CHARACTERIZATION OF BIOPOLYMER PRODUCED BY MIXED BACTERIAL CULTURE UNDER SUBMERGED FERMENTATION

A Thesis submitted to the Orissa University of Agriculture and Technology in partial fulfilment of the requirement for the degree of (*Master of Science*) *in Microbiology*

By

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CERTIFICATE-I

This is to certify that the thesis entitled **"Characterization of biopolymer produced by mixed bacterial culture under submerged fermentation"** submitted in partial fulfilment of the requirements for the award of the degree of **Masters of Science in Microbiology** to the **Orissa University of Agriculture and Technology**, Bhubaneswar, Odisha is a faithful record of bonafide and original research work carried out by ***Ms. Deepika Devadarshini*** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma. It is further certified that the assistance and help received by her from various sources during the course of investigation has been duly acknowledged.

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CERTIFICATE-II

This is to certify that the thesis entitled **"Characterization of biopolymer produced by mixed bacterial culture under submerged fermentation"** submitted by the student bearing Admission No. 11MB/16 to the Orissa University of Agriculture and Technology, Bhubaneswar, in partial fulfillment of the requirements for the award of the degree of **Masters of Science in Microbiology**, has been approved by the students advisory committee and the external examiner.

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I do here by declare that the thesis entitled **"Characterization of biopolymer produced by mixed bacterial culture under submerged fermentation"** is a record of original research work conducted by me and that no part of the thesis has been submitted before for award of any other degree or diploma of any University.

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CONTENTS

SL. NO.	PARTICULARS	PAGE NO.
CHAPTER 1.INTRODUCTION		**1-03**
	1.1 Background	1
	1.2 Biopolymer: Polyhydroxyalkanoates (PHAs)	1
	1.3 PHAsproductionby mixed bacterial culture (MBC**)**	1-2
	1.4 Objectives	2
	1.5 Plan of work	3
CHAPTER 2.REVIEW OF LITERATURE		**4-20**
2.1 Introduction		4
2.2PHAs production by mono bacterial culture		4-12
2.3PHAs production by mixed bacterial culture (MBC)		13-16
CHAPTER 3.MATERIALS AND METHODS		**21-30**
3.1 Introduction		42
3.2 Materials and Methods		42-54
3.2.1Selection of source organism and morphological characterization		42
3.2.2Culture media and chemicals		44
3.2.3Detection of PHAs granule by sudan black B staining		44
3.2.4Detection of PHAs granule by nile red staining		44
3.2.5 Antagonistic activity (in-vitro) among PHAs producing bacteria		45-47
3.2.6Optimization of growth parameters for biomass and PHAs production		48-54
3.2.6.1Culture media		
3.2.6.2pH		
3.2.6.3 Temperature		
3.2.6.4 Carbon source		
3.2.6.5 Nitrogen source		
3.2.6.6 Inoculum size		
3.2.7 PHAs production by submerged fermentation process and extraction		

by sodium hypochlorite digestion and multi-solvent method

3.2.8Structural characterization and biodegradability of PHAs 54

3.2.8.1Fourier transform infrared (FTIR) spectroscopic analysis 54

3.2.8.2BiodegradationofPHAs by composting method 54-67

CHAPTER 4.RESULTS DISCUSSION **68-95**

4.1 Morphological characterization of source bacteria 68

4.2Antagonistic activity (*in-vitro*) among PHAs producing bacteria

4.3Optimization of growth parameters for biomass and PHAs production

4.4 PHAs production by bacterial isolates

4.5 Fourier transform infrared (FTIR)spectroscopic analysis

4.6 Biodegradation of PHAs by composting method

CHAPTER 5.SUMMARY AND CONCLUSION **112-119**

5.1 Summary 112-118

5.2 Conclusion 118-119

5.3 Future outlook 119

BIBILIOGRAPHY **120-137**

APPENDIX **i-vi**

LIST OF FIGURES

SL. NO.	PARTICULARS	PAGE NO.
1.	Revival of preserved PHAs producing bacterial isolates (a) *Bacillus* sp. C1 (2013) (b) *Bacillus* sp. P1(2013b) (c) *Bacillus* sp. P2(2013) (d) *Bacillus* sp. P3(2013) (e) *Bacillus* sp. P4(2013c) (f) *Bacillus* sp. O6 (g) *Bacillus* sp. G5 (h) *Zobellella* sp. DD5	4
2.	Micrographs of PHAs producing bacterial isolates under Sudan Black staining (a) *Bacillus* sp. C1 (2013) (b) *Bacillus* sp. P1 (2013b) (c) *Bacillus* sp. P2 (2013) (d) *Zobellella* sp. O6	5
3.	Antagonistic activity of PHAs producing bacterial isolates (a) *Bacillus* sp. C1 (2013) & *Bacillus* sp. O6. (b) *Bacillus* sp. P1 (2013b) & *Bacillus* sp. O6 (c) *Bacillus* sp. P1 (2013b) & *Bacillus* sp. P2 (2013) (d) *Bacillus* sp. C1 (2013) & *Bacillus* sp. P2 (2013)	43
4.	Optimization of growth parameters such as culture media, pH, temperature, carbon source, nitrogen source and inoculum size of PHAs producing bacterial isolates.	43
5.	PHAs film produced from MBC under submerged fermentation	43
6.	FTIR of PHAs produced by MBC under submerged fermentation	43

LIST OF TABLES

SL. NO.	PARTICULARS	PAGE NO.
1.	FTIR spectra of PHAs and their corresponding annotation	55
2.	Biodegradation of PHB by composting method	63

LIST OF ABBREVIATIONS

gm	Gram
l	Litre
mg	Milligrams
min.	Minute
ml	Milliliter
NB	Nutrient Broth
v/v	Volume by Volume
Vol	Volume
w/v	Weight by Volume
%	Percentage
CFU	Colony Forming Unit
NA	Nutrient Agar
µl	Micro liter
^{o}C	Degree Celsius
PHAs	Polyhydroxyalkanoates
PHB	Poly-β-hydroxybutyrate
MSM	Minimal Salt Medium
GM	Growth Media
RPM	Revolution Per Minute
g	Gravitational Force
HDD	Hydroxydodecanoate
HTD	Hydroxytetradecanoate
µm	Micrometer
SEM	Scanning Electron Microscope
TEM	Transmission Electron Microscope
nm	Nano Meter
bp	Base Pair
ATCC	American Type of Culture Collection Centre
PCR	Polymerase Chain Reaction

CoA	Coenzyme A
M	Molarity
Temp.	Temperature
PHV	Polyhydroxyvalerate
PLA	Poly Lactic Acid
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
sp.	Species
D	Dalton
KD	Kilo Dalton
XRD	X-ray Diffraction
GC-MS	Gas Chromatography-Mass Spectroscopy
FTIR	Fourier Transform Infrared Spectroscopy
H^1 NMR	Proton Nuclear Magnetic Resonance
DSC	Differential Scanning Calorimeter
TGA	Thermo Gravimetric Analysis
UV	Ultra-Violet
Td	Decomposition Temperature
Tg	Glass Transition Temperature
Tm	Melting Temperature
Xc	Degree of Crystalinity
scl	Short chain lent
mcl	Medium chain length
lcl	Long chain length
mm	Milli Meter
mM	Milli Molar

CHAPTER: 1 INTRODUCTION

1.1 Background

The global petrochemical based plastics or polymer production has been amplified from 1.5 million tons in 1950 to 299 million tons in 2013. Rapid exploitation these non-biodegradable or recalcitrant polymers have generated huge amounts of toxic waste as well as a significant burden on their management (Yang *et al*., 2015). Thus, it is the need of the hour to replace these synthetic polymers by an alternate ecofriendly biopolymer.

1.2 Biopolymer: Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) referred to a group of biodegradable, biocompatible & bio-based polyesters accumulated as carbon and energy storage granules in the cytoplasm of bacterial cell. Their thermoplastic, biodegradability and biocompatibility properties make them a possible alternative of synthetic plastics, to prevail over environmental concerns originated from the non-biodegradable plastics (Basak *et al*., 2011; Keshavarz and Roy, 2010).

PHAs accumulation is one of the responses towards stress experienced by microbes inhabiting at diverse ecological niches (Koller *et al*., 2011). These are synthesized by more than 300 (Yang *et al*., 2011) various Gram-positive bacteria such as *B. subtilis, B. amyloliquefaciens* DSM7, *B. licheniformis*, *B. macerans*, *B. cereus* PS10, *B. circulans*, *B. megaterium* Y6, *B. coagulans*, *B. brevis*, *B. thuringiensis*, *Clostridium* sp., *Corynebacterium* sp., *Nocardia* sp., *Rhodococcus* sp., *Streptomyces* sp., *Staphylococcus* sp. and Gram-negative bacteria including *Alcaligenes latus*, *Ralstonia eutropha*, *Aeromonas hydrophila*, *P. putida* KT2440, *P. oleovorans* GPo1, (Sharma and Bajaj, 2016). PHAs can be divided into three broad classes such as short (up to C5 carbon atom), medium (C6 to C14 carbon atom) and long chain length PHAs (more than C14), based on the number of carbon atoms present in the polymer chain. More than 150 different monomers of PHAs has been reported (Li *et al*., 2016) and molecular weight of these polymers range between 2×10^2 and 3×10^3 KDa depending on the microbes and the fermentation conditions (Keshavarz & Roy, 2010). These biomaterials imitate the characteristic of synthetic polymer and recyclable to CO_2 and H_2O in the natural condition (Khanna and Srivastava, 2005). So far eight different pathways of microbial PHAs synthesis have been reported (Chen, 2010).

PHB, the most common homopolymer of PHAs synthesis starts from metabolism of glucose to generate acetyl-CoA and NADPH through the glycolytic and pentose phosphate pathways. Then, the two acetyl- CoA molecules condensed by *β*-ketothiolase (*PhaA*) into acetoacetyl-CoA and subsequently reduced to 3-hydroxybutyryl-CoA by acetoacetyl-CoA dehydrogenase (*PhaB*) using NADPH as a cofactor and finally polymerized into PHB by P (3HB) polymerase (*PhaC*) (Kalia *et al*., 2007; Rehm, 2003; Lee, 1996). These PHAs are widely used for preparation of plastics materials, medical implants, drug delivery carriers, printing and photographic materials, nutritional supplements, drugs and fine chemicals (Chen, 2009).

1.3 PHAs production by mixed bacterial culture (MBC)

However, wide-spread replacement of synthetic plastics has been limited due to their high production cost, which holds back its successful commercialization (Waltz, 2008). Thus, more efforts such as applications of high PHAs yielding bacteria, inexpensive carbon sources like agro-industrial & domestic waste (Mohapatra *et al*., 2017), genetically modified bacteria and mixed bacterial culture (MBC) are needed for cost effective biopolymer production (Reddy *et al*., 2003). As the PHAs production is parallel to microbial biomass, thus application of MBC for cost effective PHAs production has gained significant attention of researchers. In contrast to pure cultures, the use of MBC for PHAs production has an added advantage of reducing energy usage associated with eliminating the requirement to maintain axenic conditions. Though the concept of using MBC for PHAs production have been reported earlier (Cavaillé *et al*., 2016; Morgan-Sagastume *et al*., 2014; Reddy and Mohan, 2012; Keshavarz and Roy, 2010) still it is in the stage of infancy. Thus, the objective of the study is to evaluate the feasibility of MBC for PHAs production and its characterizations.

1.4 Objectives

1. Optimization of biopolymer (PHAs) production by mixed bacterial culture.
2. Structural characterization of PHAs extracted from mixed bacterial culture.
3. Study on the biodegradation of extracted PHAs under natural environment.

1.5 Plan of work

1. Revival of preserved PHAs producing bacterial isolates using Nutrient agar medium.

2. Detection of PHAs granules in the bacterial isolate by Sudan black staining and Nile red staining.
3. Evaluation of antagonistic activities among potential PHAs producing bacterial isolates.
4. Preparation of PHAs producing mixed bacterial culture using suitable culture medium.
5. Optimization of pH, temperature, carbon source and nitrogen source and inoculum size on biomass production by mixed bacterial culture.
6. Mass production of PHAs by submerged fermentation process under optimized conditions.
7. Extraction and purifications of PHAs from bacterial cell biomass through downstream processing.
8. Structural characterization of PHAs using FTIR analysis.
9. Biodegradation of extracted PHAs by open windrow composting method under natural environment.

CHAPTER: 2 REVIEW OF LITERATURE

2.1 Introduction

To provide a cut-throat compition to petrochemical based plastics, production of PHAs has to be more commercially attractive. MBC system have demonstrated a good potential for PHAs productivity and also maintained parallelism with polymer quantity and quality. The physical properties of PHAs greatly influenced by factors like, microbial strain, MBC, accumulation of biopolymer in bacterial biomass & its composition, product quantity & purity and chemicals required for its recovery (Koller *et al*., 2013). In this review, we have discussed the PHAs production by mono bacterial and mixed bacterial culture.

2.2 PHAs production by mono bacterial culture

Sindhu *et al*., (2015) reported that, the solid SSF has been reassessed an alternative to SF process for cost effective PHAs production. Moreover, the cost factor for SSF is usually lower than that of SF and the cost of raw materials for SSF would be cheap, since it uses inexpensive carbon sources. These positive factors make SSF a potential technique for PHAs production. However, this method is still in the stage of infancy for commercialization. The major drawback to address is the proper maintenance of the culture conditions under SSF. Thus, recent advances in this regard should be employed in solid-state fermentation for the PHAs production and its commercialization.

Kumar *et al*., (2015) reported that, *Bacillus thuringiensis* EGU45 produced 1.5-3.5 g/l PHAs from feed containing 1-10% CG (v/v) and nutrient broth (NB, 125 ml) without any acclimatization. *B. thuringiensis* EGU45 could produce PHAs at the rate of 1.54 g/l to 1.83 g/l, from 1% CG (v/v) on media having high nitrogen contents: (i) NB, (ii) NB + 0.5% NH_4Cl (w/v) and (iii) peptone + yeast extract + 0.5% NH_4Cl (w/v). *B. thuringiensis* EGU45 was able to produce co-polymer of P(3HB-co-3HV) with 13.4% 3HV content on high N containing feed supplemented with propionic acid.

Domínguez-Díaz *et al*., (2015) studied on PHB production by wild (OP) and three mutant strains (OPN, SOP1 and OPNA) of *Azotobacter vinelandii*. It was observed that, melting, crystallization and degradation temperatures of the PHBs initially increased with increasing the molecular weight. However, the highest molecular weight PHBs exhibited cold crystallization and reduction in melting temperature and melting enthalpy, which was determined by DSC and TGA. Uniaxial tensile tests showed that the

mechanical Young's modulus is an increasing function of molecular weight, whereas the strain at fracture decreased, denoting brittleness as molecular weight increased. Moreover, cell line exhibits any cytotoxic effect and allowed the growth of human embryonic kidney 293 cells (HEK293) on as-cast films indicating the biocompatibility as a support for cell systems.

Mohapatra *et al*., (2015) isolated PHB producing bacteria from rhizospheric soil region of sweet potato. Out of all, four bacterial isolates showed presence of PHB granules confirmed by sudan black B staining. All four strains belonged to genus *Bacillus*. Highest PHAs production (64.53%) was observed in *Bacillus* P3 under optimized condition such as temperature 37°C and pH 7. Then the potential PHAs producing isolates were tested for multiple resistances to antibiotics showing an advantageous feature for other biotechnological production of PHAs.

Altaee *et al*., (2015) isolated Gram-positive bacterium *Rhodococcus equi* from fertile soil in mineral salt media (MSM) by using 1% crude palm kernel oil (CPKO) as the carbon source. The strain has the capability to produce 38% PHB from 1.43 (g/l) DCW. The Gas chromatography GC and NMR depict the chemical structure of PHB respectively. In addition, DSC and TGA revealed the thermal properties of the polymer, where the melting temperature (*Tm*) was 173^{o}C, the glass transition temperature (*Tg*) was 2.79^{o}C and the decomposition temperature (*Td*) was 276^{o}C. Gel permeation chromatography (GPC) was used to study the molecular mass of the recovered PHB in addition to comparing the results with other studies using different bacteria and substrates, where the molecular weight was 642 kDa, to enable its usage in many applications.

Castro *et al*., (2014) studied the influence of various fed-batch strategies in the performance of metabolically engineered *Pseudomonas putida* strains, Δgcd and Δgcd-pgl, for improving production of *mcl*-PHAs using glucose as the carbon source. A fed-batch process was developed that comprised an initial phase of biomass accumulation based on an exponential feeding carbon-limited strategy. For the *mcl*-PHAs accumulation stage, three induction techniques were tested under nitrogen limitation. The substrate pulse feeding was more efficient than the constant feeding approach to promote the accumulation of the desirable product. Nonetheless, the most efficient approach for maximum PHA synthesis was the application of a dissolved-oxygen-stat feeding strategy (DO-stat), where *P. putida* Δgcd mutant strain showed a final PHAs

content and specific PHAs productivity of 67% and 0.83 g/l per hour, respectively. This *mcl*-PHAs titre is the highest value that has been ever reported using glucose as the sole carbon and energy source.

Dash *et al*., (2014) observed PHAs production by using species of *Bacillus* isolated from the rhizospheric soil region of sugarcane. Under optimized condition *Bacillus licheniformis* produces 53.01% of polyhydroxyalkanoates in MSM culture medium. The production of PHAs was found to be increased along with the increase in the cell biomass. The FTIR analysis of the extracted PHAs showed the distinct peaks corresponding to C=O groups and the spectroscopic analysis gave proper insight view for the chemical structure of PHB by reflecting the monomeric units.

Mohapatra *et al*., (2014) isolated 78 bacteria from rhizospheric soil of different plants, of which 16 isolates showed presence of PHAs granules. All the PHAs producing bacterial isolates were identified by 16S-rRNA gene sequencing belong to genus *Bacillus* such as *Bacillus flexus* MRK13, *Bacillus* sp. S4(2013b), *Bacillus* sp. P1(2013b), *Bacillus* sp. S1(2013b), *Bacillus* sp. S6(2013b), *Bacillus* sp. O1, *Bacillus* sp. P2(2013), *Bacillus* sp. P3(2013), *Bacillus* sp. P4(2013c), *Bacillus* sp. B2(2013c), *Bacillus* sp. B5(2013b), *Bacillus* sp. C1(2013), *Bacillus* sp. C3(2013), *Bacillus* sp. O6, *Bacillus* sp. S8, *Bacillus thuringiensis* RKD12. Under optimized condition *Bacillus* sp. S1 (2013b) possesses the potential for the production PHAs (80.94%) *in-vitro*.

Catone *et al*., (2014) observed short chain length PHAs production by *P. extremaustralis* revealed the presence of another PHB cluster *phbFPX*, with high similarity to genes belonging to *Burkholderiales*, and also a cluster, *phaC1ZC2D*, coding for medium chain length PHAs production (*mcl*-PHA). All *mcl*-PHA genes showed high similarity to genes from *Pseudomonas* species and interestingly, this cluster also showed a natural insertion of seven ORFs not related to *mcl*-PHA metabolism. RT-qPCR analysis showed different levels of expression for the PHB synthase, *phbC*, and the *mcl*-PHA synthases. The expression level of *phbC*, was significantly higher than the obtained for *phaC1* and *phaC2*, in late exponential phase cultures. The results of this work show the high efficiency of a foreign gene (*phbC*) in comparison with the *mcl*-PHA core genome genes (*phaC1 and phaC2*) indicating that the ability of *P. extremaustralis* to produce high amounts of PHB could be explained by the different expression levels of the genes encoding the scl and mcl PHA synthases.

Sasidharan *et al*., (2014) studied PHB production by *Vibrio* species isolated from marine sediments. Out of 828 isolates, *Vibrio* sp. BTKB33 showed 0.21 g/l of PHAs production from 193.33 mg/g of CDW. The strain was identified as *Vibrio azureus* based on phenotypic characterization and partial 16S rDNA sequence analysis. Under optimized conditions (35 °C, pH-7, 1.5 % NaCl, 120 rpm, 12 h of inoculum age, 2.5 %, magnesium sulphate, glucose and ammonium chloride) PHAs production was found to be increased to 0.48 g/l and PHAs content to 426.88 mg/g of CDW, indicating a 2.28-fold increase in production. The FTIR analysis indicates that, the extracted PHAs is PHB.

Zarei *et al*., (2013) studied the effect of reducing agent on PHAs production. It was observed that reducing agents could be effective to direct metabolic pathway to the PHAs production. The results showed that the amount of PHAs produced from fermentation medium without reducing agent and by adding reducing agent to fermentation are 0.18 g/l and 0.5 g/l respectively.

Nair *et al*., (2013) reported that polyhydroxyalkanoates are natural biopolymers produced by bacteria and many other microbes such as yeast and fungi. They observed PHAs production by *Bacillus* sp., isolated from soil sample. FTIR and XRD analysis showed the sharp and intense peaks those pertaining to PHA. Moreover, DSC analysis showed that, the compound was stable and TGA was observed that the compound was stable until 236.52°C due to the removal of adsorbed water molecules i.e., 53.12 wt % for PHAs. The weight loss observed between 276.19°C and 362.41°C, 362.41°Cand 656.14°Cand 278.19°Cand 800°C respectively, may be due to the polymer degradation.

Hungund *et al*., (2013) conducted an experiment for PHA production by using bacteria isolated from spent wash and oil mill soil. Different bacterial isolates were screened by PCR using primers designed for *Bacillus megaterium*. Isolate-4 showed amplicon similar in size to that of *Bacillus megaterium*. Based on 16S rRNA gene sequences the bacteria identified as *Paenibacillus durus.* MSM with fructose as carbon source was used for production of PHB in shake flask method. The study revealed that fructose and peptone gave better PHB accumulation and growth rates for the isolate and the reference strain.

Israni and Shivakumar, (2013) isolated and identified PHAs producing *Bacillus* sp. from different rhizospheric soils capable of producing a variety of hydrolytic enzymes. Two promising isolates: Ti1 and Ti3, from tomato rhizospheric soil samples

depicted the PHAs yield of 1.44 - 1.6 g/l and the % PHAs accumulations are of 50.58 and 50.1, respectively. Combinatorial qualitative innate enzyme based PHAs production assay revealed production of PHAs by these isolates on some of these complex substrates. Molecular phylogenetic analysis identified that Ti3 has close homology with *B. megaterium* and also it has the capability to produce PHAs in presence of starch as source of carbon.

Tanamool *et al*., (2013) studied production of PHAs by using sweet sorghum juice as a substrate by bacteria isolated from soil. PHAs production was carried out under controlled conditions at pH 7, temperature 35°C and 20 g/l of initial total sugar in the SSJ. Maximum PHAs production (1.74 g/l) was obtained with 57.62% dry cell weight. The PHAs producing bacterial isolate identified as *Bacillus aryabhattai* by 16S rRNA sequencing.

Gowda and Shivakumar, (2013) reported that *B. thuringiensis* IAM 12077 was grown in different culture conditions with glucose as carbon source (10g/l) and limitation of potassium, nitrogen, sulphur and phosphorous sources. The PHAs yields were found to be 1.61 g/l, 65.9%; 1.86 g/l, 64.1%; 1.66 g/l, 65.9% and 1.7 g/l, 68.9% dry cell weight for potassium, nitrogen, sulphur and phosphorus limitations respectively. Time course study in nitrogen deficient medium revealed that cell biomass remained almost steady from 0 hour after transfer to production medium till the end of fermentation 120 hours, PHB yield showed increase from 0.533 g/l at 0 hour to 4 g/l by 48 hours and later gradually decreased, with PHB accumulation increase from 11% to 77% by 96 hours. PCR analysis revealed that, the presence of class IV *pha* synthase in *B. thuringiensis* IAM12077 suggesting the capability of this strain to accumulate different PHAs monomers.

Thuoc *et al*., (2012) reported PHAs production by using 100 halophilic and halo-tolerant bacteria isolated from mangrove forests located in northern Vietnam. Three of the strains (ND153, ND97 and QN194) isolated from the Vietnamese forests were identified as *Bacillus*, while other five strains (QN187, ND199, ND218, ND240 and QN271) were close related to the proteo-bacterium *Yangia pacifica*. These strains were found to accumulate PHAs in noticeable amounts. Strains ND153, ND97, QN194, QN187, ND240 and QN271 synthesized PHB from glucose, whereas strains ND199 and ND218 synthesized pol (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) from this carbohydrate. With the exception of strain QN194, the strains accumulated PHBV when

a combination of glucose and propionate was included in the culture medium. The polymer yields and cell growth was higher in *Bacillus* strain ND153 and Gram-negative bacteria, strain QN271. The shake flasks method of production revealed that strain ND153 reached a maximum PHBV yield of 71 wt% and a cell dry weight (CDW) of 3.6 g/l while strain QN271 attained a maximum PHB yield of 48 wt% and a CDW of 5.1 g/l.

Gholami *et al*., (2012) studied PHAs production by using new potential extremophilic bacterial species isolated from Maharlu salt lake, Iran. Among 132 bacteria, 21 were selected as PHAs producer and identified by partial sequence of the 16S rRNA gene. The identified new novel bacterial isolates such as *Bacillus endophyticus* BCCS 011 and *Lysobacter* sp. BCCS 052 has high potential for PHAs production.

Sangakharak and Prasertsan, (2012) reported that, fifty bacterial isolates belong to *Bacillus*, *Proteus*, *Pseudomonas*, *Aeromonas*, *Alcaligenes* and *Chromobacterium*, of which 25 bacterial isolates were able to produce PHAs. The maximum PHAs were accumulated by *B. licheniformis* PHA 007 at 68.80% of dry cell weight (DCW). However moderate amount of PHAs production were observed by species of *Pseudomonas*, *Aeromonas*, *Alcaligenes* & *Chromobacterium*. *Bacillus licheniformis* PHA 007 had the highest lipase and protease activity with a hydrolysis zone of 7.9 mm and 23 mm respectively. However, the highest amylase activity was observed in *Bacillus* sp. PHA 023 at 1.4 (with a hydrolytic zone of 5.5 mm) for cost effective PHAs production different sources of cheap substrates were tested. *Bacillus cereus* PHA 008 gave the maximal yield of PHAs production (64.09% of DCW) when cultivated in an aerobically treated palm oil mill effluent (POME). In addition, the accumulation of PHAs copolymers such as 3-hydroxyvalerate and 3-hydroxyhexanoate was also observed in *Bacillus* and *Pseudomomas* sp. 012 and 045, respectively. Eight of the nine isolates accumulated a significant amount of PHAs when inexpensive carbon sources were used as substrates. Here it varied from 1.69% of DCW by *B. licheniformis* PHA 007 to 64.09% of DCW by *B. cereus* PHA 008.

Shah, (2012) isolated fifteen bacteria from soil samples of industrial area. Among all, twelve bacterial isolates were found to be the most promising PHAs accumulating bacteria confirmed by Sudan black staining. PHAs extraction was carried out by chloroform digestion method. Biochemical and 16s rRNA analysis showed that PHAs producing bacteria belong to genus *Bacillus* with maximum production of PHAs. Then

the PHAs was analysed by U.V spectrophotometer and characterized by FTIR spectroscopy.

Narayanan and Ramana, (2012) investigated production of PHB using glucose-peptone broth by *Bacillus mycoides* DFC1 strain isolated from garden soil. The central composite rotatable design was used to study the interactive effects of three variables: glucose, peptone and pH on cell growth and PHB production. The optimized medium conditions with the constraint 'to maximize' cell growth and PHB content were glucose 17.34 g/l, peptone 7.03 g/l at pH 7.3. A maximum dry cell weight of 4.35 g/l and PHB yield of 3.32 g/l amounting to 76.32 % (w/w) of dry cell weight with negligible sporulation at the end of 72 hour resulted in a significant increase (1.83–3.32 g/l) in the production of PHB in comparison to their preliminary studies.

Berekaa, (2012) studied phenotypically for synthesis and intracellular accumulation of PHAs granule in eight *Bacillus* strain. Pair of specific PCR primers was designed and applied for genotypic detection of *pha*C gene. Approximately, 760 bp DNA fragment was successfully amplified in the eight strains. Among the positive strains, *Bacillus* sp SW1-2, produced 36 g/l of the biopolymer during growth on modified E2 medium supplemented with glucose. Spectroscopic analysis by C^{13} NMR and H^{1} NMR revealed four narrow peaks (CH3; 21.2 ppm, CH2; 42.7, CH; 68.5 and C=O; 169.7 ppm) and 3 groups of signals (2.45, 2.58 and 5.2 ppm) identical and characteristic to PHB respectively. Moreover, the amplified PCR fragment, from genomic DNA of *Bacillus* sp SW1-2, was cloned in pGEM-T-Easy vector and sequenced with universal T7 and SP6 primers. The sequence showed 99% identity to *pha*C gene for PHA synthase of many *B. megaterium* strains deposited in Gene-Bank.

Brigham *et al.*, (2012) studied PHB production and mobilization in *Ralstonia eutropha* but in only a few instances has PHB production been explored in relation to other cellular processes. The global gene expression of wild-type *R. eutropha* throughout the PHB cycle: growth on fructose, PHB production using fructose following ammonium depletion, and PHB utilization in the absence of exogenous carbon after ammonium was resupplied. These results confirm or lend support to previously reported results regarding the expression of PHB-related genes and enzymes. Global gene regulations during PHB production are strongly reminiscent of the gene expressions pattern observed during the stringent response in other species. These results indicate that the stringent responses are

required for PHB accumulation in *R. eutropha*, helping to elucidate a thus-far-unknown physiological basis for this process.

Goh and Tan, (2012) isolated15 PHAs producing bacterial strains from Antarctic soils and subjected to production with a medium supplemented with 0.5% (w/v) sodium octanoate or glucose. 16S rRNA gene sequence analysis revealed that the isolated PHAs-producing strains were mainly *Pseudomonas* sp. and a few were *Janthinobacterium* sp. All the isolated *Pseudomonas* strains were able to produce *mcl*-PHA using fatty acids as carbon source, while some could also produce *mcl*-PHA by using glucose. The *Janthinobacterium* strains could only utilize glucose to produce PHB. A *Pseudomonas* isolate, UMAB-40, accumulated PHAs up to 48% cell dry mass by utilizing fatty acids as carbon source. This high accumulation occurred at between 5^{o}C to 20^{o}C and then decreased with increasing temperatures. Highly unsaturated *mcl*-PHA was produced by UMAB-40 from glucose.

Wu *et al*., (2011) used a proteomic approach to investigate accumulation of PHB in *Bacillus thuringiensis*, a well-known biopesticides. This strategy is used to increase the synthesis and accumulation of PHB by the bacterial isolate. Under heat stress condition, the bacterium adjusted its metabolism by up or down regulation that enhances continuous accumulation of PHB inside the bacteria.

Kunasundari & Sudesh, (2011) summarized the efficiency of different methods of downstream processing for extraction of PHAs and their quality. These include solvent extraction, chemical digestion, enzymatic treatment and mechanical disruption, supercritical fluid disruption, flotation techniques, use of gamma irradiation and aqueous two-phase system. Though economical PHAs production depends on the cost of both upstream and downstream processing, thus for cost effective eco-biopolymer production this analysis is highly indispensable.

Yang *et al*., (2011) reported the extraction of PHAs from bacteria such as *Ralstonia eutropha* and *Escherichia coli* under various conditions. Most detergents tested recovered extremely pure PHAs and the amount depends on the percentage of biopolymer accumulated in the cell. Detergents including linear alkyl benzene sulfonic acid (LAS-99) extracted significant amount of PHAs with high degree of purity. Moreover, less amount of detergent was needed for the extraction of PHAs as compared to SDS. LAS-99 also has the advantage of being biodegradable and environmentally

safe. Chemical extraction of PHAs with detergents could potentially eliminate the risk of eco-pollution, thus making industrial PHAs production economic and eco-friendly.

Selvakumar *et al*., (2011) studied PHAs production from halophillic archae and bacterial strain, *Haloarculamari smortui* MTCC 1596. The strain has capability to produce high amounts of PHAs in the presence of excess carbon and limited nitrogen source in the medium. The intracellular PHAs compounds were isolated by osmotic cell lysis method using distilled water. This method was found to be simple and cost effective for cell lysis and it can be able to reduce the high downstream processing expenditure of conventional PHAs extraction procedure. Optimization of PHAs was carried out by altering the carbon sources and found that, sodium acetate to be the potential source for higher production of PHAs.

Wang & Binging, (2011) reported PHB production using different microbial strains such as *Ralstonia eutropha* (ATCC 17699), *Alcaligenes latus* (ATCC 29712) and *Alcaligenes latus* (ATCC 29712) based on their growth kinetics. *A. latus* (ATCC 29714) was found to have the highest μm (0.38 ± 0.01/hour) and significantly (P≤ 0.05) lower Td (1.80 ± 0.05/hour) and was thus selected to perform PHB fermentations. Two-stage batch fermentations with 3 time points (14th hour, 16th hour or 18th hour) for introducing nitrogen limited media and fed-batch fermentations with similar time points for initiating feeding of nitrogen limited media, were conducted using synthetic media with sucrose as the only carbon source. Two-stage batch fermentation with introduction of nitrogen limitation at 16th hour with the second stage ending at 26th hour post induction of nitrogen limitation was found to be the optimal fermentation mode for PHB production and was further investigated for fermentation of sugar beet juice. The fermentation process with synthetic media resulted in a dry cell weight of 7.88 ± 0.10 g/l, PHB yield coefficient relative to cell dry weight (Yp/x) 0.47 ± 0.12, productivity 0.13 ± 0.04 g/l/hour and PHB content 48.42 ± 7.06%. PHB yield based on sugar consumed was 0.71 ± 0.14. The values for PHB content and the maximum productivity from 2 stage batch fermentation using sugar beet juice adjust for sugar and nutrient content with partial were 38.66 ± 7.28% and 0.22 g/l/hour, respectively, those were both significantly higher (P≤0.05) than those obtained by other two strategies.

Sangkharak & Prasrtsan, (2008) observed PHB production by using three halotolerant bacterial strains such as *Rhodobacter sphaeroides* ES16 and the two mutant strains of *R. sphaeroides* ES16, namely N20 and U7 in glutamate-malate. The mutant

strains N20 and U7 were found to accumulate PHB (53.9 and 42.0% of DCW) 3.6 and 2.8 times higher than the wild type strain (19.5% of DCW) respectively. *R. sphaeroides*N20 were selected for studies on the effects of nutrient and environmental conditions on PHB accumulation. The optimal condition was 4 g/L acetate, 0.02 g/L $[NH_4]_2SO_4$, C/N ratio of 6:1, 1.0 g/L K_2HPO_4, 1.0 g/L KH_2PO_4 and3% NaCl with initial pH at 7.0. Under optimized condition, the maximum PHB accumulation increased from 53.9% to 88% of DCW and 9.11 ± 0.08 g/L biomass, 8.02 ± 0.10 g/L PHB concentration were achieved after 60 hours of cultivation at 37ºC.

Rehman *et al*., (2007) isolated sixteen bacteria from sugarcane molasses soil, sewage water, of which nine bacterial isolate showed presence of PHAs granule confirmed by Sudan black staining. These nine PHAs producers belong to the genus *Pseudomonas*, *Enterobacter*, *Citrobacter*, *Bacillus* and *Escherichia*. PHAs production was optimized for different growth parameters such as nitrogen concentration, pH and temperature. It is observed that, bacterial strains US1 and M1 accumulated up to 30% PHAs of their cell dry weight. Bacterial strain US1 was identified by 16S rRNA gene analysis as *P. aeruginosa* (DQ455691). *Pha C* gene responsible for PHA synthesis was confirmed by PCR amplification. Five strains from nine PHAs producers gave positive results on PCR. *PhaC* gene fragment of US1 was sequenced and submitted to Gene-Bank under the accession number DQ455690. Then *phbc* synthase enzyme amino acid sequence showed homology using the protein BLAST at 129–132 sites with different PHA synthases of the *Pseudomonas* species.

Liu *et al*., (2007) investigated PHB production using a Gram positive soil bacteria *Corynebacterium glutamicum*. In this study, an *E. Coli*, *C.glutamicum* shuttle expression plasmid harbouring PHB synthesis genes, *phbCAB* from *Ralstonia eutropha*, was constructed under the Ptrc promoter. *C. glutamicum* harbouring this plasmid accumulated 3-13% PHB with a weight average molecular mass of 125,400 and a polydispersity of 11.3 when grown on glucose. PHB synthesis related enzyme activities including beta-ketothiolase (PhbA), acetoacetyl-CoA reductase (*PhbB*) and PHB synthase (*PhbC*) were found to be constitutively produced independent of IPTG. L-Glutamate production increased 39–68% in two *C. glutamicum* strains harbouring PHB synthesis genes compared with their parent strains in shake flask experiments. In fermenter studies, the recombinant produced approximately 23% more L-glutamate compared with that of the wild type, and yielded less intermediate metabolites or by-

products including beta-ketoglutarate, L-glutamine and lactate. These results suggested that then expression of *phbCAB* genes in *C. glutamicum* could help regulate glutamate production metabolism.

Sheu *et al*., (2000) studied screening of PHA producing bacteria isolated from the environment using colony PCR and semi-nested PCR technique. 38 PHA positive strains were isolated and their phenotype was further confirmed by Nile blue A staining assay. 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. This protocol is suitable for screening large numbers of environmental isolates. The PHAs accumulation ability of well-separated colonies isolated from environmental samples can be directly validated by PCR with no further culturing or chromosomal DNA extraction procedures. In addition to its application to the screening of wild-type isolates, the individual PCR-amplified product is also suitable as a specific probe for PHA operon cloning.

2.3 PHAs production by mixed bacterial culture (MBC)

Ntaikou *et al*., (2018) analyzed the accumulation capacity, thermal properties, molecular masses, mechanical properties of PHAs produced from acidified waste glycerol (AWG) and its derivatives via an enriched MBC isolated from soil. Among all the substrates the non-acidified waste glycerol has been noticed for showing lowest accumulation capacity. However, average molecular weight was quite high as 1.8×106 Da. Besides this, the thermal and mechanical behavior of PHAs was found to be affected by their monomeric composition.

Montiel-jarillo *et al*., (2017) conducted PHAs production through an enriched MBC at different pH under feast-famine condition. PHAs production reached to maximum of 36% with a production rate of 0.16 Cmol X^{-1} $h^{-1}$when there no control on pH. PHAs production was also evaluated at variable nutrients concentration (pH ranging 8.8 to 9.2) and the results indicated the higher PHAs production of 51% g PHAs g^{-1} VSS under nitrogen limitation.

Janarthan *et al*., (2016) examined the PHAs production through a MBC by taking fermented whey permeates as substrate. They also characterized the organisms in MBC as *Flavisolibacter* and *Zoogloea* through 16S rRNA sequencing. PHA accumulation capacity of the community was increased to population flux during enrichment and acetic acid and propionic acids are found to be the main component of final polymer.

This community adaptation suggests that mixed culture PHA production is a robust process.

Cui *et al.,* (2016) Studied PHAs production from MBC separately by feding them acetate sodium, glucose and starch as an enriching carbon source, followed by long term exposure to aerobic dynamic feeding (ADF) periods. The PHA production capacity, kinetics and stoichiometry of the enrichments, the PHA composition, and the microbial diversity and community composition were explored to determine carbon and enrichment correlations. The highest PHA content of 64.7% and 60.5% cell dry weight (CDW) was observed in MBCs enriched by acetate sodium and glucose. High-throughput sequencing revealed that non-PHA bacteria survived alongside PHA storing bacteria, even under severe F-F selective pressure. The community diversity and composition was studied which revealed that acetate-enriched MBC was dominated by *Pseudomonas* and *Stappia*, glucose enriched MBC was dominated by genus of *Oceanicella*, *Piscicoccus* and *Vibrio* and the starch-enriched MBC was conquered by *Vibrio* genus.

Bianca *et al*., (2016) compared the PHAs accumulation through MBC by taking two fermented cheese wheys (FCW). FCW1 composed of lactic, acetic and butyric acids in the proportion of 58/16/26 (% COD Organic Acid (OA) and FCW2 composed of acetic, propionic, butyric, lactic and valeric acids in the proportion of 58/19/13/6/4 (% CODOA). Between the two substrates FCW2 showed higher PHA accumulation i.e, 0.42 ± 0.03 mg than FCW1 (0.24 ± 0.02 mg). PHAs obtained from FCW2 has an advantage due to the presence of 40% of 3-hydroxyvalerate (HV) and 60% of HB unlike from FCW1 which was composed of exclusively of 3-hydroxybutyrate (HB).

Carvalho *et al*., (2014) studied the impact of operational changes on the microbial community and the associated process performance of PHA producing MBCs by taking fermented molasses as substrate. The organisms in the MBC were identified as *Azoarcus* and *Thauera.* Higher PHAs production and lower biomass growth yields were noticed by enrichriching *Azoarcus* as compared to *Thauera*, while the *Thauera* abundance was parallel to higher hydroxyvalerate (HV) fractions. High PHA production was found by enrichment of *Azoarcus* as the primary biomass fraction with *Thauera* as a minor fraction and composition of the extracted polymer also revealed high HV content which add advantage to the mechanical properties of the biopolymer.

Jia *et al*., (2014) reported the PHAs production by MBC using sludge fermentation liquid (SFL) as a substrate. This lab-scale study suggests that, the maximum PHAs production was found in stimulated SFL (S-SFL) like 59.18% and dropped to 23.47% DCW in actual SFL (L-SFL). However, the pilot scale integrated system comprised of anaerobic fermentation reactor (AFR), a ceramic membrane system (CMS) and a PHAs production bio-reactor (PHAsR) gave the highest PHAs production of 59.47% DCWwith the maximal PHAs yield coefficient (YP/S) of 0.17 g PHAs/gCOD. The above result concluded that VFA containing SFL was suitable for PHAs production.

Duque *et al*., (2013) demonstrated PHAs production from mixed bacterial cultures (MBC) by employing three-stage process. In the research they used cheese whey (CW) and sugar cane molasses (SCW) as cheap substrates, which gave products such as acetate & butyrate and propionate & valerate respectively. Among the two substrates the selected culture attained a maximum PHAs content of 65% with fermented SCM and they also demonstrate a tailoring polymer composition by mixing CW and SCM in equal volume proportions.

Koumelis *et al*., (2013) isolated some micro-organisms from soil and investigated about the effect of nitrogen limitation on PHAs production from MBC by using a synthetic medium with mixed volatile fatty acids was used as carbon source. It was shown that PHAs yields measured either as g PHAs/g VSS or g PHAs/g t-COD consumed were higher when the reactor was operated in nitrogen limitation mode. The 1H-NMR characterization of the polymer revealed that 3HB was the dominant monomer, ranging from 62% to 91%, whereas 3HV was also detected in all cases and 4HB in one case. Thermal characterization gave confirmation about the copolymeric nature of the product with a molecular weight of 77.104 Da to 180.104 Da. Finally, the dominants micro-organisms were also characterized via various biochemical and phenotypical tests followed by 16S r-RNA sequencing.

Reddy and mohan, (2012) demonstrated the PHAs production using wastewater as substrate and mixed culture as biocatalyst. They observed highest PHAs accumulation at higher substrate load (OLR3, 40.3% of dry cell weight (DCW)), low nitrogen (N1, 45.1% DCW) and low phosphorous (P1, 54.2% DCW) conditions. With optimized nutrient conditions production efficiency increased by 14%. Fractional

composition of PHA showed co-polymer [poly (b-OH) butyrate-co-poly (b-OH) valerate, P3(HB-co-HV)] contains PHB (88%) in more concentration compared to PHV (8%). Dehydrogenase and phosphatase enzymatic activities were also monitored during process operation. Good substrate degradation (as COD) of 75% was registered during PHA production. The 16S rRNA sequencing showed the dominance of Firmicutes (71.4%) and Proteobacteria (28.6%), which are known to involve in PHAs accumulation and waste treatment.

Dobroth *et al*., (2011) investigated the PHAs production by MBC using crude glycerol (CG). The extracted PHAs were found to be polyhydroxybutyrate (PHB). This study revealed that PHB synthesis was stimulated by a macronutrient deficiency and intracellular concentrations remained relatively constant over an operational cycle, with microbial growth occurring concurrent with polymer synthesis. The average molecular weight was found to be from 200–380 kDa, while thermal properties compared well with commercial PHB. By considering the pilot scale production, it was estimated that a 38 million L (10 million gallon) per year biodiesel operation could potentially produce up to 19 metric ton (20.9 ton) of PHB per year**.**

Albuquerque *et al*., (2010) analyzed the effect of the influent substrate concentration (30e60 Cmmol VFA/L) on the selection of a PHAs storing culture using fermented sugar molasses. They observed that increase in the carbon substrate concentration from 30 and 45 Cmmol VFA/L to 60 Cmmol VFA/L, resulted in growth limitation however, influent substrate concentration of 45 Cmmol VFA/L showed the best PHA-storing capacity. Besides this the selected MMC was highly enriched in PHA-storing organisms (88%) with a maximum PHA content of 74.6%.

Johnson *et al*., (2009) reported the requirement of nutrients for efficient PHAs producing mixed culture, by investigating the influence of different degrees of carbon and nitrogen limitation under an acetate-fed feast–famine sequencing batch reactor (SBR). High acetate uptake rates were marked in carbon-limited SBRs (medium C/N ratios 6–13.2 Cmol/Nmol), while nitrogen limited SBRs (medium C/N ratios 15–24 Cmol/Nmol) were marked by high ammonia uptake rates.The PHAs storage capacity in a nitrogen-limited SBR operated at 0.5 d SRT decreased significantly over less than 5 months operation. Optimum PHAs production and biomass accumulation requires a

carbon limiting condition and nutrient deficient waste waters, which makes way for selection of a stable PHAs storing biomass with a high storage capacity.

Salehizadeh & Loosdrecht, (2004) studied accumulation of PHAs in various microbes as carbon/energy or reducing-power storage material. The biodegradability of PHAs makes it a efficient substitute of petroleum based polymer. Thus, to reduce the production cost a great effort has been devoted to develop better bacterial strains and more efficient fermentation/recovery processes. The researchers also analyzed the accumulation of PHA by mixed cultures occurs under transient conditions mainly caused by intermittent feeding and variation in the electron donor/acceptor presence. This work reviews the development of PHA research which includes metabolism and various mechanisms for PHA production by mixed cultures; kinetics of PHA accumulation and conversion; effects of carbon source and temperature on PHA production using mixed cultures; PHA production process design; and characteristics of PHA produced by mixed cultures.

CHAPTER: 3 MATERIALS AND METHODS

3.1 Introduction

PHAs represent a group of biopolymers that could be accumulated as carbon and energy sources by several of bacterial genera. As their thermoplastic properties are comparable with the petroleum-based plastics, the pilot scale production of PHAs has become a burning topic in today's plastics industry. Process economics of industrial PHAs production study reveals that use of high yielding strain, mixed microbial culture, inexpensive carbon sources in upstream and least quantity of solvent in downstream processing can make the process cost affordable (Reddy *et al.*, 2003; Gomma, 2014; Mohapatra *et al.*, 2014). Thus, use of mixed microbial culture (MMC) is an interesting substitute in order to reduce the current PHAs production costs. In this research work, we analyzed the quality and quantity of PHAs produced under submerged fermentation process by using mixed bacterial culture (MBC).

3.2 Materials and Methods

3.2.1 Selection of source organism and morphological characterization

In our preceding work (Mohapatra *et al.*, 2014) sixteen Gram positive rhizospheric *Bacillus* species were evaluated for PHAs production. Among all the strains C1, O6, P1 and P2 were found to maintain their PHAs production activity even after 3 years of preservation at -20^{o}C. The selected bacterial isolates were revived in the nutrient agar (NA) medium and incubated at 37^{o}C for 24 hours followed by Gram staining to observe their cellular morphology.

3.2.2 Culture media and chemicals

Most of the culture media used for the revival of bacteria and production of PHAs were prepared as per the manufacturer's instruction by using double distilled water. Culture media and chemicals required for this research work were procured from Hi-media laboratories private limited, Mumbai. The organic solvents and standard used in this research work were procured from Merck bioscience and Sigma-Aldrich chemicals respectively.

3.2.3 Detection of PHAs granule by Sudan black B staining

Screening of the PHAs producing bacterial isolates were conducted by Sudan black B staining method (Schlegel *et al*., 1970). This method is used for detection of PHB (the most common homopolymer of PHAs) granule in the cytosol of bacteria. However, before screening, the bacterial isolates were induced to accumulate PHAs granule by growing in nitrogen limiting mineral salt medium (MSM) (NaCl 10.0 g/l, KH_2PO_4 2.5 g/l, K_2HPO_4 2.5 g/l, $(NH_4)_2SO_4$ 3.38 g/l, yeast extract 2 g/l, Na_2HPO_4 1.5 g/l, $MgSO_4.7H_2O$ 0.2 g/l, glucose 20.0 g/l, $CaCl_2$ 0.052 g/l and agar agar 20.0 g/l) and incubated at 37°C for 48 hr. Then, smear was made on a clean glass slide. After drying, cells were deposited on a glass slide and heat fixed followed by 0.3% (w/v in 70% ethanol) of Sudan black B staining for 15 minutes. The slide was washed gently with distilled water followed by Gram's decolourizer for few second and then counterstained with safranin (5% w/v in de-ionized water) for 10 seconds. The slides were then washed gently, dried and observed under light microscope (1000X, Leica DM5000B).

3.2.4 Detection of PHAs granule by Nile red staining

The highly specific Nile red viable colony staining technique (Spiekermann *et al*., 1999) was also conducted for detection of PHB granule present in the bacterial cytosol. Briefly, selected bacterial isolates were streaked on nitrogen limiting MSM containing 0.2% Nile red solution (dissolve in DMSO) and incubated at 37°C for 48 hr. Plates were observed under UV-transillumination with excitation wave length of 312 nm.

3.2.5 Antagonistic activity (*in-vitro*) among PHAs producing bacteria

Antagonistic activity among PHAs producing bacterial strains were studied by agar well diffusion technique. This test was conducted by taking fresh culture of selected bacterial isolates. Fresh culture of the bacterial isolate was lawn cultured on NA plate and then 10µl of another bacterial suspension was placed in the agar well (prepared using gel puncture). Then, the plate was incubated at 37°C for 24 hours to observe the zone of inhibition (Khokhar, *et al*., 2011). This experiment was conducted in triplicates and the qualitative results were noted by observation. Then, mixed bacterial culture or consortium was prepared by taking desired bacterial isolates.

3.2.6. Optimization of growth parameters for biomass and PHAs production

3.2.6.1 Culture media

Presence of various nutrients in the culture media that regulates bacterial growth. Therefore, 100 ml of nitrogen limiting (glucose to nitrogen ratio 6:1) minimal salt media (MSM) and modified growth medium (GM) was taken in different Erlenmeyer flask and 10 ml of 24 hours fresh culture (MBC) was dispensed and incubated at 37°C for 24 hours at 120 rpm. Then comparative bacterial biomass production was estimated by measuring the OD_{600} in UV-Vis spectrophotometer (λ35, Perkin-Elmer) and the optimized culture media was determined.

3.2.6.2 pH

This test was carried out using optimized culture medium (MSM). For the observation of optimum pH, 100 ml of MSM was taken in different Erlenmeyer flask and pH was adjusted from 5-9 with help of 1N HCl, 1N NaOH and digital pH meter. 10 ml of 24 hours fresh culture (MBC) was dispensed and incubated at 37°C for 24 hours at 120 rpm. Then comparative bacterial biomass production was evaluated by measuring the OD_{600} in UV-Vis spectrophotometer (λ35, Perkin-Elmer) and the optimum pH was determined.

3.2.6.3 Temperature

This test was carried out using optimized culture medium (MSM) and pH (7.0). To study the optimum temperature for biomass production, 100 ml of MSM was taken in different Erlenmeyer flask and temperature was adjusted at 23°C, 30°C, 37°C and 42°C. 10 ml of 24 hours fresh culture (MBC) was dispensed and incubated at 37°C for 24 hours at 120 rpm. Then comparative bacterial biomass production was evaluated by measuring the OD_{600} in UV-Vis spectrophotometer (λ35, Perkin-Elmer) and the optimum temperature was determined.

3.2.6.4. Carbon source

This test was carried out using optimized culture medium (MSM), pH (7.0) and temperature 37ºC. Thus, 100 ml of MSM was taken in different Erlenmeyer flask and various carbon sources such as dextrose, fructose, sucrose, glucose and maltose were added. 10 ml of 24 hours fresh culture (MBC) was dispensed and incubated at 37°C for

24 hours at 120 rpm. Then comparative bacterial biomass production was evaluated by measuring the OD_{600} in UV-Vis spectrophotometer (λ35, Perkin-Elmer) and the best carbon source was determined.

3.2.6.5. Nitrogen source

This test was carried out using optimized culture medium (MSM), pH (7.0), temperature 37ºC and carbon source (sucrose). Thus, 100 ml of MSM was taken in different Erlenmeyer flask and different nitrogen sources such as sodium nitrate, peptone, ammonium chloride, ammonium sulphate and yeast extract were added. 10 ml of 24 hours fresh culture (MBC) was dispensed and incubated at 37°C for 24 hours at 120 rpm. Then comparative bacterial biomass production was evaluated by measuring the OD_{600} in UV-Vis spectrophotometer (λ35, Perkin-Elmer) and the finest nitrogen source was determined.

3.2.6.6. Inoculum size

Likewise to find out the optimum inoculum size for higher biomass production, the test was conducted using optimized culture medium (MSM), pH (7.0), temperature 37ºC, carbon source (sucrose) and nitrogen source (ammonium chloride). For optimization of inoculums size 100 ml of optimized culture medium (MSM) was taken in different Erlenmeyer flask and different concentrations such as 5%, 10%, 15% and 20% of inoculum (24 hours fresh mixed bacterial culture) was added, followed by incubation at 37°C for 24 hours at 120 rpm. Moreover, comparative bacterial biomass production was evaluated by measuring the OD_{600} in UV-Vis spectrophotometer (λ35, Perkin-Elmer) and the optimum inoculum size was determined.

3.2.7. PHAs production by submerged fermentation process and extraction by sodium hypochlorite digestion and multi-solvent extraction method

One-stage batch cultivation in shake flasks method was conducted for accumulation of PHA granule in the bacterial cell. The mixed bacterial culture was subjected to PHA production under optimized growth conditions (Mohapatra *et al*., 2014). Briefly, 1L of MSM medium with pH 7.0 was taken in an Erlenmeyer flask and 10% of inoculum (overnight mixed bacterial culture containing $1.5x10^8$cells/ml) was added. The mixed bacterial culture was incubated in shaker incubator at 37ºC for 72 h

with an agitation rate of 150 rpm. Then, PHAs extraction was carried out by following different method.

The harvested mixed bacterial cells were centrifuged at 10,000 rpm for 15 min and dried over night at 50°C. The cell pellets were suspended in a mixture of sodium hypochlorite & water solution (3:1) and incubated at 37°C for 1hour for complete digestion of non PHA materials. The bacterial cell suspension was centrifuged with the same condition to collect PHAs granules and the supernatant was discarded. The sediment was washed twice with 10 ml of distilled water and centrifuged with the same condition. The PHAs granules in the sediment were washed twice with acetone, methanol and diethyl ether in 1:1:1 ratio respectively. Then, the pellets were collected in chloroform, placed in water bath at 60°C for 10min and then centrifuged with the same condition. The pellets were separated by filtration and the filterate was evaporated at 70-80°C in a water bath to obtained PHAs film. Then, the weight of PHAs film was determined (Mohapatra *et al*., 2016). Moreover, PHAs production was also quantified using the following formula:

$$\text{PHAs production } (\%) = \frac{\text{Weight of PHAs}}{\text{Cell biomass (DCW)}} \times 100$$

3.2.8 Structural characterization and biodegradability of PHAs

3.2.8.1 Fourier transform infrared (FTIR) spectroscopic analysis

Extracted PHAs sample was mixed with 2% potassium bromide (KBr). The mixtures were compressed into translucent sample discs and fixed in the FTIR spectrometer (Perkin-Elmer RX I) for analysis (Dash *et al*., 2013) infrared spectra for the sample was recorded in the transmission mode from 4,000 to 400 cm^{-1} to check the presence of functional group (Lau *et al*., 2011).

3.2.8.2. Biodegradation of PHAs by composting method

The extracted PHAs film was subjected to biodegradation in natural environment (Mohapatra *et al*., 2016). Briefly, 0.106 gm of PHAs film was composted 10cm below the surface of soil having environmental temperature and pH 35°C and 7.2 respectively. Then, the PHAs film was visually inspected for changes in their morphology and weight loss at different time interval such as 7, 14, 21 and 30 days respectively. Change in surface structure of PHAs film was analyzed using stereomicroscopic analysis.

CHAPTER:4 RESULTS AND DISCUSSION

4.1 Morphological characterization of source bacteria

The genera *Bacillus* are preferred over other Gram negative bacteria by several industries and academia because of their availability in nature, faster growth rate, genetical stability, capability to grow by utilizing inexpensive carbon sources and to produce endotoxin free PHAs (Mohapatra *et al*., 2017).In light of above the preserved PHAs producing *Bacillus* sp. C1 (2013) (KF626477), *Bacillus* sp. P1 (2013b) (KF626468),*Bacillus* sp. P2 (2013) (KF626472),*Bacillus* sp. P3 (2013) (KF626473), *Bacillus* sp. P4 (2013c) (KF626474), *Bacillus* sp. O6 (KF626479) and *Bacillus* sp. G5(KP172548)were selected for the present research work (Fig. 1). Besides this a Gram negative bacterium *Zobellella* sp. DD5 (KX258951) was also selected. Out of all, four bacterial isolate such as *Bacillus* sp. C1 (2013) (KF626477), *Bacillus* sp. P1 (2013b) (KF626468),*Bacillus* sp. P4 (2013c) (KF626474) and *Bacillus* sp. O6 (KF626479) accumulated PHAs granules in their cytosol even after 1-3 years of preservation, which was confirmed from Sudan black (Fig. 2) and Nile red staining. All these four bacterial isolates are energetically stable, thus PHAs synthesis ability of the bacterial isolates were not gone even after 3 years of preservation at low temperature. (Mohapatra *et al*., 2016).

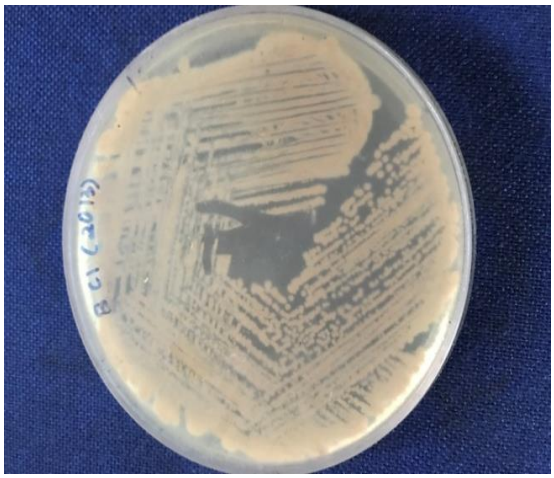


Fig.1a: *Bacillus* sp. C1(2013)

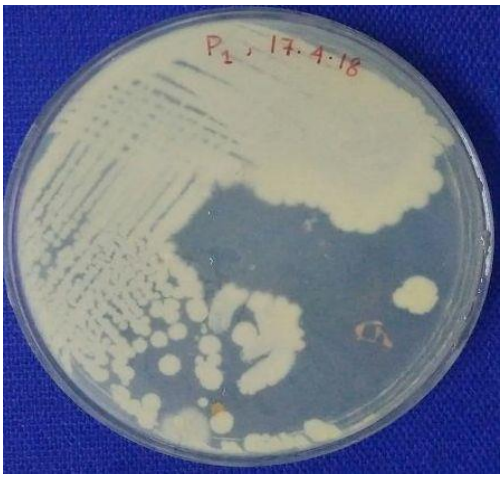


Fig.1b: *Bacillus* sp. P1(2013b)

Fig.1c: *Bacillus* sp. P2 (2013)

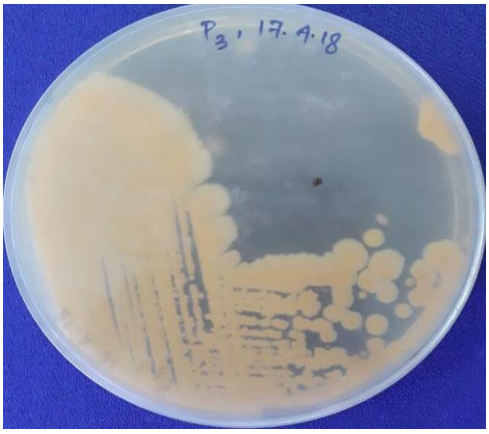


Fig.1d: *Bacillus* sp. P3 (2013)

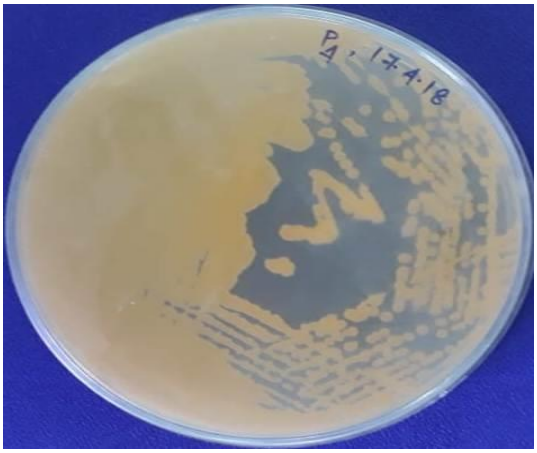


Fig.1e: *Bacillus* sp. P4 (2013c)

Fig.1f: *Bacillus* sp. O6

Fig.1g: *Bacillus* sp. G5

Fig. 1h: *Zobellella* sp. DD5

Fig. 1: Revival of preserved PHAs producing bacterial isolates

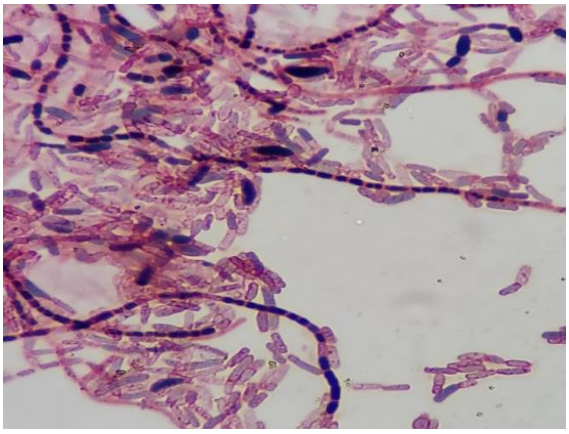


Fig.2a: *Bacillus* sp. C1(2013)

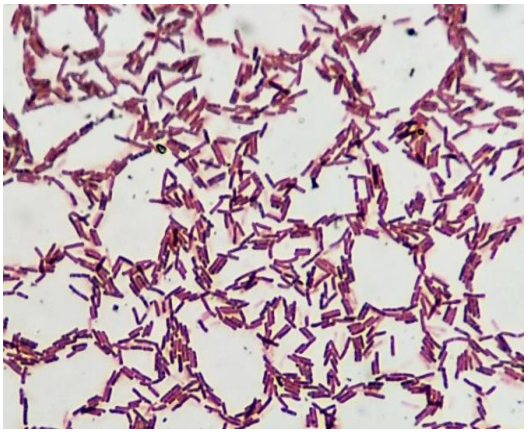


Fig.2b: *Bacillus* sp. P1(2013b)

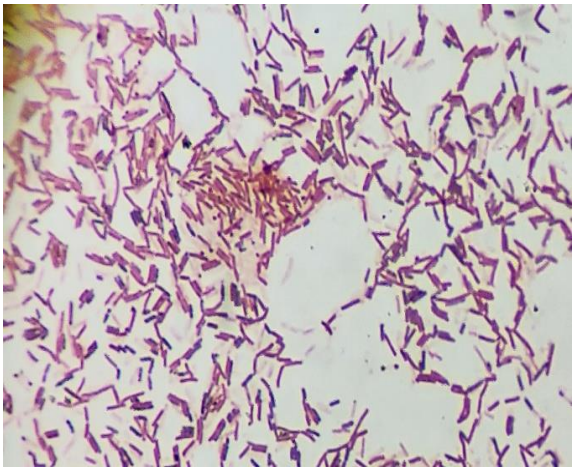


Fig.2c: *Bacillus* sp. P2 (2013)

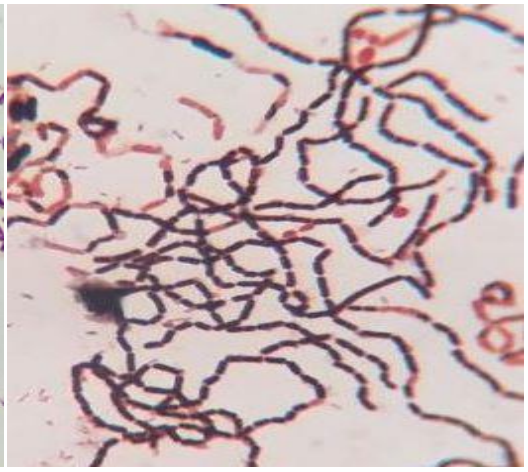


Fig.2d: *Bacillus* sp. O6

Fig. 2: Micrographs of PHAs producing bacterial isolates under Sudan Black staining

4.2 Antagonistic activity (*in-vitro*) among PHAs producing bacteria

After screening, these PHAs producing bacterial isolates were subjected for antagonistic activity study. This study has is highly essential for PHAs production by mixed bacterial culture. Antagonism was performed between *Bacillus* sp. C1 (2013) &*Bacillus* sp. O6, *Bacillus* sp. C1 (2013) &*Bacillus* sp. P1 (2013b), *Bacillus* sp. C1 (2013) &*Bacillus* sp. P2 (2013), *Bacillus* sp. O6 &*Bacillus* sp. P1 (2013b), *Bacillus* sp. O6 & *Bacillus* sp. P2 (2013) and *Bacillus* sp. P1 (2013b) &*Bacillus* sp. P2 (2013). Among the six selected pair, *Bacillus* sp. C1 (2013) and *Bacillus* sp. O6 showed compatibility against each other as confirmed from antagonistic activity(Fig. 3). However, other bacterial isolates showed a high degree of inhibition to each other. Similar result was also obtained by author (Bianca *et al*., 2016) while evaluated PHAs

production by enriched mixed microbial culture of activated sludge. This might be due to production of secondary metabolites such as antibiotics, toxins, pigments and immune modulatory substances (Velusamy and Gnanamanickam, 2008).

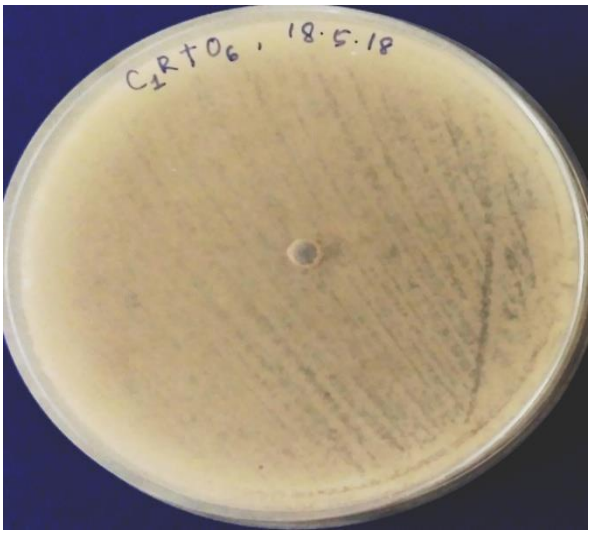


Fig. 3a*: Bacillus* sp. C1 (2013) &*Bacillus* sp.O6.

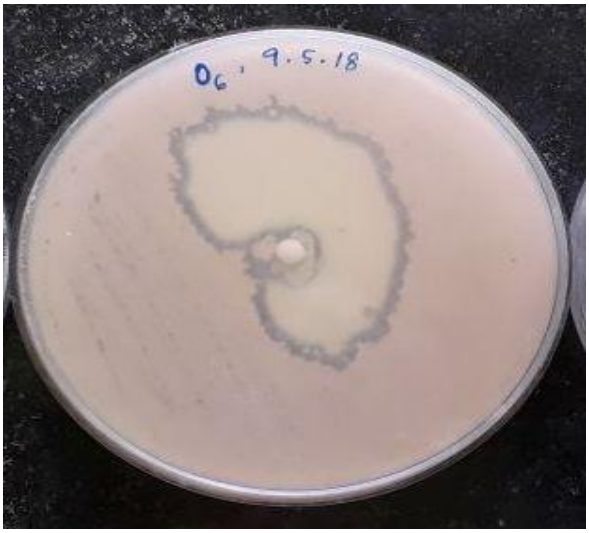


Fig. 3b: *Bacillus sp. P1 (2013b) & Bacillus sp. O6*

Fig. 3c: *Bacillus* sp. P1(2013b) & *Bacillus* sp.P2(2013)

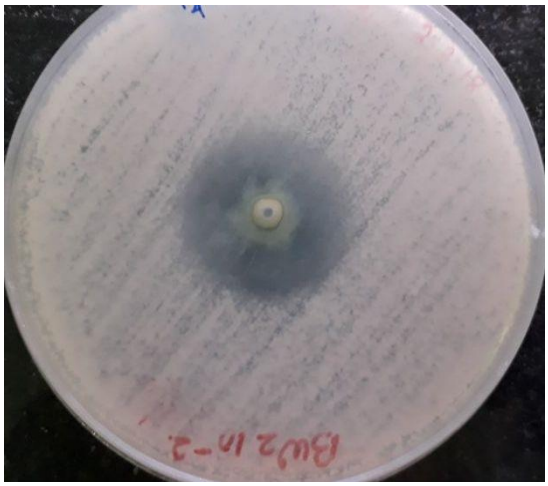


Fig. 3d: *Bacillus* sp. C1 (2013) & *Bacillus* sp P2(2013)

Fig. 3: Antagonistic activity of PHAs producing bacterial isolates

4.3 Optimization of growth parameters for biomass and PHAs production

As *in vitro* PHAs synthesis is parallel to microbial biomass production, thus optimization of growth parameters are highly essential.Initially culture medium such asgrowth medium (GM)and minimal salt medium (MSM) were taken for optimization with respect to biomass production.The statistical outcome revealedthat MSM was more suitable for biomass production than GM. Similarly, other growth parameters such as pH, temperature, carbon source, nitrogen source and inoculums size were optimized (Fig. 4). The mixed bacterial culture wasfound to produce maximum cell biomass at pH 7.0, temperature 37ºC, 10% inoculum size, sucrose as carbon and ammonium chloride as nitrogen source. The optimum pH and temperature for higher biomass production by mixed bacterial culture were 7.0 and 37ºC respectively as reported earlier (Shalin *et al*., 2014; Colombo *et al*., 2016; Jarillo *et al*., 2017).Similarly, sucrose and ammonium chloride were found to be the best carbon and nitrogen source as per the literature available in the public domain (Zhang *et al*., 1994; Colombo *et al*., 2016). However, during this study the C: N ratio was maintained at 6:1 to obtained higher cell biomass. Most of the bacterial isolates were produced higher cell biomass in this C:N ratio(Sangkharak and Prasertsan, 2008).

The above result revealed that the mixed bacterial culture containing *Bacillus* sp. C1 (2013) and *Bacillus* sp. O6 depicted a higher cell biomass production at pH 7.0 and temperature 37˚C as these bacteria were isolated from environmental samples. Furthermore, sucrose is the most easily assimilable carbon source as it is the industrially important carbon source for microbial fermentation (Sabri *et al*., 2012).In the same way ammonium chloride was found to be the best nitrogen source for biomass production which ultimately leads to high PHAs production. Under nitrogen restrictive condition, the unavailability of nitrogen pool leads to the decrease in NADPH consumption followed by blocking of amino acid synthesis causing the accumulation of excess NADPH in the cells, which eventually direct the enhanced PHAs synthesis in nitrogen-deficient cells (Singh & Mallick, 2008).

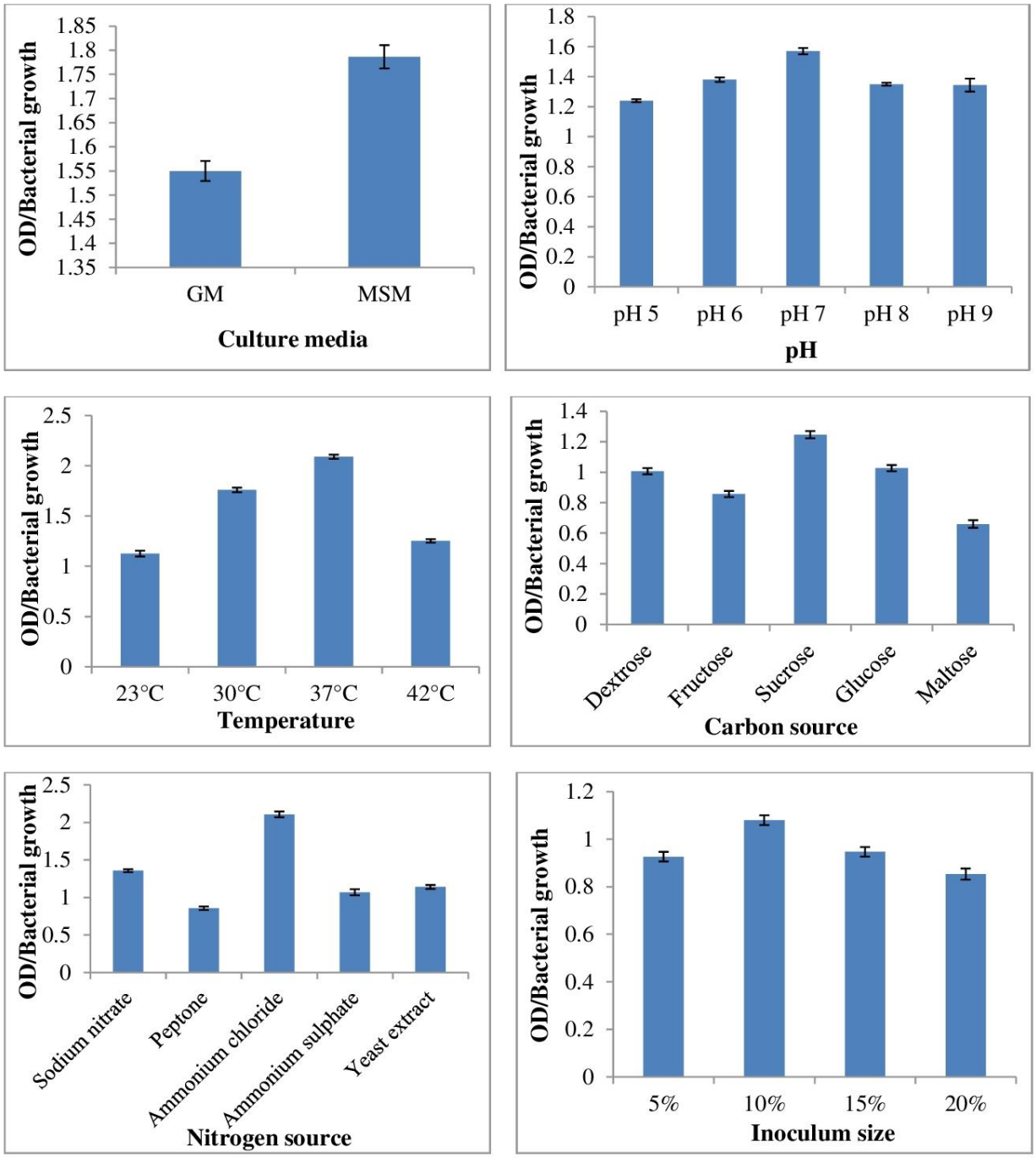


Fig. 4: Optimization of growth parameters such as culture media, pH, temperature, carbon source, nitrogen source and inoculum size of PHAs producing bacterial isolates.

4.4 PHAs production by bacterial isolates

Under optimized conditions, the mixed bacterial culture produced 1.5 g/L of PHAs (Fig. 5) in 72 hours and the rate of PHAs production was decreased after 72 hours. This result is similar with the early findings of Colombo *et al*., (2016), Dobroth *et al*., (2011) and Jia *et al*., (2014). In this study two different species of *Bacillus* were used in consortium because of their advantage over other bacterial species for PHAs production due to their absence of lipopolysaccharides external layer which makes the extraction much easier, its capability of growing in cheap raw materials and high growth rate in comparison to other bacteria (Khiyami *et al*., 2011). In addition to this they also displayed high level of compatibility during their growth. *Bacillus* species are also capable of utilising agro-industrial and other waste materials as their carbon source due to the presence of variety of hydrolytic enzymes (Israni and Shivakumar, 2013).

Fig. 5: PHAs film produced from MBC under submerged fermentation

4.5 Fourier transform infrared (FTIR) spectroscopic analysis

FTIR spectra of the PHAs produced under submerged fermentation from mixed bacterial culture showed six intense absorption bands at 1719.76 cm^{-1} (C=O stretch), 1379.14 cm^{-1} (CH_2,CH_3& -CH_3), 1261.54 cm^{-1} (–CH_3& C-O stretch), 1226.71 cm^{-1} (C-O stretch), 1130.74 cm^{-1} (C-N & C-O stretch) and 1099.96 cm^{-1} (C-N, C-O stretch) (Table 1). The high intense absorption band, was obtained at 1719.76 cm^{-1} corresponding to the (C = O stretch) ester carbonyl group, which has the characteristic of PHB (Fig. 6).Moreover, PHB is the most common homopolymer of PHAs. The extracted PHB has

56% purity.Similar outcomes was also obtained by researchersShalin *et al*., (2014) and Reddy & Mohan, (2012), Thus, IR spectroscopic analysis gave proper insights for the chemical structures of the PHB by reflecting the monomeric units.

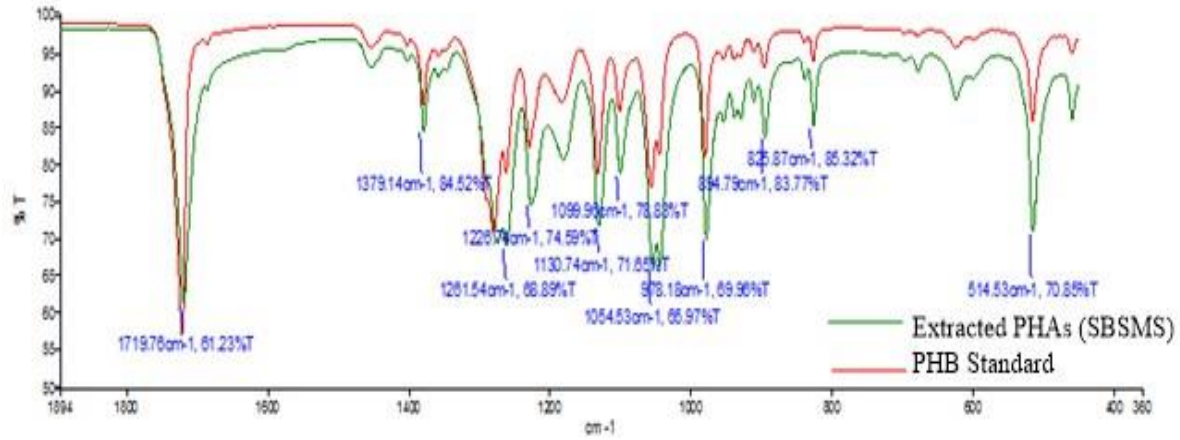


Fig. 6:FTIR of PHAs produced by MBC under submerged fermentation

Table 1: FTIR spectra of PHAs and their corresponding annotation

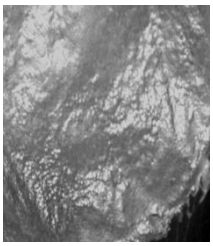
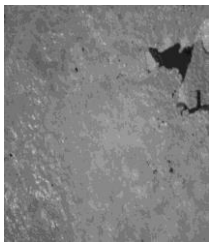
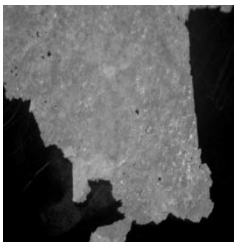
Peaks (cm^{-1})	**1719.76**	**1379.14**	**1261.54**	**1226.71**	**1130.74**	**1099.96**
Bonds	**C=O stretch**	**CH_2& CH_3,-CH_3**	**-CH_3,C-O stretch**	**C-O stretch**	**C-N,C-O stretch**	**C-N,C-O stretch**
Functional groups	**Alpha, Beta-unsaturated esters**	**Alkanes**	**Alkanes, Carboxylic acids & Esters**	**Carboxylic acids, Esters & Ether**	**Aliphatic amine, Alcohol & Ethers**	**Aliphatic amine, Alcohol & Ether**

4.6 Biodegradation of PHAs by composting method

PHB film produced by MBC under submerged fermentation was found to be biodegradable as the data revealed from open windrow composting (Table 2). Different weight loss dynamics were observed during degradation of extracted PHAs, which can be estimated by a decrease in the polymer weight during composting. Moreover, the PHB extracted from MBC degraded within 21 days. Similar to our results, Shalin *et al*., (2014) also reported the 20% retention of PHAs weight after 35 days of incubation.

However, Sridewi *et al*. (2006) reported the degradation of PHB within 7 days. In our work, the variation in degradation of biofilm is due to the variation in surface roughness and properties of different monomer of PHB (Wang *et al*., 2014; Tang *et al*., 2009; Mohanty *et al*., 2014). Rough surface (McAllister *et al*., 1993) and crystalline nature (Spyros *et al*., 1997) of the PHAs film enhances better attachment of microbes, leading to degradation of polymer to CO_2 & water in aerobic and CO_2 & methane in anaerobic condition (Varsha & Savitha., 2011; Knoll *et al*., 2009; Boyandin *et al*., 2012; Reddy *et al*., 2003).

Table 2: Biodegradation of PHAs by composting method

Production process	**1st day**	**7th day**	**14th day**	**21st day**
Submerged fermentation	 (0.106g)	 (0.020g)	 (0.018g)	-

CHAPTER: 5 SUMMARY AND CONCLUSION

5.1 Summary

The genera *Bacillus* are preferred by several industries and academia due to their genetic stability, short generation time, utilize inexpensive carbon sources and produce endotoxin free PHAs as compared to Gram negative bacteria (Mohapatra *et al*., 2015; Mohapatra *et al*., 2017). Owing to the fact of various industrial and pharmaceutical applications of PHAs, in this study the PHAs production was observed from MBC using species of *Bacillus*.

In light of above eight different preserved PHAs producing *Bacillus* sp. C1 (2013) (KF626477), *Bacillus* sp. P1 (2013b) (KF626468), *Bacillus* sp. P2 (2013) (KF626472), *Bacillus* sp. P3 (2013) (KF626473), *Bacillus* sp. P4 (2013c) (KF626474), *Bacillus* sp. O6 (KF626479) and *Bacillus* sp. G5 (KP172548) and one Gram negative bacterium *Zobellella* sp. DD5 (KX258951) were selected for the present research work. Out of all, four bacterial isolate such as *Bacillus* sp. C1 (2013) (KF626477), *Bacillus* sp. P1 (2013b) (KF626468), *Bacillus* sp. P4 (2013c) (KF626474) and *Bacillus* sp. O6 (KF626479) accumulated PHAs granules in their cytosol even after 3 years of preservation.

Antagonism was performed between *Bacillus* sp. C1 (2013) & *Bacillus* sp. O6, *Bacillus* sp. C1 (2013) & *Bacillus* sp. P1 (2013b), *Bacillus* sp. C1 (2013) & *Bacillus* sp. P2 (2013), *Bacillus* sp. O6 & *Bacillus* sp. P1 (2013b), *Bacillus* sp. O6 & *Bacillus* sp. P2 (2013) and *Bacillus* sp. P1 (2013b) & *Bacillus* sp. P2 (2013). Among the six selected pair, *Bacillus* sp. C1 (2013) and *Bacillus* sp. O6 showed compatibility against each other, however other bacterial isolates showed a high degree of inhibition to each other.

With the view of *in vitro* PHAs synthesis is parallel to microbial biomass production, thus various growth parameters were optimized. The culture media optimization results revealed that MSM was more suitable for biomass production than GM. Similarly, other growth parameters such as pH, temperature, carbon source, nitrogen source and inoculums size were optimized. The mixed bacterial culture was found to produce maximum cell biomass at pH 7.0, temperature 37^{o}C, 10% inoculum size, sucrose as carbon and ammonium chloride as nitrogen source.

Under optimized conditions, the mixed bacterial culture produced 1.5 g/L of PHAs (Fig. 5) in 72 hours and the rate of PHAs production was decreased after 72 hours. Moreover, 1.5 g/L of PHAs was extracted from MBC using sodium hypochlorite and multi-solvent extraction method.

Besides this, PHAs produced from MBC also depicted high intense absorption bands in between 1719-1720 indicating presence of carbonyl ester (C=O) group of PHB, the most common homopolymer of PHAs. Thus, IR spectroscopic analysis gave proper insights for the chemical structures of the PHB by reflecting the monomeric units. The extracted PHB has 56% purity. PHAs film was found to be biodegradable within 21 days as revealed from open windrow composting. Different weight loss dynamics (0.106gm to 0.018gm) were observed during degradation of PHAs film from 1 to 21 days.

5.2 Conclusion

In this small piece of research, we focoused on quality and quantity of PHAs produced by mixed bacterial culture containing *Bacillus* sp. C1 (2013) and *Bacillus* sp. O6. under submerged fermentation process. Moreover, 1.5g/L of PHB was produced by MBC under submerged fermentation. However, the quality of PHB in terms of purity was 56%. This PHB film is biodegradable within 21 days. For validation of this research work in pilot scale further research is highly indispensable.

5.3 Future outlook

1. Structural and thermal characterization of the PHB produced by MBC under submerged fermentation.
2. Evaluation of biocompatibility of the PHB in terms of cytotoxicity test.
3. Possible application of PHB produced by MBC under submerged fermentation.

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