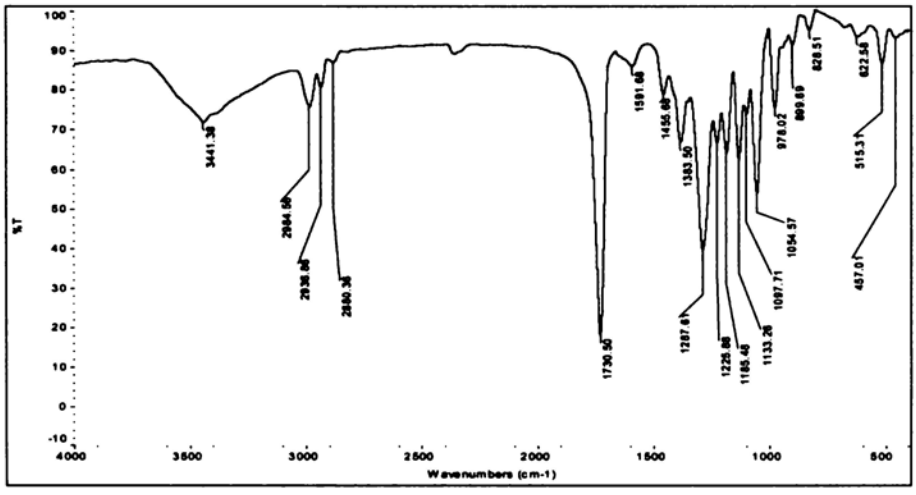


Fig. 4.44 FTIR spectrum of PHB (Sigma)

4.9.2 ^{13}C NMR analysis

The purified PHA was analyzed by ^{13}C NMR to elucidate the structure of the biopolymer. The monomeric units in the PHA from strain BTKB33 were

identified by analyzing its ^{13}C spectra and comparing it with the NMR spectra of other PHAs available in literature.

The signals of the ^{13}C spectra of purified PHA produced (Fig. 4.45) corresponded to the small chain length structures.

The NMR spectra of the extracted BTKB33 PHA correlated with the specific peaks corresponding to carbon atoms in the PHB structure such as C=O, CH_2, CH and CH_3 groups. Each specific peak was indicated as carbon atom in PHA structure (Table 4.18). The carbonyl group of polyhydroxybutyrate is indicated by the chemical shift of 166.73 ppm. Doi *et al.*, 1989 reported the resonance of a methyl group with a chemical displacement at 21.97 ppm, which corresponds to a terminal methyl of hydroxybutyrate. The bands at 67.16 ppm and 37.75 ppm correspond to peaks of carbon atoms of the CH and CH_2 groups respectively. All these clearly point to the presence of polyhydroxybutyrate as the scl-PHA in *Vibrio* sp. BTKB33.

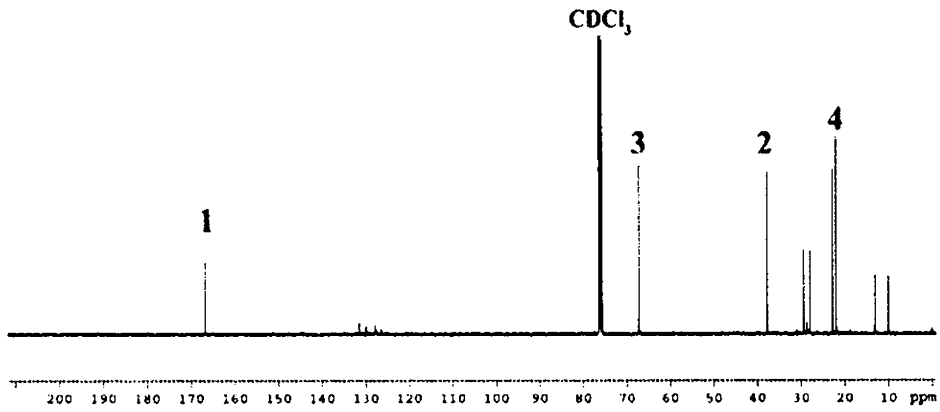
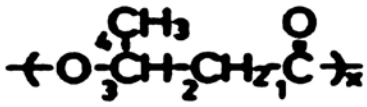


Fig. 4.45 ^{13}C NMR spectrum of purified PHA extracted from *Vibrio* sp. strain BTKB33

Table 4.18 Chemical shift and peak number of extracted purified PHA from *Vibrio* sp. BTKB33



Chemical shift (ppm)	Peak number	Carbon position
166.73	1	C=O
37.75	2	CH_2
67.16	3	CH
21.97	4	CH_3

4.9.3 Differential scanning calorimetry (DSC)

The thermogram obtained from DSC analysis of standard PHB (Fig. 4.46) indicated a melting temperature (Tm) of 171.77°C as reported in literature . The Tm obtained for PHA produced by *Vibrio* sp. BTKB33 was 135.76°C as represented in Fig 4.47 suggesting that it may be a semi-crystalline polymer.

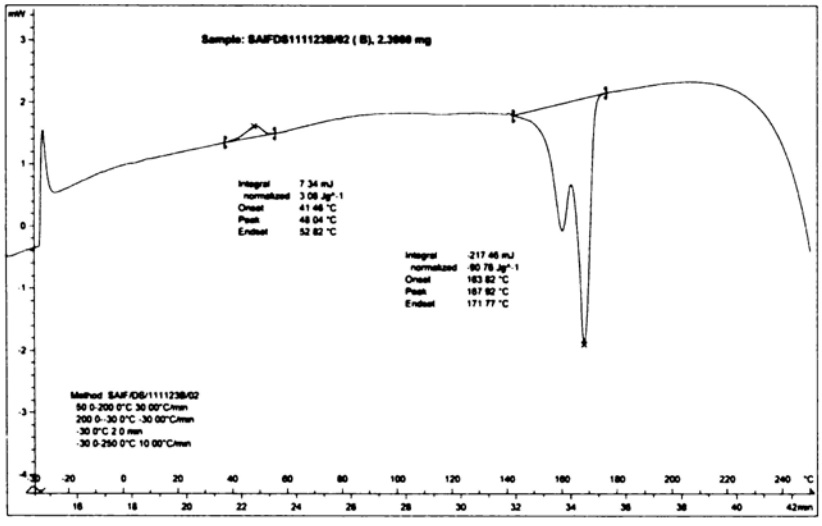


Fig 4.46 The thermogram obtained by DSC of standard PHB (Sigma)

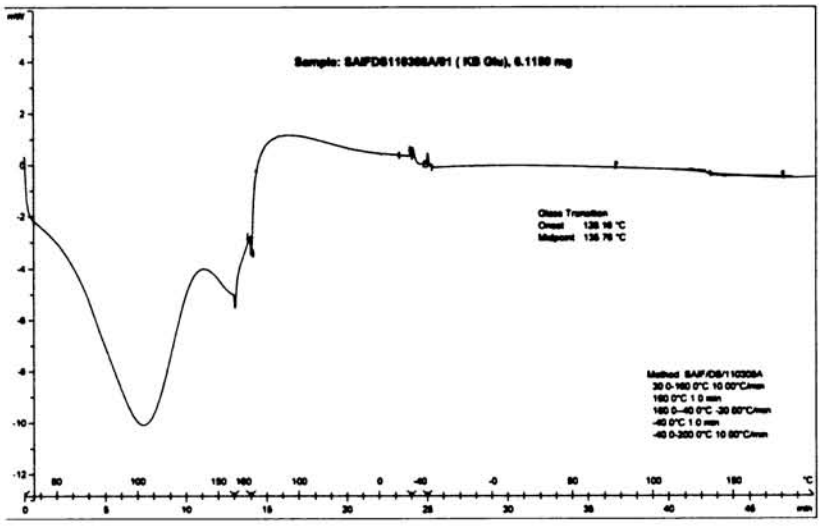


Fig 4.47 The thermogram obtained by DSC of PHAs of *Vibrio* sp. BTKB33

4.9.4 Thermogravimetric analysis (TGA)

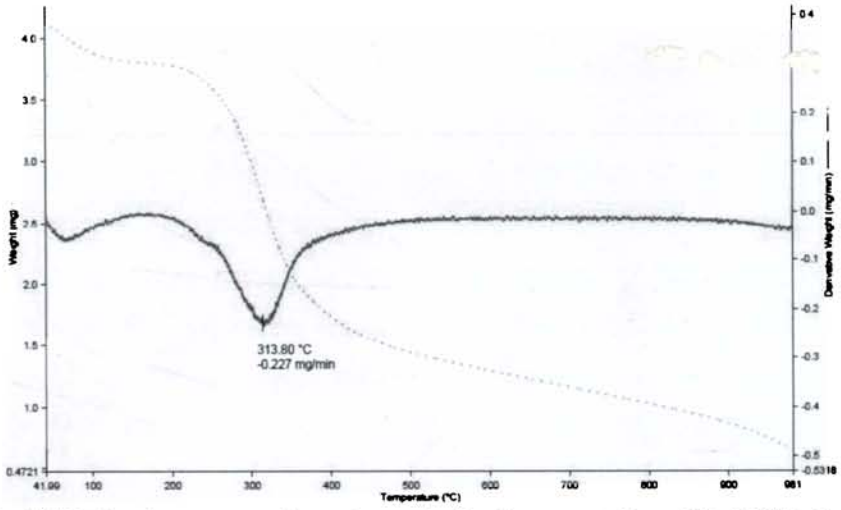


Fig 4.48 The thermogravimetric analysis of extracted purified PHA from *Vibrio* sp. strain BTKB33.

Thermogravimetric analysis showed that this polymer is degraded at 313.80 °C (Fig. 4.48), indicating that the biopolymer has good thermal stability.

5 Discussion

Sampling and identification of PHA accumulating vibrios from marine benthic environments

Marine benthic environments are known to harbor diverse groups of microorganisms, flora and fauna. Knowledge on the biodiversity and economically important flora and fauna from this environment available, even today, is considerably miniscule. Consequently this environment represents a largely untapped source for isolation of novel microorganisms for novel functions. This highly diverse and challenging environment was therefore considered to be the most ideal resource for the isolation of novel PHAs-accumulating *Vibrio* strains with diverse industrial applications.

Until recently, there are only few reports on marine PHAs producing microorganisms (Arun *et al.*, 2009; Ayub *et al.*, 2004; Berlanga *et al.*, 2006; Chien *et al.*, 2007, Lopez *et al.*, 2008; Odham *et al.*, 1986; Rawte and Mavinkurve, 2004; Sun *et al.*, 1994; Weiner, 1997). Some marine bacteria have already been exploited for the industrial production of biopolymers (Lee *et al.*, 2001; Weiner, 1997). Vibrios were among the first reported strains of PHAs producers from marine sediments (Baumann *et al.*, 1971; Oliver and Colwell, 1973) and are reportedly a dominant flora among the commensally heterotrophic bacteria in Cochin backwaters and near-shore areas of west coast of India (Chandrasekaran *et al.*, 1984). They are ubiquitous to the aquatic environment, found free or in association with the aquatic organisms, occurring as they do in estuaries, marine sediments, coastal waters and aquaculture settings worldwide (Barbieri *et al.*, 1999; Heidelberg *et al.*, 2002; Thompson *et al.*, 2001; Urakawa *et al.*, 2000; Vandenberghe *et al.*, 2003; Venter *et al.*, 2004).

In the present study high incidence of PHA accumulating *Vibrio* sp. was observed. Sixty two percent of the *Vibrio* sp. were identified as PHA accumulators during the preliminary screening by Nile blue plate assay method, chosen

primarily for its simplicity and sensitivity in detecting PHA in bacteria (Kranz *et al.*, 1997), wherein the stain diffuses into the cytoplasm of the cells and subsequently into the PHA inclusion resulting in fluorescence under UV light and could therefore be directly used to detect the PHA component in the growing bacterial colonies (Spiekermann *et al.*, 1999). Microbial mats were reported to be potential sources for isolation of PHA accumulating strains (Berlanga *et al.*, 2006; Lopez-Cortes *et al.*, 2008; Rothermich *et al.*, 2000). There are also several reports of PHA accumulating vibrios from marine sediments (Arun *et al.*, 2009; Chien *et al.*, 2007; Rawte and Mavinkurve, 2004; Wei *et al.*, 2011b). The results obtained from the current study are completely in accord with these previous findings, suggesting the possibility of these environments as an excellent source for the isolation of novel PHA accumulating strains.

The occurrence of several extracellular enzymes like chitinase (Itoi *et al.*, 2007; Suginta *et al.*, 2000), alginate lyase (Fu *et al.*, 2007), pectinase (Samuel *et al.*, 1981), protease (Fenical *et al.*, 1993; Maureen *et al.*, 1977; Young *et al.*,1982) and lipase (Giudice *et al.*, 2006; Maureen *et al.*, 1977) have been reported in vibrios. *Vibrio* strains isolated from the intestine of the highly toxic wild puffer fish *Fugu vermicularis radiatus* were reported to produce toxins, that are useful in neurophysiological and neuropharmacological studies (Lee *et al.*, 2000). These studies therefore indicate that marine bacteria, especially vibrios, may also be significant and important sources of other valuable bioactive molecules.

In the marine benthic environments, all life forms are subject to perpetual competition and stress. It is therefore not surprising that the organisms that live in the sea produce an enormous range of biological activities, producing biomolecules ranging from antagonistic substances, enzymes and biopolymeric substances, to name a few. From the previous studies it was reported that the accumulation of PHA enhanced the survival ability of microorganisms under adverse environmental conditions (Ayub *et al.*, 2004; Wang and Bakken, 1998; Kadouri *et al.*, 2005; Lopez *et al.*, 1995). The relation between PHA accumulation

and stress were discussed previously by many researchers (Ayub *et al.*, 2004; Lopez *et al.*, 1995, 1998; López-Cortés *et al.*, 2008; Zhao and Chen, 2007). Lopez *et al.* (1995) reported that accumulation of PHB increases the survival capabilities of bacteria in homogeneous aquatic microcosms. PHB accumulation increased survival of *Bacillus megaterium* in water (Lopez *et al.*, 1998). Nickels *et al.* (1979) observed that the PHB accumulation can be induced in the estuarine detrital microbiota under unbalanced growth conditions such as limitation of critical factors in the presence of carbon and energy sources. In the studies of Lopez-Cortes and his co-workers (2008) on PHA producing bacteria from polluted marine sediments, it was proposed that PHA not only functions as a storage material, but also as a mechanism to cope with stressed and imbalanced nutrient environments.

Phenotypic characterization of potential PHA accumulating vibrios

The diversity of phenotypic characters shown by PHA accumulating vibrios aided in their identification upto species level. The results of biochemical tests, extracellular enzyme screening and antibiotic profiling, can all be used as a reference dataset for species level identification studies of genus *Vibrio* from benthic environments (Alsina and Blanch, 1994; Colwell, 1970; Ottaviani *et al.*, 2003).

Hemolysis on blood agar plates is an indication of the potential for pathogenesis in bacteria. Only 6 strains, viz., BTPT3, BTTN7, BTKB33, BTTC26, BTTN33 and BTMV10 out of 80 tested on blood agar plates were non hemolytic. The non-pathogenic nature of the bacterium is one of the major criterion for selection of microbes for large scale production of any product (Byrom, 1992) and this helped to narrow down the selection of the production organism in this study.

It may be noted that this study revealed that PHAs accumulating vibrios isolated from marine sediments, were producers of several hydrolytic extracellular

enzymes; 93.75% were lipase producers, 88.75% were amylase producers, 88.75% were DNase producers, 87.5% were gelatinase producers, 65% were caseinase producers, 20% were cellulase producers and 5% were pectinase producers, although alginase, xylanase and phosphatase activity were not detected in this study.

Alginase activity is rare among *Vibrio* sp. and was not seen even in the species like *V.alginolyticus, V.parahemolyticus* and *V.harveyi*. Species of vibrios are reported to be producers of gelatinase, amylase, lipase and DNase (Furniss *et al.,* 1978) and the present study also reports similar results. However it is interesting that multiple hydrolytic enzymatic activities have been detected in all the tested strains. Extracellular hydrolytic enzymes such as amylases, caseinases, lipases, DNases, pectinases, cellulases and gelatinases can find diverse application in food industry, as feed additive, in biomedical sciences and in chemical industries (Bhat M, 2000; Jayani *et al.,* 2005; Kulkarni *et al.,* 1999; Pandey *et al.,* 1999,2000; Rao *et al.,* 1998).

The mineralization process in the marine environments are performed mainly by bacterial extracellular enzymes (Belanger *et al.,* 1997). The arsenal of enzymes with these microorganisms gives them an edge to survive under adverse and stressful environmental conditions and in addition play a pivotal role in the recycling of organic carbon and nitrogen compounds (Belanger *et al.,* 1997), providing suitable carbon sources. Since *Vibrio* sp. are the dominant heterotrophic bacteria in the marine environments along the west coast of India (Chandrasekharan *et al.,* 1984), the results of the present study suggests a significant role for vibrios in the mineralization process that happens in the marine benthic environments. These are also indications that vibrios play a key role in nutrient cycling in aquatic environments by taking up dissolved organic matter (Sherr and Sherr, 2000). As flagellates selectively graze on vibrios, these bacteria may contribute immensely to the cycling of organic matter in aquatic settings (Beardsley *et al.,* 2003).

The results suggests that survival of these microbes under adverse conditions like those in the benthic environments, may be dependent not only on their ability to utilize diverse and complex organic material as carbon source by expressing multitude of hydrolytic enzymes but also their capability to accumulate PHA. These abilities can be weighted in, while selecting an industrial production organism. The current findings can also aid in taxonomic classification of members under genus *Vibrionaceae* using extracellular enzyme production as yet another variable (Alsina and Blanch, 1994; Colwell 1970; Ottaviani *et al.,* 2003), and can indicate wider scope for utilization of these vibrios for production of these industrial enzymes as well on a large scale after intensive investigation. The amylase producing strains of *Vibrio* sp. obtained in the present study were used in studies on microbial biodegradation of starch/dextrin-plastic blends (Anna *et al.,* 2008, 2010; Zeena *et al.,* 2010a,b; 2011a,b) and polyvinyl alcohol-plastic blends (Vidya *et al.* 2010; 2011a,b,c,d) implying at the scope of applications.

The PHA accumulating *Vibrio* sp. showed resistance to ampicillin, vancomycin and rifampicin. The incidence of resistance in *Vibrio* sp. towards ampicillin, vancomycin and rifampicin were already reported for *Vibrio* sp. isolated from water and fishery samples along south west coast of India (Manjusha *et al.,* 2005). A higher MAR Index of ≥ 0.3 shown in this study, may be due to anthropogenic effect on the aquatic settings, like persistent use of antibiotics to combat diseases in human and other life forms (Manjusha *et al,* 2005). In the previous studies only 16% isolates from south west coastal regions of India, showed multiple antibiotic resistance patterns (Bernard 2006). The present study observed a high rate of MAR Index in marine vibrios compared to previous findings, confirming fears that multiple antibiotic resistances are increasing by the day (Threlfall *et al.*, 1997).

The higher MAR index pattern along with occurrence of PHA accumulation and multiple hydrolytic extracellular enzyme production ability of

the *Vibrio* sp. inhabiting marine benthic environments gives once again point to the highly stressful conditions in these environments.

16 S rDNA sequence analysis

16S rRNA gene sequence analysis is a reliable method for identifying unknown bacterial isolates. The data generated using the universal 16S rRNA gene segment primers is of great accuracy and reproducibility. This method is less time consuming compared to the conventional phenotypic identification schemes. A 16S rRNA gene sequence similarity of ≥97% is a reasonable level for grouping bacteria into species (Hagstrom *et al.*, 2000).

16S rRNA gene sequence analysis helped to identify 4 species within the 24 selected PHA producing *Vibrio* sp. - seven *V.parahaemolyticus*, seven *V.alginolyticus*, six *V. azureus* and four *V. harveyi* strains. Several species belonging to genus *Vibrio* including *V. natriegens*, *V. nereis* and *V. harveyi* have been reported to produce PHAs (Arun *et al.*, 2009; Boyandin *et al.*, 2008; Chien *et al.*, 2007; Rawte and Mavinkurve, 2004; Sun *et al.*, 1994; Weiner, 1997; Miyamoto *et al.*, 1998).

Phylograms drawn with selected strains helped to study their relatedness or variability. Neighbour joining method was successfully used for this purpose. All the trees were rooted. It was clear from the phylogram of PHA accumulating *Vibrio* sp. that all the strains despite being quite diverse, formed a large clade, but they grouped separately from the related taxa included in this study.

Intra species variation was also analyzed within the three major groups of PHA accumulating vibrios, namely *Vibrio alginolyticus* (7 isolates), *Vibrio parahemolyticus* (5 isolates) and *Vibrio azureus* (7 isolates) with *Photobacterium* sp. (Accession no. D21226.1) as an outgroup. *Vibrio* strains showed a distinct pattern of relatedness among themselves. It was previously reported that the intraspecies variation of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* were quite diverse among themselves with respect to 16S rRNA gene (Bernard 2006).

Intraspecies variation was observed among *Vibrio azureus,* with all 7 strains forming a clade, including strain BTKB33, but separately within this group.

The divergence observed in the phylogentic analysis using 16S rDNA sequences above is also visible in the results of the biochemical analysis, the antibiotic pattern and the exoenzyme profile of these *Vibrio* sp. in the present study. The phenotypic characters exhibited by these marine benthic environmental vibrios are equally diverse and different, despite identification as belonging to the same species.

Production organism-*Vibrio* sp. strain BTKB33

The strain selected for optimization of bioprocess variables by submerged fermentation for enhancing PHA production and PHA content was *Vibrio* sp. BTKB33, with PHA production of 0.21 g/l and PHA content of 19.3% (% of CDW) under unoptimised condition.

The *Vibrio* sp. strain BTKB33 showed close relatedness to *V. azureus* strain VPMP45 (Accession no. 663915.1) (99% identity) by partial 16S rDNA sequence homology. A 16S rRNA gene sequence similarity of ≥97% is a reasonable level for grouping bacteria into species (Hagstrom *et al.*, 2000). The phenotypic characterisation results also confirmed the species level identification of strain BTKB33 as *V. azureus* (Yoshizawa *et al.*, 2009). The nucleotide sequence has been deposited in the GenBank database under accession number HM346671. It was noted that this is the first report of PHA accumulating *V. azureus*. The phylogenetic tree showing relationship of the strain with related taxa showed its relationship with other *V.azureus* strains. In the phylogram, the strain BTKB33 grouped with the *V.azureus* strains to form a major clade. Within the clade, the strain grouped with *V. azureus* strain VPMP45 and showed divergence from the rest.

The strain BTKB33 was non-hemolytic on blood agar indicating its non-pathogenic nature. PCR screening is a rapid method to detect the presence of

desired gene/genes and was employed to screen for three established virulence genes normally found in vibrios. The virulent genes like *toxR, zot* and *ctxA* were absent in strain BTKB33 and this supports its use as production organism. *V. azureus* are nonpathogenic vibrios (Yoshizawa *et al.,* 2009) and therefore normally do not harbor the other virulence genes associated with pathogenicity in vibrios. Strain BTKB33 produced biotechnologically important hydrolytic exoenzymes like caseinase, amylase, gelatinase and DNase suggesting possible use of diverse carbon source for PHA production in large scale. The stability, safety, higher growth and PHA accumulation rates, range of utilizable carbon sources, etc; were among the key factors for the suitability of a bacterium for industrial production for PHAs in large scale (Byrom, 1992). Phase contrast microscopy indicating the brightly refractile cytoplasmic inclusions and Sudan black staining of lipophilic inclusion in *Vibrio* sp. BTKB33; both suggest accumulation of PHAs. From the growth curve it was clear that the organism BTKB33 had a very short generation time of 8.61 minutes with the specific growth rate of 0.001465, which is faster than that of *E.coli* (Edens *et al.,* 1997). Short generation times are essential criterion for any production organism. The log phase (exponential phase) of the bacterium started by 3^{th} hour of incubation and lasted upto 15 hours. These features are contributing and supporting factors in its selection as a production organism.

Molecular characterisation of PHA synthase gene

PHA synthase, considered a crucial enzyme in all PHA synthesis pathways (Anderson & Dawes, 1990; Madison and Huisman, 1999; Rehm and Steinbuchel, 1999), are included as a class of highly versatile enzymes, but are not specific to only one type of hydroxyalkanoic acid (Steinbuchel *et al.,* 1992). The PHA synthases can be broadly categorized into three different types based on their primary amino acid sequences and *in vivo* substrate specificities (Rehm & Steinbuchel, 1999). Among these, Class I PHA synthases are important as they are

preferentially active towards coenzyme A thioesters of various scl 3-HA comprising three to five carbon atoms. They include PHB, PHV, PHBV etc and gene responsible for PHA synthase enzyme is termed *phaC* gene. The relationship between structure and function of PHA synthase has been discussed based on the primary structures, because no crystal structure of PHA synthase has been available (Rehm, 2003).

Small chain length (scl) PHA synthase gene was detected by colony and seminested PCR indicating presence of Class I PHA synthase gene (scl-PHA synthase gene) in the test organisms. Medium chain length (mcl) PHA synthase gene could not be detected in the tested *Vibrio* strains. Earlier reports on PHAs extracted from *Vibrio* sp. indicated scl-PHAs coded by Class I PHA synthase gene (Chien *et al.*, 2007; Wei *et al.*, 2011b); the results of the present study concur with the previous studies.

Vibrio sp. including *V.alginolyticus, V.cholerae, V.furnissi, V.splendidus, V. parahaemolyticus* and *V. harveyi* have been reported to harbor the class I PHA synthase gene (NCBI database). The sequence analysis of the gene product from BTKB33 by BLAST showed 93% similarity with *phaC* gene of *Vibrio alginolyticus* strain 40B (Accession no. NZ ACZB01000148.1). This study allowed us to determine that the 231bp partial sequence of *phaC* gene obtained from *Vibrio* sp. strain BTKB33 is a polyhydroxyalkanoate synthase class I, specifically a polyhydroxybutyrate polymerase. This report is highly significant since the sequence of *phaC* gene obtained is the first deposit in GenBank from *Vibrio azureus,* a species of genus *Vibrio*. As the review of *V.azureus* has not included the determination of PHA production (Yoshizawa *et al.*, 2009), this report also adds to the description of this organism.

The multiple sequence alignment of the deduced partial sequence of *phaC* gene of *Vibrio* sp. BTKB 33 with those included in the analysis, clearly showed the conserved regions of the protein within the genus *Vibrio*, if only in this short

stretch. This multiple sequence alignment also allowed us to look at remarkable differences in the *phaC* gene from vibrios and other bacteria in the study.

Submerged fermentation (SmF) for the production of polyhydroxyalkanoates by *Vibrio* sp. BTKB33

Boyandin *et al.* (2008) pointed out the ability of luminous bacteria of different taxa including *Vibrio* sp. to synthesize PHAs as storage macromolecules. Despite the common practice of exploiting the diversity of bacteria in the environment for the industrial production of novel compounds, there are very few reports that have explored the potential of industrial production of PHAs by bacteria (Chen *et al.*, 2000; Reddy *et al.*, 2003). Factors inducing PHA synthesis are species specific; for some PHA producers it is deficiency of biogenic elements like nitrogen, phosphates etc in the medium whereas for others this is oxygen deficiency (Braunegg *et al.*, 1998). Optimization of different physical and chemical parameters influencing PHA production like sodium chloride concentration, aeration rate, incubation period, initial pH, incubation temperature, N and P content, various carbon and nitrogen sources etc in *Vibrio* sp. were discussed previously (Arun *et al.*, 2009; Belova, 1997; Chien *et al.*, 2007; Rawte and Mavinkurve, 2004; Sun *et al.*, 1994).

Time of maximum PHA production is an important factor in industrial production of PHAs. The incubation period for PHA production depends on the characteristics of the strain and growth rate. In the present study the PHA production commences after 12 h of incubation and reached maximum at 36 h. After 36 h incubation the PHA production decreased with incubation period, which may be due to utilization of accumulated PHAs by the bacterium (Benoit *et al.*, 1990; Nam and Ryu, 1985). PHA accumulated by organisms is utilized as a carbon source for their survival and can therefore cause lowering of PHA accumulation at higher incubation periods (Santhanam and Sasidharan 2010; Yamane *et al.* 1996). The cell dry weight of the *Vibrio* sp. BTKB33 increased

with incubation time upto 42 h of incubation and then decreased. The rapid drop in cell dry weight (CDW) was due to the cell growth decline phase. In marine vibrios PHAs accumulation begins at late log phase, reaching maximum at stationary phase of growth (Rawte and Mavinkurve, 2004).The maximum accumulation of PHAs was during exponential phase in *Rhodopseudomonas palustris* KU003 (Ramachander *et al.*, 2010), *Rhodobacter sphaeroides* ES 16 (Sangharak and Praserstan, 2008) and *A. latus* ATCC 29712 (El-Sayed *et al.*, 2009). *Ralstonia eutropha* accumulated PHB at the stationary phase (Madison and Huisman, 1999). In the studies of Ramadas *et al.* (2009) on PHA accumulation in *Bacillus sphaericus* NCIM 5149, the incubation period upto 28th hour favoured PHA accumulation and after that the cell started to utilize the accumulated PHAs (Benoit, 1990; Nam and Ryu, 1985).

Temperature is an important influencing factor on microbial activity, speeding up enzymatic reaction and can influence the metabolism of bacteria (Wei et al., 2009). The effect of incubation temperature is a critical parameter that has to be controlled in PHA production studies. Maximum PHAs production by BTKB33 was found to be at incubation temperature of 37°C (0.36 g/l). It was found that PHA production decreased drastically at higher temperatures and little/no PHA production was observed at lower temperature below 25°C. The effect of incubation temperature on PHA production are known to vary from one genus to another. The optimum temperature for PHA production in *Cupriavidus taiwanensis* (Wei *et al.*, 2011a) was 30°C, while high temperature of 45°C favoured PHA production in *Haloferax mediterranei* (Lillo and Valera, 1990). In the studies on effect of temperature in PHA accumulation in activated sludges by Wei and co-workers (2009), it was inferred that a lower temperature was suitable for PHA synthesis. Under phosphorous limitation phase, PHA synthesis was maximum at 19°C and 10°C in the studies of Wei *et al.* (2009) and Chinwetkitvanich *et al.* (2004) respectively.

Agitation is required for PHA production in *Vibrio* sp. BTKB33 since PHA production was lowered in static culture conditions. It was observed that PHA production increases with increase in agitation speed and maximum PHA production was observed at 120 rpm. The increase in PHA production could be attributed to increasing oxygen transfer rate and due to increased surface area of contact with media components. Lower agitation speed would affect the dissolved oxygen in the medium. The drastic reduction of dissolved oxygen with respect to air saturation was previously reported in *Halomonas boliviensis* LC1, resulting in the reduction of the polymer production (Quillaguaman *et al.*, 2005). However at higher agitation speed i.e, at 150 rpm, the PHA production by BTKB33 was lower than the value obtained at 120 rpm. This may be due to increase in shear stress on the organism due to higher agitation, thereby causing reduction in growth and PHA production. The CDW increased with increase in agitation speed and maximum CDW measurement was at 150 rpm. In the studies on optimization of agitation speed in PHA production in halotolerant *Rhodobacter sphaeroides* U7 (Kemavongse *et al.*, 2008), the optimum agitation speed for growth was 300 rpm while it was 200 rpm for PHA production.

In the present study PHAs accumulation was dramatically reduced at acidic pH, while alkaline pH supported PHA accumulation. Maximum PHAs production was observed at an initial pH of 7 and the same similar observations were reported previously in the studies of PHA accumulation in *Vibrio* sp. (Arun *et al.*, 2009; Rawte and Mavinkurve, 2004). The influence of pH of cultivation medium on PHA production was also reported in *Cupriavidus taiwanensis* (Wei *et al.*, 2011a), *Haloferax mediterranei* (Lillo and Valera 1990), *Alcaligenes latus* (Palleroni and Palleroni, 1978) and in *Bacillus sphaericus* NCIM 5149 (Ramadas *et al.*, 2009). The optimum pH for maximum cell dry weight measurement and PHA content was also observed at pH 7. Little PHA or no production was observed at pH lower than 6 and these results were in accord with the previous works of Rawte and Mavinkurve (2004) on PHA accumulating vibrios.

The induction of PHA accumulation due to application of stress during fermentation has been reported in several studies (Ayub *et al.,* 2004; Obruca *et al.,* 2010; Wang *et al.,* 2005; Zhao and Chen, 2007). In *Rhizobium* DDSS-69 cultures, NaCl stress resulted in the accumulation of PHB (Natarajan *et al.,* 1995). In the present study the PHA production was found to be maximum at 1.5% NaCl concentration. Since the bacterium was isolated from marine environments, NaCl concentration may play an important role in its PHA production (Chien *et al.,* 2007). These results were in complete agreement with the studies of Wei *et al.* (2011b) on PHA production in *Vibrio* sp. BM-1, where they reported increase in PHA production with increase in NaCl concentration upto 1.8 %, and thereafter a drastic decrease with further increase in NaCl concentration. There were previous reports that PHA production in certain *Vibrio* sp was enhanced by the increase in concentration of NaCl in medium (Arun *et al.* 2009; Chien *et al.* 2007), and was maximum at 30% NaCl concentration (Arun *et al.* 2009).

Selection of suitable medium is other important parameter for PHA production. Depending on the culture conditions that favor PHA accumulation, bacteria that are used for the production of PHA can be grouped into two. The first group that requires limitation of essential nutrients such as nitrogen and oxygen and presence of excess carbon source for the efficient synthesis of PHA and the second which has no requirement for nutrient limitation and can accumulate PHA during exponential growth phase (Khanna and Srivastava, 2005; Lee, 1996). The nitrogen limiting minimal medium formulated by Sun *et al.* (1994) favored maximum PHA production in this study. It was clear that the *Vibrio* sp. BTKB33 belonged to the first group of bacteria requiring limitation of essential nutrients such as nitrogen, oxygen and presence of excess carbon source for the efficient synthesis of PHA. In the present study, it was clear that PHA production was enhanced by stress developed as a result of nitrogen deficiency in the medium.

Pre inoculum age is another major bioprocess variable for PHA production. Production of PHA by BTKB33 was maximum with 12h old seed, but

showed a steady decline with further increase in age of seed. The log phase of strain BTKB33 (exponential phase) was between 3-15 h. It was reported that in marine vibrios, PHAs accumulation begins at late log phase, reaching maximum at stationary phase of growth (Rawte and Mavinkurve, 2004). The maximum accumulation of PHAs was during exponential phase in *Rhodopseudomonas palustris* KU003 (Ramachander *et al.,* 2010), *Rhodobacter sphaeroides* ES 16 (Sangharak and Praserstan, 2008) and *A. latus* ATCC 29712 (El-Sayed *et al.,* 2009). In *Ralstonia eutropha*, PHB accumulation was at the stationary phase (Madison and Huisman, 1999).

Santhanam and Sasidharan (2010) pointed out that optimization of initial microbial load is very important for production studies of PHA. In the current study the PHA production of the bacterium changes with inoculum concentration indicating a correlation between inoculum concentration and PHA production. The PHA production increased with increase in inoculum concentration upto 2.5% initial inoculum concentration. One of the reasons for this affect may be that higher inoculum of bacterial cells rapidly utilized the already accumulated intra cellular PHA granules as carbon and energy source (Santhanam and Sasidharan 2010; Yamane *et al.* 1996). This may be the reason why strain BTKB33 did not accumulate more PHAs with increase in initial inoculum concentration above 2.5%. In organisms like *Alcaligenes eutrophus*, *Alcaligenes latus* and *Pseudomonas oleovorans*, PHAs accumulation was lower at high concentration (5%) of inoculum (Yamane *et al.* 1996). Similar report was found in *Bacillus sphaericus* NCIM 5149 (Ramadas *et al.,* 2009). Vijayendra *et al.* (2007) in their studies on optimisation studies of PHA production in *Bacillus* sp. CFR 256 reported that 1% inoculum is favorouble for PHA production. In some cases like in *Bacillus* sp. higher initial inoculum concentration (10%) was required to produce PHA (Valappil *et al.* 2007).

Nitrogen limitation is necessary to trigger PHA production and generally ammonia is used as the critical control factor for uncoupling the growth of cells

and PHA production (Wang and Lee, 1997). All the nitrogen sources tested in this study enhanced PHA production, with NH_4Cl as the best inorganic nitrogen source. NH_4Cl as the inorganic nitrogen source for maximum PHA production was also reported in *Alcaligenes eutrophus* (Koutinas *et al.*, 2007), *Rhodopseudomonas palustris* KU003 (Ramachander *et al.*, 2010) and *Sinorhizobium fredii* (Liangqi *et al.*, 2006). Ammonium sulphate was the prefered inorganic nitrogen source for PHA accumulation in certain *Vibrio* sp. (Arun *et al.*, 2009) and in *Pseudomonas* sp. LDC-5 (Saranya and Shenbagarathai, 2010).

All the organic nitrogen sources tested in this study improved PHA production compared to control. The PHA production and PHA content was found to be maximum in medium having yeast extract. Urea favoured maximum cell dry weight. It was reported earlier in *B. subtilis* 25 and in *B. megaterium* 12, that peptone as nitrogen sources favoured maximum PHA production (Yuksekdag *et al.*, 2004). The PHA production medium with yeast extract as complex N source for PHA production by marine vibrios has been reported earlier (Wei *et al.*, 2011b).

Among the media ingredients, the inorganic salts play an important role in PHA accumulation (Lee *et al.*, 2007). Effect of inorganic salts like $MgSO_4$/$MgCl_2$ in the monomer composition in the PHAs is reported earlier (Lee *et al.*, 2007). In *Delftia acidovorans,* it was found that the concentration of Mg^{2+} affected the biosynthesis of P(3HB-co-4HB) by affecting glucose uptake from the culture medium. In the present study $MgSO_4$ followed by $CaCl_2$ favored maximum PHA production. A positive influence of $CaCl_2$ supplementation in PHA production was reported earlier (Saranya and Shenbagarathai, 2010). There were also reports on the negative effect of adding mineral salts like $MgSO_4.7H_2O$ in PHB production medium in *Vibrio* sp. BM-1 (Wei *et al.*, 2011b).

Suitable carbon source is considered to be very important for high production of PHA (Santhanam and Sasidharan, 2010). Glucose (2%) was found to be the ideal carbon source for PHA accumulation (0.26 g/l) in *Vibrio* sp.

BTKB33. Glucose as an ideal C- source in PHA accumulation in *Vibrio* sp. has been reported in previous studies (Arun *et al.* 2009; Rawte and Mavinkurve 2002). *Alcaligenes eutrophus and Alcaligenes latus* were reported to accumulate high concentrations of PHA in medium with glucose. But in cases, like *P. oleovorans* ATCC 29347, higher yield of PHA was obtained with n-alkanes as C source for PHA production (Santhanam and Sasidharan, 2010).

In the present study, another likely carbon source supporting PHA production more than other substrates was glycerol. This result point toward the use of a cheap industrial byproduct like glycerol in industrial production of PHAs from *Vibrio* sp. strain BTKB33 in future. Glycerol was already reported as an ideal carbon source source for PHA production in certain vibrios (Chien *et al.* 2007; Wei *et al.,* 2011b). PHA production using biodiesel waste glycerol by *Halomonas* sp. KM-1 Strain (Yoshikazu and Sei-ichi, 2010) and *Ralstonia eutropha* (Taidi *et al.,* 1994) have already been reported. The strain BTKB33 bearing a significant exoenzyme profile, can be manipulated in future for utilization of diverse substrates as C source for PHA production. Since this strain demonstrated the ability to utilize all the tested carbon sources for PHA production, its impact on altering the PHA composition can also be factored in future studies. Carbon source and its concentrations are the key factors altering the PHA compositions in bacteria (Chai *et al.,* 2009).

Statistical approach in optimization of bioprocess variables in PHA production by *Vibrio* sp. strain BTKB33

The traditional ''single-factor" optimization is a shotgun approach, where each parameter is considered to be insensitive to the other process variables involved. In fermentation processes, where operating variables interact and influence each others effects on the response, it is essential that the optimization method should account for these interactions in determining a set of optimal experimental conditions (Sen and Swaminathan, 1997). Plackett-Burman design

(PBD) offers an effective screening procedure and computes the significance of a large number of factors in one experiment, which is time saving and maintains convincing information on each component (Sharma and Satyanarayana, 2006). The Plackett-Burman design has been frequently used for screening of process variables that have the greatest impact on the process (Plackett and Burman, 1946). Usage of PBD in bioprocess variable optimization for microbial production of PHAs is well reported (Mokhtari-Hosseini *et al.,* 2009; Khosravi-Darani *et al.,* 2004).

Response-surface methodology (Khuri and Cornell 1987; Montgomery 1991) is an empirical statistical technique employed for multiple regression analysis by using quantitative data obtained from properly designed experiments to solve multivariate equations simultaneously. The graphical representations of these equations are called response surfaces, which can be used to describe the individual and cumulative effect of the test variables on the response and to determine the mutual interactions between the test variables and their subsequent effect on the response (Sen and Swaminathan, 1997). Statistical design of experiments by the application of factorial experimental design in maximizing PHA production by optimizing major bioprocess variables is well reported (Khanna and Srivastava, 2005; Kemovangse *et al.,* 2008; Lee and Gilmore, 2006; Nikel *et al.*, 2005; Ramadas *et al.,* 2010; Vijayendra *et al.,* 2007). PBD was more suitable for screening of process variables in PHB production by *Ralstonia eutropha* (Khosravi-Darani *et al.,* 2004).

Medium that could support maximum PHA production by *Vibrio* sp. strain BTKB33 was optimized employing statistical approach. Initially process variables were optimized using Plackett-Burman design and in the second stage, Response surface methodology was adopted towards selection of optimal variables and understanding the probable interaction among the significant variables. Effect of individual parameters studied in Plackett-Burman design testified that incubation period, pH, tryptone, inoculum concentration, $MgSO_4$ and Glucose

effected an enhancement in PHA production, whereas yeast extract, process temperature, ammonium chloride, KH_2PO_4 and sodium chloride concentration recorded a negative effect on PHA production when there was an increase in the value of variable. From the results of PBD in the current study, it was inferred that among the eleven variables screened, only three variables, viz. NaCl, temperature and incubation period were found to be the most significant. Experimental data obtained from the RSM studies on *Vibrio* sp. BTKB33, demonstrated the effect of three selected bioprocess variables on PHA production and PHA, showing strong dependence on the presence and levels of selected factors. It was noted that PHA production varied between 0.02 and 0.62 g/l, while PHA content varied between 1.73 and 60.27 (% of CDW) under the experimental conditions studied.

The RSM studies were analyzed with two responses, PHA concentration (g/l) and PHA content (%). The ANOVA analysis of PHA production and PHA content yielded values of Prob>F less than 0.05, suggesting that the model terms were significant, with a P value of 0.0001 and an F value of 3.87 and 4.10 respectively. The goodness of fit model was determined by coefficient (R^2 = 0.8327, 0.8404 response of PHA concentration and PHA content, respectively) which implied that the sample variation of more than 83.27% and 84.04% (from the response of PHA concentration and PHA content respectively) was attributed to the variables and only 16.73 % and 15.96 % (from the response of PHA concentration and PHA content, respectively) of the total variance could not be explained by the model. It was observed that all the selected parameters were significant, and varied levels of interactions were recorded for the variables in their cumulative effect on PHA production and PHA content. The adequate precision that measures the signal-to-noise ratio was 6.276 and 7.201 for the response of PHA concentration and PHA content respectively. The ratio greater than 4 is desirable as it indicates an adequate signal. Thus, this model could be used to navigate the design space.

The pair wise interactions among the factors in terms of PHA production and PHA content under the optimized conditions were assessed by examining the response surfaces. The three parameters optimized included NaCl concentration (1.26% w/v), Incubation period (35.97 h), and temperature (30.02°C). In the RSM studies, lower NaCl concentration favored PHA production and these results were in agreement with the studies of Wei *et al.* (2011b) on PHA production in *Vibrio* sp. BM-1. As *Vibrio* sp. strain BTKB33 is a marine benthic isolate the optimization of NaCl concentration assumed importance for PHA production studies. The present study also revealed the crucial role of optimization of NaCl concentration in PHA accumulation studies. This finding reflected the necessity of controlling the salinity of the culture within a proper range to prevent high osmotic stress, thereby improving polymer production (Wei *et al.,* 2011a). Another bioprocess variable optimized was the time of incubation. The incubation period for PHA production depends on the characteristics of the strain and growth rate. In the statistical optimization studies, incubation period of 36h favored PHA production by *Vibrio* sp. BTKB33. The lower incubation period for PHA accumulation of 36 h obtained was therefore significant as a lower incubation period is an important criteria for the industrial production of PHAs. *R.eutropha* ATCC 17697 commenced PHA production only after 60 h incubation (El-Sayed et al., 2009). In *Alcaligenes latus* (Grothe *et al.,* 1999), PHA production needed 3-4 days growth, while 48 h was required for *Paracoccus seriniphilus* E71 strain isolated from a polluted marine microbial mat (Lopez-Cortes *et al.,* 2010). Incubation temperature is an important bioprocess variable influencing microbial activity, effecting speed of enzymatic reaction and thereby influencing the metabolism of bacteria (Wei *et al.,* 2009). The effect of incubation temperature on PHA production can vary from one genus to another (Lillo and Valera 1990; Wei *et al.* 2011b). In the present study lowering the incubation temperature favored PHA production. Wei and co-workers (2009) inferred that a lower temperature is suitable for PHA synthesis. Under phosphorous limitation phase, PHA synthesis

was maximum at 19°C (Wei *et al.*, 2009) and 10°C (Chinwetkitvanich *et al.* 2004) in activated sludge. Wei *et al.* (2009) observed a lower optimum temperature under nitrogen limitation phase compared to phosphorous limitation phase, while studying the effect of temperature in PHA accumulation in activated sludge, which indicates the importance of studying the interaction between temperature and other bioprocess variables in PHA production.

Validation of the deduced response surface model based on previous experiments was carried out in shake flask under conditions predicted by the model. The experimental values were found to be very close to predicted values, and hence the model was successfully validated.

Optimized conditions of major bioprocess variables for the PHA production and PHA content in *Vibrio* sp. BTKB33 after statistical analysis were: NaCl 1.26% (w/v); Temperature 30.02°C; Incubation period 36 h; pH 7.5; glucose concentration 3% (w/v); Ammonium Chloride 0.08% (w/v); Yeast Extract 0.08% (w/v); KH_2PO_4 0.08% (w/v); $MgSO_4$ 0.01% (w/v); Tryptone 0.3% (w/v) and Inoculum concentration 5.5% (v/v). The time course studies using *Vibrio* sp. strain BTKB33 under final optimized conditions revealed that PHA production increased rapidly during initial stages of fermentation and maximum PHA production was recorded at 36 h with PHA production of 0.8 g/l and PHA content of 69%. However with further progress of fermentation, the PHA production declined with incubation period. Similar pattern was observed in case of PHA content determination in the strain. The PHA accumulation and PHA content per CDW of the *Vibrio* sp. strain BTKB33 obtained after statistical analysis of bioprocess variables using PB-Design and RSM studies under optimized condition was nearly 4 times higher than that obtained under the unoptimized conditions.

Characterization of extracted purified PHAs

The functional groups of the extracted PHA was confirmed as C=O groups by the presence of bands at 1736.90 cm^{-1} in FT-IR spectroscopy of

extracted PHAs from *Vibrio* sp. BTKB33 and this result confirmed the presence of PHAs in this strain (De Smet *et al.*, 1983; Hong *et al.*, 1999; Kansiz *et al.*, 2000; Shamala *et al.*, 2003). In these polymers, the carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighboring monomer (Madison and Huisman, 1999). The FTIR spectra of the purified PHAs extracted from *Vibrio* sp. BTKB33 were compared with the FTIR spectrum of commercial PHB (sigma) and with previous results available in literature (Castillo *et al.*, 1986; De Smet *et al.*, 1983; Hong *et al.*, 1999; Kansiz *et al.*, 2000) and it is inferred that the spectra of the *Vibrio* sp. BTKB33 were showing maximum resemblance with the polyhydroxybutyrate (PHB).

The FTIR results are in agreement with those obtained from the molecular characterization of PHA synthase genes in Vibrios and both revealed the presence of polyhydroxybutyrate (PHB). The purified PHA from BTKB33 was analyzed by ^{13}C NMR to elucidate the structure of the biopolymer. The identity of the monomeric units in the PHA was obtained on comparison of the ^{13}C spectra with those of other PHAs available in literature. The NMR spectra of the extracted PHA correlated with the specific peaks corresponding to carbon atoms in the PHB structure (Doi *et al.*, 1989; Kemavongsa *et al.*, 2008) such as C=O, CH_2, CH and CH_3 groups and it was inferred that the extracted PHA from the *Vibrio* sp. BTKB33 is PHB. This concured with the results of FTIR analysis and molecular characterization of PHA synthase gene in *Vibrio* sp. strain BTKB33.

Chien *et al.* (2007) in their studies on PHA accumulation in vibrios from marine sediments, had reported that *Vibrio* sp. harbored PHB and no other type of PHA, pointing out that the diversity of PHAs produced in marine environments may not be as versatile as found in other environments. The present study also reports only PHB accumulation in the *Vibrio* sp. tested.

In Differential Scanning Calorimetric (DSC) analysis, the melting temperature (Tm) of extracted purified PHA is found to be 135.76°C, which was slightly lower than the reported value for PHB. T_m depends on the molecular

weight of the polymer, so lower grades will have lower melting points than expected. The Tm value reported for PHB is between 174.73 - 177°C and that of polypropylene is 176°C (Madison and Huisman 1999; Pal and Paul 2002). The thermograms of standard PHB (Sigma) indicated a melting temperature (Tm) of 171.77°C as reported in literature (Furukawa *et al.,* 2005; Loo *et al.,* 2005; Matsusaki *et al.,* 2000). Highest Tm value for an mcl-PHA reported so far was 77.62°C reported by Liu and his co-workers (2011).

Thermogravimetric Analysis (TGA) measures the amount and rate of change in the weight of a material as a function of temperature or time in a controlled atmosphere. TGA showed that this polymer extracted from BTKB33 was degraded at 313.80°C, indicating that the biopolymer has good thermal stability. The thermal stability of the PHA extracted from BTKB33 was slightly higher than reported thermal stability value of PHB in literature (Xu *et al.,* 2006). DSC analysis and TGA results point towards the possibility of addition of slight impurities like hydroxy valerate (HV) monomers in the polymer (Loo *et al.*, 2005; Matsusaki *et al.,* 2000).

The molecular characterization studies of *phaC* gene, FTIR and NMR analysis of purified polymer extracted from *Vibrio* sp. strain BTKB33 revealed the presence of polyhydroxybutyrate (PHB), smallest known scl-PHA. PHB is the main candidate scl-PHAs displaying a similar degree of crystallinity and melting point (*T*m) as polypropylene (PP), being stiffer and more brittle than PP; but its copolymerization with HV monomers reduces its stiffness and increases its toughness, giving a product with desirable properties for commercial applications. The PHB, is ubiquitous in nature and are found in trace amounts in human plasma (Madison and Huisman, 1999). As it is biocompatible, it finds several applications in medicine and pharmacy (Aebischer *et al.,* 1988; Baptist and Ziegler, 1965; Bowald and Johansson-Ruden 1990, 1997; Hocking and Marchessault, 1994; Holmes, 1985; Ljungberg *et al.,* 1999; Williams and Martin, 2002).

The present study discussed PHA accumulation in *Vibrio* sp., their phenotypic and genotypic variation, the high incidence of multiple hydrolytic enzymes and higher MAR index. A potential non-pathogenic production strain, *Vibrio* sp. strain BTKB33 with a short generation time and with ability to utilize diverse carbon sources, was selected for PHA production studies under submerged fermentation conditions. Major bioprocess variables enhancing PHA production were optimized using statistical approaches. The biopolymer accumulated in the strain was characterized as PHB employing phenotypic and molecular characterization approaches.

6 Summary

The marine environment has proved to be substantial resource for novel bacteria producing enzymes and biopolymer. Sediment samples from marine benthic environments including estuaries, salt pans, mangroves and beaches were used to isolate 828 strains of *Vibrio* sp. employing routine biochemical tests of which 68% were identified to be PHA accumulators. In this study several strains of fast-growing, easy to cultivate, PHA producing *Vibrio* sp. were identified.

Phenotypic characterization of PHA accumulating strains involved biochemical tests, exo-enzyme screening and antibiotic susceptibility tests. Highly diverse phenotypic variations were shown by PHA accumulating vibrios. Eighty strains were selected for further studies based on their PHA accumulating ability, of which only six were non hemolytic on blood agar plates.

The benthic PHA producing vibrios produced several hydrolytic exoenzymes - caseinase, amylase, lipase, gelatinase and DNase. A few also produced extracellular cellulase and pectinase. The ability to express extracellular hydrolytic enzymes enhance their survival in the natural environments, and gives inkling regarding their role in the mineralization process in the benthic environments; at the same time indicating their capability in utilizing diverse, complex organic C-source in industrial production of PHA.

The strains of *Vibrio* sp. tested had a MAR index ≥ 0.3 with resistance to vancomycin, rifampicin and ampicillin. Their exoenzyme profile and antibiotic resistance pattern, along with their PHA accumulation ability, only point towards the stressful habitats of these microbes.

The secondary screening segregated 24 strains with PHA accumulation more than 0.04g/l. These 24 strains of *Vibrio* sp. producing ≥ 0.04 g/l of PHA were characterized upto species by partial 16S rDNA sequence analysis, followed by the submission of the sequences to the GenBank database. These strains were

identified as *V. parahaemolyticus* (N=7), *V. alginolyticus* (N=7), *V.azureus* (N=6), and *V. harveyi* strains (N=4).

The phylogram constructed based on the partial 16S rDNA sequence of isolated *Vibrio* sp. and related taxa, showed the close relationships among species of the genus *Vibrio.* It was clear from the phylogram that all the strains were diverse, but claded together as a large clade, while they grouped separately from the related taxa. Intra species variation was also analyzed with three major groups of PHA accumulating vibrios obtained in this study, namely *Vibrio alginolyticus* (7 isolates), *Vibrio parahemolyticus* (5 isolates) and *Vibrio azureus* (7 isolates).

The preliminary and secondary screening helped to select *Vibrio* sp. strain BTKB33 with maximum PHA accumulation (0.21 g/l), having CDW of 1.07 g/l and PHA content of 19.3 % of CDW out of the 24 selected strains as production organism for further fermentation studies. The production strain BTKB33 was identified as *V. azureus* by 16S rDNA sequence analysis and phenotypic characterization studies. The nucleotide sequence has been deposited in the GenBank database under accession number HM346671. The phylogenetic tree based on partial 16S rDNA sequence of *Vibrio* sp. BTKB33 and related *Vibrio* sp. showed the divergence of the strain within related taxa. The strain BTKB33 was non hemolytic in blood agar and negative when screened for a few virulent genes like *toxR, zot* and *ctxA* screened by PCR, indicating its non-pathogenic nature. Although other screens may be required, this supports its application as production organism in large scale industrial production of PHAs. Strain BTKB33 also produced multiple extracellular enzymes like caseinase, amylase, gelatinase and DNase and had a MAR index of 0.4. The growth curve of this organism showed that it had a very short generation time of 8.61 minutes with the specific growth rate of 0.001465. The log phase (exponential phase) of the bacterium started by 3rd hour of incubation and lasted upto 15 hours. Phase contrast microscopy indicated the brightly refractile cytoplasmic inclusions and Sudan black staining of

lipophilic inclusion in *Vibrio* sp. BTKB33; both suggesting accumulation of PHAs.

The presence of Class I PHA synthase gene (scl-PHA synthase gene) was confirmed in the test organisms using colony PCR and semi-nested PCR which gave 406bp and 496bp products respectively. None of the tested organisms were positive for medium chain length (mcl) PHA synthase gene by PCR. The presence of small chain length (scl) PHA synthase gene was indicated in *Vibrio* sp. strain BTKB33.The sequence analysis of the *phaC* gene product from BTKB33 by BLAST showed maximum identity (93% similarity) with *phaC* gene of *Vibrio alginolyticus* strain 40B (Accession no. NZ ACZB01000148.1). This result is significant, as the sequence of *phaC* obtained from the current study, is the first deposit in GenBank from the *Vibrio azureus* species of the genus *Vibrio*. The partial *phaC* sequence of *Vibrio* sp. strain BTKB33 indicated that it was a class I poly-(3-hydroxyalkanoate) synthase, specifically a poly-beta-hydroxybutyrate polymerase.

The alignment of the deduced 76 amino acid peptide sequence of *phaC* gene from *Vibrio* sp. strain BTKB33 with other reported *phaC* genes available in NCBI database, showed the similarity within this 76 amino acid stretch with that of the related *Vibrio* sp. and also the divergence with regard to other bacteria. This 76 amino acid sequence was analyzed using the putative conserved domains search service (RPS-BLAST) and it was found that the partial *phaC* gene of strain BTKB33 comes under conserved domain model TIGR01838, which represents the class I subfamily of PHA synthase.

The submerged fermentation for the production of polyhydroxyalkanoates by *Vibrio* sp. strain BTKB33 for the optimization of bioprocess variables for PHA production by *Vibrio* sp. strain BTKB33 employing "one-factor-at-a-time" method was done and the results are as summarized in table 5.1.

Table 6.1 Optimized bioprocess variables for PHA production by *Vibrio* sp. strain BTKB33 employing "one-factor-at-a-time" method

Sl no.	Bioprocess variables tested	Condition showing maximum PHA concentration (g/l).	Condition showing maximum CDW (g/l)	Condition showing maximum PHA content (% of CDW)
1	Incubation period	36h (0.21 g/l)	42h (0.9g/l)	12h (29.5 %)
2	pH	pH 7 (0.22 g/l)	pH 7 (1.13g/l)	pH (19.3 %)
3	NaCl concentration	1.5% (0.23 g/l)	2 % (1.3 g/l).	1.5% (18 %)
4	Agitation	120 rpm (0.24 g/l)	150 rpm (1.47 g/l)	120 rpm (18 %)
5	Temperature	35°C (0.25 g/l)	35°C (1.39 g/l).	35°C (17.7 %).
6	Media	PHA minimal medium outlined by Sun *et al.* 1994 (0.26 g/l)	Nutrient medium (2.1 g/l)	PHA minimal medium outlined by Sun *et al.* 1994 (16%)
8	Age of inoculum	12h (0.31 g/l).	12h (1.5 g/l).	12h (20%)
10	Inorganic N-source	NH_4Cl (0.32 g/l)	$NaNO_3$ (2.85 g/l)	NH_4Cl (24 %)
5	Organic N - source	Yeast extract (0.33 g/l)	Urea (2.1 g/l)	Yeast extract (25.9 %)
11	Inorganic salts	$MgSO_4$ (0.36 g/l)	$CaCl_2$ (2.8 g/l)	$MgSO_4$ (22.5%)
9	Inoculum conc.	2.5 % (0.37 g/l)	10 % (2.2 g/l).	2.5% (25)
12	Carbon source	Glucose (0.48 g/l)	Glycerol (1.38 g/l)	Glucose (42.7 %)

A 2.29 fold increase in PHA production and 2.22 fold increase in PHA content was observed after the "one factor at a time" optimization method of major bioprocess variables of submerged fermentation by *Vibrio* sp. strain BTKB33.

A statistical approach was followed to optimize medium supporting maximum PHA production by *Vibrio* sp. strain BTKB33, where initially process variables were optimized using Plackett-Burman design and in the second stage, Response surface methodology was adopted for selection of optimal variables to understand the probable interaction among these significant variables.

From the PB-Design, the variables that greatly influenced PHA production were incubation time, temperature and NaCl concentration. Effect of individual parameters studied in PBD testified that incubation period, pH, tryptone, inoculum concentration, $MgSO_4$ and Glucose effected an enhancement in PHA production, whereas yeast extract, process temperature, ammonium chloride, KH_2PO_4 and sodium chloride concentration recorded a negative effect on PHA production when there was an increase in the value of variable.

In the RSM studies employing Box-Behnken design, the three dimensional response surface model to study the interactions among bioprocess variables like temperature, incubation period and NaCl conc., predicted maximum PHA production (upto 0.64 g/l) and PHA content (64.56%) and the model was successfully validated.

Optimized conditions of major bioprocess variables of the medium for the PHA production and PHA content in *Vibrio* sp. BTKB33 after statistical analysis were: NaCl 1.26% (w/v); temperature 30.02°C; incubation period 35.97 h; pH 7.5; glucose concentration 3% (w/v); ammonium chloride 0.08% (w/v); yeast extract 0.08% (w/v); KH_2PO_4 0.08% (w/v); $MgSO_4$ 0.01% (w/v); tryptone 0.3% (w/v) and inoculum concentration 5.5% (v/v).

Final time course study under optimized conditions for PHA production by *Vibrio* sp. BTKB33, gave PHA concentration and PHA content of 0.79 g/l (a 3.76 fold increase) and 69 % (3.58 fold increase) respectively at 36h incubation.

The FTIR spectrum and NMR analysis identified the extracted purified PHAs in *Vibrio* sp. strain BTKB33 as polyhydroxybutyrates (PHB), smallest PHAs having wide application in domestic, industry and medicine. The FTIR and

NMR results are in agreement with the molecular characterization of PHA synthase gene in strain BTKB33. In the DSC analysis, the melting temperature (Tm) of extracted purified PHA was found to be 135.76°C and had a higher degradation temperature of 313.80°C in the thermogravimetric analysis.

6 Conclusion

The incidence of PHA accumulation in *Vibrio* sp. isolated from marine sediments was observed to be high, indicating that the natural habitat of these bacteria are stressful. Considering their ubiquitous nature, the ecological role played by vibrios in maintaining the delicate balance of the benthic ecosystem besides returning potential strains, with the ability to elaborate a plethora of extracellular enzymes for industrial application, is significant. The elaboration of several hydrolytic enzymes by individuals also emphasize the crucial role of vibrios in the mineralization process in the marine environment. This study throws light on the extracellular hydrolytic enzyme profile exhibited by vibrios. It was concluded that apart from the PHA accumulation, presence of exoenzyme production and higher MAR index also aids in their survival in the highly challenging benthic environments. The phylogenetic analysis of the strains and studies on intra species variation within PHA accumulating strains reveal their diversity.

The isolate selected for production in this study was *Vibrio* sp. strain BTKB33, identified as *V.azureus* by 16S rDNA sequencing and phenotypic characterization. The bioprocess variables for PHA production utilising submerged fermentation was optimized employing one-factor-at-a-time-method, PB design and RSM studies. The statistical optimization of bioprocess variables revealed that NaCl concentration, temperature and incubation period are the major bioprocess variables influencing PHA production and PHA content. The presence of Class I PHA synthase genes in BTKB33 was also unveiled. The characterization of *phaC* genes by PCR and of the extracted polymer employing FTIR and NMR analysis revealed the presence of polyhydroxybutyrate, smallest known PHAs, having wider domestic, industrial and medical application. The strain BTKB33 bearing a significant exoenzyme profile, can thus be manipulated

in future for utilization of diverse substrates as C- source for PHA production. In addition to BTKB33, several fast growing *Vibrio* sp. having PHA accumulating ability were also isolated, revealing the prospects of this environment as a mine for novel PHA accumulating microbes. The findings of this study will provide a reference for further research in industrial production of PHAs from marine microorganisms.

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APPENDIX I

Reagents

Oxidase reagent

Tetramethyl p-phenenylenediaminedihydrochloride	1 g
Isoamyl alcohol	100 ml

Nile Blue A solution

Nile blue A (Himedia, Mumbai)	0.25 mg
Dimethylsulfoxide (DMSO)	1.00ml

Tris-Acetate EDTA (50 X)

Tris base	242 g
0.5 M EDTA (pH 8.0)	100 ml
Glacial acetic acid	57.1 ml

Distilled water to 1000 ml
The stock solution was diluted to 1X for gel runs.

Glycerol Loading Dye (6X)

Bromophenol blue	0.26 g
Glycerol	30 ml

Ethidium Bromide (10 mg/ml)

Ethidium Bromide	0.1 g
Distilled water	10 ml

The 10 mg/ml of solution was kept in dark bottle and stored at 4°C. For staining agarose gels, a working solution of 0.5 µg/ml was made.

Proteinase K (10 mg/ml)

Proteinase K	5 mg
Sterile distilled water	0.5 ml

Stored at -20^{0}C in 50 µl aliquots.

Sodium dodecyl sulphate (SDS) – 10%

SDS	10 g
Distilled water	100 ml

Sodium dodecyl sulphate (SDS) – 1%

SDS	1 g
Distilled water	100 ml

Sodium hypochlorite solution (NaOCl) – 30%

NaOCl	30ml
Distilled water	70 ml

Disodium ethylenediamine tetraacetete (EDTA) - 0.5 M

EDTA	186.1 g
Distilled water	1000 ml
NaOH	~20 g

EDTA was dissolved in 800 ml of distilled water and stir vigorously on a magnetic stirrer. Adjusted pH t0 8.0 using NaOH pellets and made the final volume to 1000 ml. Autoclaved before use.

Tris EDTA (TE) buffer

Tris-HCl	10 mM	2 ml 1 M Tris-HCl (pH 8.0)
EDTA (pH 8.0)	1 mM	0.4 ml 0.5 M EDTA
Distilled water to 200 ml		

Sodium Acetate - 3 M

Sodium Acetate•$3H_2O$	408.3 g
H_2O	800 ml

Adjusted the pH to 5.2 with glacial acetic acid or adjusted the pH to 7.0 with dilute acetic acid. Adjused the volume to 1 liter with H_2O. Dispensed into aliquots and sterilized by autoclaving.

Media

Nutrient Medium (Himedia, Mumbai, India)

Peptic digest of animal tissue	0.5 % (w/v)
Sodium chloride	0.5 % (w/v)
Beef extract	0.15 % (w/v)
Yeast extract	0.15 % (w/v)
pH (25°C)	7.4±0.2

Dissolved 1.3g of media (Himedia, Mumbai, India) in 100ml of distilled water. Mixed well, autoclaved at 15 lbs pressure for 15 minutes and cooled to 50-55°C. When used as solid agar medium, 2.0% agar (w/v) was added to the medium for agar plate preparation.

Mueller -Hinton agar (Himedia, Mumbai, India)

Beef, dehydrated infusion	30.0 % (w/v)
Casein hydrolysate	1.75 % (w/v)
Starch	0.15 % (w/v)
pH (25°C)	7.4±0.2

Dissolved 38g of media (Himedia, Mumbai, India) in 100ml of distilled water and add 2g agar to it and heated to boiling. Mixed well, autoclaved at 15 lbs pressure for 15 minutes and cooled to 50-55°C. The sterile medium was poured into sterile plates and the plates were stored at 4°C .

PHA production medium (Sun *et al.*, 1994)

NaC1	1 % (w/v)
Na_2HPO_4	0.37 % (w/v)
KH_2PO_4	0.1% (w/v)
$(NH_4)_2HPO_4$	0.05 % (w/v)
$MgSO_4.7H_20$	0.02 % (w/v)
Glycerol	2 % (v/v)
Bactotryptone	0.5 % (w/v)
Yeast extract	0.05 % (w/v)
pH	7±0.3

The medium was prepared in distilled water, autoclaved at 121°C and cooled to 60°C. The glycerol and $MgSO_4.7H_20$ were autoclaved separately. The cooled medium was added with previously sterilized glycerol and $MgSO_4.7H_2O$, the contents were mixed well to dissolve before inoculation.

In case of Nile Blue A plate agar preparation, Nile Blue A solution was added to agar medium to give a final concentration of 0.5 $\mu g/ml^{-1}$, the contents were mixed well and the medium was poured onto sterile petri dishes. When used as solid agar medium, 2.0% agar (w/v) was added to the medium for agar plate preparation.

Blood agar medium (Brown, 1919)

Enzymatic digest of Casein	1.5 % (w/v)
Enzymatic digest of animal tissue	0.4 % (w/v)
Yeast extract	0.2 % (w/v)
Starch	0.1 % (w/v)
Sodium Chloride	0.5 % (w/v)
Agar	1.4 % (w/v)
Final pH: 7.0 ± 0.2 at 25°C	

Suspended 42 g of the blood agar base in one liter of purified water. Heated the agar base with frequent agitation and boiled for one minute to completely dissolve the medium. Autoclaved the medium at 121°C for 15 minutes and prepared 10% blood agar by aseptically adding the appropriate volume of sterile defibrinated blood to melted sterile agar medium, cooled to 45 - 50°C and poured into sterile petriplates.

Zobell Marine Broth (Himedia, Mumbai, India)

Peptic digest of animal tissue	0.5 % (w/v)
Yeast extract	0.1 % (w/v)
Ferric citrate	0.01 % (w/v)
Sodium chloride	1.945 % (w/v)
Magnesium chloride	0.88 % (w/v)
Sodium sulphate	0.324 % (w/v)
Calcium chloride	0.18 % (w/v)
Potassium chloride	0.055 % (w/v)
Sodium bicarbonate	0.016 % (w/v)
Potassium bromide	0.008 % (w/v)
Strontium chloride	0.0034 % (w/v)
Boric acid	0.0022 % (w/v)
Sodium silicate	0.0004 % (w/v)
Sodium fluoride	0.00024 % (w/v)
Ammonium nitrate	0.00016 % (w/v)
Disodium phosphate	0.0008 % (w/v)

Dissolved 4.025g of media (Himedia, Mumbai, India) in 100ml of distilled water. Mixed well, autoclaved at 15 lbs pressure for 15 minutes and cooled to 50-55°C. When used as solid agar medium, 2.0% agar (w/v) was added to the medium.

Starch agar Medium (Furniss *et al.* 1979)

Dissolved 1.3g of Nutrient media (Himedia, Mumbai, India) and 2.0% agar (w/v) in 100ml of distilled water. Mixed well, autoclaved at 15 lbs pressure for 15 minutes and cooled to 50-55°C. The soluble starch (0.1%) was sterilized separately and added to the agar media at bearable temperature just before pouring onto petriplates.

Gelatin agar plate (Furniss *et al.* 1979)

Dissolved 1.3g of Nutrient media (Himedia, Mumbai, India) and 2.0% agar (w/v) in 100ml of distilled water. Mixed well, autoclaved at 15 lbs pressure for 15 minutes and cooled to 50-55°C. The gelatin (0.1%) was sterilized separately and added to the agar media at bearable temperature just before pouring onto petriplates.

DNA agar plate (Zierdt *et al.*, 1970)

DNA (Himedia, Mumbai)	0.2% (w/v)
Tryptone (Difco)	2% (w/v)
NaCl	0.5% (w/v)
Agar	1.5% (w/v)

The medium was prepared in distilled water, autoclaved at 121°C and cooled to 60°C. The DNA (0.2%) was autoclaved separately. The cooled medium was added with previously sterilized DNA, the contents were mixed well to dissolve and the medium was poured onto sterile petridishes.

JG agar medium (Jayasankar and Graham, 1970)

K_2HPO_4	0.05 % (w/v)
$MgSO_4.\ 7H_20$	0.01 % (w/v)
NaCl	0.02 % (w/v)
$CaCl_2.2H_2O$	0.02 % (w/v)
$FeCl_3.6H_20$	0.001 % (w/v)
Yeast extract	0.1 % (w/v)
Pectin (HiMedia, India)	0.5 % (w/v)
Agar	2 % (w/v)

The medium was prepared in distilled water, adjusted to pH 7, autoclaved at 121°C and cooled to 60°C. The pectin (0.5%) was autoclaved separately. The cooled medium was added with previously sterilized pectin, the contents were mixed well to dissolve and the medium was poured onto sterile petridishes.

Xylan agar plates (Anuradha *et al.*, 2007)

Xylan	0.7 % (w/v)
Yeast extract	0.1 % (w/v)
NaCl	0.5 % (w/v)
K_2HPO_4	0.1 % (w/v)
$MgSO_4$	0.02 % (w/v)
$CaCl_2$	0.01 % (w/v)
Na_2CO_3	1 % (w/v)

The medium was prepared in distilled water, adjusted to pH 7, autoclaved at 121°C and cooled to 60°C. The Na_2CO_3 (1%) was autoclaved separately. The cooled medium was added with previously sterilized Na_2CO_3, the contents were mixed well to dissolve and the medium was poured onto sterile petridishes.

Casein agar plate (Kazanas 1968)

Casein	1.5 % (w/v)
Beef extract	0.3 % (w/v)
Peptone	0.5 % (w/v)
NaCl	3 % (w/v)
Agar	2 % (w/v)

The medium was prepared in distilled water, autoclaved at 121°C and cooled to 60°C. The Casein (1.5%) was sterilized separately. To prevent the casein from precipitating, the base solution and casein were both tempered to 50°C before mixing. The cooled medium was added with previously sterilized Casein, the contents were mixed well to dissolve and the medium was poured onto sterile petridishes.

Tween 80 agar plate (Furniss *et al.*, 1979).

Peptone	1 % (w/v)
NaCl	1 % (w/v)
$CaCl_2.H_2O$	0.01 % (w/v)
Agar	2 % (w/v)
Tween 80	1 % (v/v)
Distilled water	100ml

The medium was prepared in distilled water, adjusted to pH 7, autoclaved at 121°C and cooled to 60°C. The Tween 80 (1%) was autoclaved separately. The cooled medium was added with previously sterilized Tween 80 (1%), the contents were mixed well before inoculation.

Sodium alginate agar plate (Furniss *et al.*, 1979).

Zobell's Marine medium	5.51 % (w/v)
Sodium alginate	2 % (w/v)
Agar	2 % (w/v)

The medium was prepared in distilled water, autoclaved at 121°C and cooled to 60°C. The Sodium alginate (2%) was sterilized separately. The cooled medium was added with previously sterilized Sodium alginate, the contents were mixed well to dissolve and the medium was poured onto sterile petridishes. The solution was mixed carefully to avoid forming bubbles. Plates were poured while the medim was still hot because alginic acid solution are very viscous and the viscocity increases as they cool.

Carboxymethylcellulose agar plate (Apun *et al.*, 2000)

Yeast Extract	0.2 % (w/v)
KH2PO4	0.1 % (w/v)
MgSO4	0.5 % (w/v)
Carboxymethylcellulose (CMC)	0.5 % (w/v)
Agar	2 % (w/v)

The medium was prepared in distilled water, adjusted to pH 7, autoclaved at 121°C and cooled to 60°C. The CMC (0.5%) was autoclaved separately. The cooled medium was added with previously sterilized CMC, the contents were mixed well to dissolve and the medium was poured onto sterile petridishes.

NBRIP medium (Nautiyal 1999)

Glucose	0.1 % (w/v)
$Ca_3(PO_4)_2$	0.5 % (w/v)
$MgCl2.6H_2O$	0.5 % (w/v)
$MgSO4.7H_2O$	0.025 % (w/v)
KCl	0.02 % (w/v)
$(NH_4)_2SO_4$	0.01 % (w/v)
Agar	2 % (w/v)

The medium was prepared in distilled water, adjusted to pH 7, autoclaved at 121°C and cooled to 60°C. The $Ca_3(PO_4)_2$ (0.5%) was autoclaved separately. The cooled medium was added with previously sterilized $Ca_3(PO_4)_2$, the contents were mixed well to dissolve and the medium was poured onto sterile petridishes.

PHA production medium (Ramdas *et al.*, 2009)

$(NH_4)_2SO_4$	0.2 % (w/v)
KH_2PO_4	0.2 % (w/v)
Na_2HPO_4	0.06 % (w/v)
$MgSO_4 .7H_2O$	0.02 % (w/v)
Yeast extract	0.02 % (w/v)
Glycerol	1 % (w/v)
Trace element solution	0.01 % (v/v)

The composition of trace element solution is as follows

H_3BO_3	0.001% (w/v)
$MnSO_4.H_2O$	0.002% (w/v)
$CuSO_4$	0.001% (w/v)
$ZnSO_4. 7H_2O$	0.01% (w/v)
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.002% (w/v)

The medium was prepared in distilled water, adjusted to pH 7, autoclaved at 121°C and cooled to 60°C. The glycerol and trace element solution was autoclaved separately.

The sterilized glycerol and microelements stock solution was added to the cooled medium and the contents were mixed well before inoculation.

E2 minimal medium (Lagaveen *et al.*, 1988)

$NaNH_4HPO_4.4H_2O$	0.35% (w/v)
$K_2HPO_4.3H_2O$	0.75% (w/v)
KH_2PO_4	0.37% (w/v)
$MgSO_4.7H_2O$	0.35 % (w/v)
Yeast extract	0.04% (w/v)
Glycerol	2% (w/v)
Microelements stock solution	2% (v/v)

Composition of microelements stock solution dissolved in distilled water (1 ml)

$FeSO_4.7 H_2O$	2.78 mg
$MnCl_4.4 H_2O$	1.98 mg
$CoSO_4.7 H_2O$	2.81 mg

$CaCl_2.2\ H_2O$	1.47 mg
$CuCl_2.2\ H_2O$	0.17 mg
$ZnSO_4.7H_2O$	0.29 mg

The medium was prepared in distilled water, adjusted to pH 7, autoclaved at 121°C and cooled to 60°C. The glycerol and microelements stock solution was autoclaved separately. The sterilized glycerol and microelements stock solution was added to the cooled medium and the contents were mixed well before inoculation.

APPENDIX II

Table 4.2 The exoenzyme profile of PHAs accumulating *Vibrio* sp. from marine benthic sediments(N= 80)

sl no	CODE	Cas	Amy	Lip	Pect	Cell	Xyl	Alg	DNA	Gel	Phos
1	BTKB32	+	+	+	-	-	-	-	+	+	-
2	BTKB33	+	+	-	-	-	-	-	+	+	-
3	BTTV19	+	+	+	-	-	-	-	+	+	-
4	BTTV31	+	+	+	-	-	-	-	+	+	-
5	BTTN22	+	+	+	-	-	-	-	-	+	-
6	BTSB13	+	+	+	-	-	-	-	+	+	-
7	BTTN4	+	+	+	-	-	-	-	+	+	-
8	BTTN16	+	+	+	-	-	-	-	+	+	-
9	BTTC7	-	+	+	-	-	-	-	-	+	-
10	BTTC26	-	+	+	-	-	-	-	+	+	-
11	BTTN3	-	+	+	-	-	-	-	+	+	-
12	BTTN25	-	+	+	-	-	-	-	+	-	-
13	BTPT3	-	+	+	+	-	-	-	-	+	-
14	BTTV25	-	+	+	-	-	-	-	+	-	-
15	BTPT5	+	+	+	-	-	-	-	+	-	-
16	BTTV26	-	+	+	-	-	-	-	+	+	-
17	BTTV30	+	+	+	-	-	-	-	+	+	-
18	BTTV6	-	-	+	-	-	-	-	+	-	-
19	BTTV22	-	-	+	-	-	-	-	+	-	-
20	BTTV14	+	+	+	-	+	-	-	+	+	-
21	BTTV21	-	+	+	-	-	-	-	+	+	-
22	BTPT1	-	+	+	-	-	-	-	+	+	-
23	BTPT2	-	+	+	-	-	-	-	-	+	-
24	BTTV24	-	+	+	-	-	-	-	+	+	-
25	BTTV4	-	+	+	-	-	-	-	+	+	-
26	BTTV29	-	+	+	+	-	-	-	+	+	-

sl no	CODE	Cas	Amy	Lip	Pect	Cell	Xyl	Alg	DNA	Gel	Phos
27	BTTV10	-	+	+	-	-	-	-	+	+	-
28	BTTV7	-	+	+	-	-	-	-	+	+	-
29	BTPT4	-	+	+	-	-	-	-	+	+	-
30	BTED48	-	+	+	-	-	-	-	+	+	-
31	BTED54	-	+	+	-	-	-	-	+	+	-
32	BTKB25	+	+	-	-	-	-	-	+	+	-
33	BTED17	-	+	+	-	+	-	-	+	+	-
34	BTKB27	+	+	+	-	-	-	-	-	+	-
35	BTKB15	+	-	+	-	-	-	-	+	-	-
36	BTED51	+	+	+	-	-	-	-	-	+	-
37	BTED56	-	+	+	-	-	-	-	-	+	-
38	BTKB16	-	-	+	-	-	-	-	+	+	-
39	BTKB21	-	+	+	-	+	-	-	+	+	-
40	BTKB2	+	+	+	-	+	-	-	+	+	-
41	BTED50	+	+	+	-	-	-	-	+	+	-
42	BTKB4	+	+	+	-	-	-	-	+	+	-
43	BTKB14	+	+	+	-	+	-	-	+	-	-
44	BTKB5	+	+	+	-	+	-	-	+	+	-
45	BTED47	+	+	+	-	-	-	-	+	+	-
46	BTED59	+	+	+	-	-	-	-	+	+	-
47	BTTV40	+	+	+	-	-	-	-	+	+	-
48	BTKB3	+	+	+	-	-	-	-	+	-	-
49	BTKB31	+	+	+	-	+	-	-	+	+	-
50	BTED49	+	+	+					+	+	-
51	BTKB18	+	+	+	-	+	-	-	+	+	-
52	BTED58	+	+	+	-	-	-	-	+	+	-
53	BTKB20	+	+	+	-	+	-	-	+	+	-
54	BTKB23	+	+	+	-	-	-	-	+	+	-

sl no	CODE	Cas	Amy	Lip	Pect	Cell	Xyl	Alg	DNA	Gel	Phos
55	BTED46	+	+	+	-	-	-	-	+	+	-
56	BTTV33	+	-	-	-	-	-	-	+	+	-
57	BTTR45	-	+	-	-	-	-	-	+	+	-
58	BTTR36	+	-	+	-	-	-	-	-	+	-
59	BTTN26	-	+	+	-	-	-	-	+	+	-
60	BTTC4	-	-	+	-	-	-	-	+	+	-
61	BTTC10	+	+	+	-	-	-	-	-	+	-
62	BTTC12	+	-	-	-	-	-	-	+	+	-
63	BTTC1	+	-	+	-	-	-	-	+	+	-
64	BTTN7	+	+	-	+	+	-	-	+	+	-
65	BTTN18	+	+	+	+	-	-	-	+	+	-
66	BTSB12	+	+	+	-	-	-	-	+	+	-
67	BTTR46	+	+	+	-	+	-	-	+	+	-
68	BTTR50	+	+	+	-	-	-	-	+	-	-
69	BTTR39	+	+	+	-	-	-	-	+	+	-
70	BTTR40	+	+	+	-	+	-	-	+	+	-
71	BTTC27	+	+	+	-	+	-	-	+	+	-
72	BTSB4	+	+	+	-	+	-	-	+	+	-
73	BTMV2	+	+	+	-	+	-	-	+	+	-
74	BTMV3	+	+	+	-	-	-	-	+	-	-
75	BTMV5	+	+	+	-	+	-	-	+	+	-
76	BTMV8	+	+	+	-	-	-	-	+	+	-
77	BTMV9	+	+	+	-	-	-	-	+	+	-
78	BTMV10	+	+	+	-	-	-	-	+	+	-
79	BTMV15	-	+	+	-	-	-	-	+	+	-
80	BTMV7	-	+	+	-	-	-	-	+	+	-

+ = positive; - = negative; Cas - caseinase; Amy - amylase; Lip - lipase; Pect - pectinase; Cell-cellulase; Xyl - xylanase; Alg - alginase; DNA - DNase; Gel - gelatinase; Pho - Phosphatase

Table 4.3. Antibiogram of PHAs accumulating *Vibrio* sp. from marine benthic sediments

Sl no	CODE	V	Am	Co	C	T	Tr	Az	Cf	R	G	MAR INDEX
1	BTKB32	R	R	S	S	R	S	S	S	R	S	0.4
2	BTKB33	R	R	S	S	R	S	S	S	R	S	0.4
3	BTTV19	R	R	S	S	S	S	S	S	R	S	0.3
4	BTTV31	R	R	S	S	S	S	S	S	R	S	0.3
5	BTTN22	R	R	S	S	S	S	S	R	R	S	0.3
6	BTSB13	R	R	S	S	S	S	S	S	R	S	0.3
7	BTTN4	R	R	S	S	S	S	S	R	R	S	0.3
8	BTTN16	R	R	S	S	S	I	I	S	R	S	0.3
9	BTTC7	R	R	S	S	S	S	S	R	R	S	0.3
10	BTTC26	R	R	S	S	S	S	S	S	R	S	0.3
11	BTTN3	R	R	S	S	S	S	S	R	R	S	0.3
12	BTTN25	R	R	S	S	S	S	S	R	R	S	0.3
13	BTPT3	R	R	S	S	I	S	S	S	R	S	0.3
14	BTTV25	R	R	S	S	S	S	S	S	R	S	0.3
15	BTPT5	R	R	S	S	S	S	S	S	R	S	0.3
16	BTTV26	R	R	S	S	S	S	S	S	R	S	0.3
17	BTTV30	R	R	S	S	S	S	S	S	R	S	0.3
18	BTTV6	R	R	S	S	S	I	S	S	R	S	0.3
19	BTTV22	R	R	S	S	S	R	S	S	R	S	0.4
20	BTTV14	R	R	S	S	S	S	S	S	R	S	0.3
21	BTTV21	R	R	S	S	S	S	S	S	R	S	0.3
22	BTPT1	R	R	S	S	S	I	S	S	R	S	0.3
23	BTPT2	R	R	S	S	S	S	S	S	R	S	0.3

Sl no	CODE	V	Am	Co	C	T	Tr	Az	Cf	R	G	MAR INDEX
24	BTTV24	R	R	S	S	S	S	S	S	R	S	0.3
25	BTTV4	R	R	S	S	S	S	S	S	R	S	0.3
26	BTTV29	R	R	S	S	S	S	S	S	R	S	0.3
27	BTTV10	R	R	S	S	S	S	S	S	R	S	0.3
28	BTTV7	R	R	S	S	R	S	S	S	R	S	0.4
29	BTPT4	R	R	S	S	S	S	S	S	R	S	0.3
30	BTED48	R	R	S	S	S	S	S	S	R	S	0.3
31	BTED54	R	R	S	S	S	S	S	I	R	S	0.3
32	BTKB25	R	R	S	S	S	S	S	S	R	S	0.3
33	BTED17	R	R	S	S	S	R	S	S	R	S	0.4
34	BTKB27	R	R	S	S	S	S	S	S	R	S	0.3
35	BTKB15	R	R	S	S	I	S	S	S	R	S	0.3
36	BTED51	R	R	S	S	S	R	S	S	R	R	0.5
37	BTED56	R	R	S	S	S	S	S	S	R	S	0.3
38	BTKB16	R	R	S	S	S	S	S	S	R	S	0.3
39	BTKB21	R	R	S	S	S	S	S	S	R	S	0.3
40	BTKB2	R	R	S	S	S	R	S	S	R	S	0.4
41	BTED50	R	R	S	S	I	S	S	S	R	S	0.3
42	BTKB4	R	R	S	S	S	S	S	S	R	S	0.3
43	BTKB14	R	R	S	S	S	I	S	S	R	S	0.3
44	BTKB5	R	R	S	S	S	S	S	S	R	S	0.3
45	BTED47	R	R	S	S	S	S	I	I	R	S	0.3
46	BTED59	R	R	S	S	S	I	S	S	R	S	0.3
47	BTTV40	R	R	S	S	R	S	S	S	R	S	0.4
48	BTKB3	R	R	S	S	S	S	I	I	R	S	0.3

Sl no	CODE	V	Am	Co	C	T	Tr	Az	Cf	R	G	MAR INDEX
49	BTKB31	R	R	R	S	S	R	S	S	R	S	0.5
50	BTED49	R	R	S	S	S	S	S	S	R	S	0.3
51	BTKB18	R	R	S	S	S	S	S	S	R	S	0.3
52	BTED58	R	R	S	S	S	S	S	S	R	S	0.3
53	BTKB20	R	R	R	R	S	R	S	S	R	R	0.7
54	BTKB23	R	R	S	S	S	S	S	S	R	S	0.3
55	BTED46	R	R	S	S	S	S	S	S	R	S	0.3
56	BTTV33	R	R	S	S	I	R	S	I	R	I	0.4
57	BTTR45	R	R	S	S	S	S	S	S	R	S	0.3
58	BTTR36	R	R	S	S	S	S	S	S	R	S	0.3
59	BTTN26	R	R	S	S	S	S	S	S	R	S	0.3
60	BTTC4	R	R	S	S	S	S	S	S	R	S	0.3
61	BTTC10	R	R	S	S	S	S	S	S	R	S	0.3
62	BTTC12	R	R	S	S	S	S	S	S	R	S	0.3
63	BTTC1	R	R	S	S	S	S	S	R	R	S	0.4
64	BTTN7	R	R	S	S	S	S	S	S	R	S	0.3
65	BTTN18	R	R	S	S	S	S	S	S	R	S	0.3
66	BTSB12	R	R	S	S	S	S	S	S	R	S	0.3
67	BTTR46	R	R	S	S	S	S	S	S	R	S	0.3
68	BTTR50	R	R	S	S	S	S	S	S	R	S	0.3
69	BTTR39	R	R	S	S	S	S	S	S	R	S	0.3
70	BTTR40	R	R	S	S	S	S	S	S	R	S	0.3
71	BTTC27	R	R	S	S	S	S	S	S	R	S	0.3
72	BTSB4	R	R	S	S	S	S	S	S	R	S	0.3
73	BTMV2	R	R	S	S	S	S	S	S	R	S	0.3

Sl no	CODE	V	Am	Co	C	T	Tr	Az	Cf	R	G	MAR INDEX
74	BTMV3	R	R	S	S	S	S	I	I	R	S	0.3
75	BTMV5	R	R	S	S	R	R	S	S	R	R	0.6
76	BTMV8	R	R	S	S	S	I	I	I	R	S	0.3
77	BTMV9	R	R	S	S	S	S	I	S	R	S	0.3
78	BTMV10	R	R	S	S	S	S	S	S	R	S	0.3
79	BTMV15	R	R	S	S	S	S	S	S	R	S	0.3
80	BTMV7	R	R	S	S	S	S	S	S	R	S	0.3

R = resistant; I = intermediate; S = sensitive; V = Vancomycin (30mcg/disc); Am = Ampicillin (10mcg/ disc); Co = Co-Trimoxazole (25mcg/disc); Cb = Carbenicillin (100mcg/disc); T = Tetracyclin (30mcg/disc), Tr = Trimetroprim (5mcg/disc), At = Azithromycin (30mcg/disc), Cf = Ciprofloxacin (5mcg/disc); R = Rifampicin (5mcg/disc); G = Gentamycin (10mcg/disc). The result was interpreted as resistant, intermediate or sensitive based on the size of the inhibition zones around each disc as provided by the manufacturer (Himedia). (Performance standard for antimicrobial disc susceptibility tests, 2005).

LIST OF PUBLICATIONS

a. Peer Reviewed

1. **Raghul SS**, Sarita GB (2011): Extracellular enzyme profile and seasonal distribution of extracellular enzyme production in *Vibrio* sp. inhabiting marine benthic environments along coastal waters of India. Indian J. Geo-Marine Sci. **40(6),** 826-833.

2. Vidya F, **Raghul SS**, Bhat SG, Thachil ET (2011): Effect of cobalt stearate and vegetable oil on uv and biodegradation of linear low-density poly(ethylene)-poly(vinyl alcohol) blends. Polym. from Renewable Resour. **2(4),** 131-148.

3. Vidya F, **Raghul SS**, Bhat SG, Thachil ET (2011): Characterisation of linear low-density polyethylene / poly(vinyl alcohol) blends and their biodegradability by *Vibrio* sp. isolated from marine benthic environment. J. Appl. Polym. Sci., DOI 10.1002/app.34155.

b. **Full paper in proceedings of National/ International Symposiums/ Conferences/ Seminars.**

1. **Raghul SS**, Siju MV, Sarita GB (2011): Genotypic approach in characterisation of polyhydroxyalkanoates (PHAs) accumulation in *Vibrio* sp. isolated from marine sediments. *In Proceedings of National symposium on Emerging Trends in Biotechnology* organised by Department of biotechnology, CUSAT, Cochin, pp. 69-70 (1-2 September 2011). ISBN-978-93-80095-30-1

2. **Raghul SS**, Smitha S, Sarita GB (2008): Exploration of marine benthic vibrios as a promising source of bioactive molecules. *In Proceedings of the National Seminar on Bioactive compounds from the marine organisms* edited and Published by Dept. of Marine biology, Microbiology and biochemistry, CUSAT, Cochin, India pp 56-66 (14-15 March 2008)

3. Zeena PH, Anna DKF, Julie J, **Raghul SS**, Thomas K, Sarita GB (2010): Thermal properties of partially biodegradable LDPE/Dextrin blends. In *Proceedings of International Conference on Advances in*

Polymer Technology organised by Dept. of Polymer Science and Rubber Technology, Cochin University of Science and Technology, Cochin, India, pp 219-222 (26-27 February 2010)

4. Anna DKF, Zeena PH, Julie J, Thomas K, **Raghul SS**, Sarita GB (2010): Partially biodegradable LLDPE-starch blends. In *Proceedings of International Conference on Advances in Polymer Technology* organised by Dept. of Polymer Science and Rubber Technology, Cochin University of Science and Technology, Cochin, India, pp. 6-12 (26-27 February 2010) .

5. Vidya F, **Raghul SS**, Sarita GB, Eby TT (2010): Microbial Degradation Studies on Linear Low- Density Poly(ethylene)- Poly(vinyl alcohol) Blends Using Vibrios. In *Proceedings of International Conference on Advances in Polymer Technology* organised by Dept. of Polymer Science and Rubber Technology, Cochin University of Science and Technology, Cochin, India, pp. 1-5 (26-27 February 2010).

6. Anna DKF, Zeena PH, Thomas K, **Raghul SS**, Sarita GB (2008): The effect of amylase producing vibrios from the benthic environment on biodegradation of low density polyethylene-starch blends. In *Proceedings of International Conference on Advances in Polymer Technology* organised by Dept. of Polymer Science and Rubber Technology, Cochin University of Science and Technology (25-27 September 2008)

c. **Poster presentations in National/ International Symposiums/ Conferences/ Seminars.**

1. Vidya F, **Raghul SS**, Sarita GB, Eby TT (2011) Biodegradation of linear low density poly(ethylene)-poly(vinyl alcohol) blends aided by pro-oxidant additives. 2nd International conference on Material for the future organized by Government Engineering College, Trichur, Kerala, India (23- 25th February 2011).

2. Zeena PH, **Raghul SS**, Sarita GB, Thomas K (2011) Dextrin filled low density polyethylene films: Mechanical properties, melt flow indices and water absorption. 2nd International conference on Material for the future organized by Government Engineering College, Trichur, Kerala, India (23- 25th February 2011).

3. Zeena PH, Anna DKP, **Raghul SS**, Sarita GB, Thomas Kurian (2011) Investigation on Tapioca starch filled low density polyethylene films. Functional polymers, NIT Calicut, India, pp. 66 (28- 30th January 2011).

4. Vidya F, **Raghul SS**, Sarita GB, Eby TT (2010) Study on the Degradation of Linear Low-Density Poly(ethylene)-Poly(vinyl alcohol) Blends in the Presence of Cobalt Stearate and Vegetable oil. the Conference on polymer science and engineering-emerging dimensions organized by university institute of chemical engineering and technology, Punjab university, Chandigarh, India (26 -27th November 2010)

5. Zeena PH, Anna DKF, **Raghul SS**, Thomas K, Sarita GB (2010) Biodegradation of Environment –friendly low density polyethylene-dextrin blends. In proceedings of Polymer Science and Engineering: Emerging Dimensions (PSE-2010) (26-27th November 2010)

6. **Raghul SS**, Siju MV, Satheesh MK, Sarita GB (2009) Characterization and diversity of Polyhydroxyalkanoates producing vibrios inhabiting the marine benthic environments. International Symposium on Marine Ecosystems: Challenges and opportunities (MECOS, 09), Cochin, India (9-12th February 2009).

7. **Raghul SS**, Sarita GB (2008) Polyhydroxyalkanoates accumulating Vibrios isolated from marine benthic environment as a good candidate for bioplatic production. International Conference on Biodiversity Conservation and Management, BIOCAM 2008 organised by Rajiv Gandhi Chair in Contemperory studies, CUSAT, Cochin, India (3-6th February 2008).

d. GenBank Submissions

1. GenBank accession number - GU904004 ; 16S rRNA gene from *Vibrio* sp. BTTN18- **Raghul SS**, Sarita BG, Smitha S and Jeena A.
2. GenBank accession number - GU904005; 16S rRNA gene from *Vibrio* sp. BTTC10- **Raghul SS**, Sarita BG, Beena SP and Siju VM
3. GenBank accession number - GU904006 ;16S rRNA gene from *Vibrio* sp. BTTC27- **Raghul SS**, Sarita BG and Vijaya A

4. GenBank accession number - HM346666 ; 16S rRNA gene from *Vibrio* sp. BTTN22- **Raghul SS**, Sarita BG, Linda L and HelvinV

5. GenBank accession number - HM030800; 16S rRNA gene from *Vibrio* sp. BTPT1- **Raghul SS** and Sarita BG

6. GenBank accession number - HM030801; 16S rRNA gene from *Vibrio* sp. BTSB4F - **Raghul SS** and Sarita BG

7. GenBank accession number - HM030802; 16S rRNA gene from *Vibrio* sp. BTTR39- **Raghul SS** and Sarita BG

8. GenBank accession number - HM030803;16S rRNA gene from *Vibrio* sp. BT TR40- **Raghul SS** and Sarita BG

9. GenBank accession number - HM346667; 16S rRNA gene from *Vibrio* sp. BTTV21- **Raghul SS** and Sarita BG

10. GenBank accession number - HM346665; 16S rRNA gene from *Vibrio* sp. BTTN7- **Raghul SS** and Sarita BG

11. GenBank accession number - HM346664; 16S rRNA gene from *Vibrio* sp. BTTC26 - **Raghul SS** and Sarita BG

12. GenBank accession number - HM346663; 16S rRNA gene from *Vibrio* sp. BTSB12 - **Raghul SS** and Sarita BG

13. GenBank accession number - HM346662; 16S rRNA gene from *Vibrio* sp. BTPT4- **Raghul SS** and Sarita BG

14. GenBank accession number - HM346661; 16S rRNA gene from *Vibrio* sp. BTPT3- **Raghul SS** and Sarita BG

15. GenBank accession number - HM346660; 16S rRNA gene from *Vibrio* sp. BTPT2- **Raghul SS** and Sarita BG

16. GenBank accession number - HM346671; 16S rRNA gene from *Vibrio* sp. BTKB33- **Raghul SS** and Sarita BG

17. GenBank accession number - HM346670; 16S rRNA gene from *Vibrio* sp. BTKB32- **Raghul SS** and Sarita BG.

18. GenBank accession number - HM346659; 16S rRNA gene from *Vibrio* sp. BTMV15- **Raghul SS** and Sarita BG

19. GenBank accession number - HM346658; 16S rRNA gene from *Vibrio* sp. BTMV10- **Raghul SS** and Sarita BG

20. GenBank accession number - HM346657; 16S rRNA gene from *Vibrio* sp. BTED48- **Raghul SS** and Sarita BG

21. GenBank accession number - HM346656; 16S rRNA gene from *Vibrio* sp. BTED47- **Raghul SS** and Sarita BG

22. GenBank accession number - HM346655; 16S rRNA gene from *Vibrio* sp. BTED17- **Raghul SS** and Sarita BG

23. GenBank accession number - HM346669; 16S rRNA gene from *Vibrio* sp. BTTV30- **Raghul SS** and Sarita BG

24. GenBank accession number - HM346668; 16S rRNA gene from *Vibrio* sp. BTTV25- **Raghul SS** and Sarita BG

25. GenBank accession number - GU904007; 16S rRNA gene from *Vibrio* sp. BTTV4- **Raghul SS** and Sarita BG

26. GenBank accession number- HM030819; 16S rRNA gene from *Bacillus licheniformis* strain BTHT8- **Raghul SS** and Sarita BG

27. GenBank accession number - JQ781052; PHA synthase, class I family, partial gene sequence from *Vibrio* sp. BTKB33- **Raghul SS** and Sarita BG