ISOLATION AND CHARACTERIZATION OF BACTERIA FROM DOMESTIC AND INDUSTRIAL WASTE MATERIALS FOR BIOPOLYMER PRODUCTION

A
Dissertation Submitted For the Award of the Degree of

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IN
BOTANY

BY

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CERTIFICATE

This is to certify that the research work embodied in this thesis entitled ***"Isolation and characterization of bacteria from domestic and industrial waste materials for biopolymer production"*** *submitted by* ***Farhana Islam Khan*** *was carried out under my supervision in the Laboratory of Microbiology, Department of Botany, University of Dhaka. This is further to certify that the research work presented here is an original work and suitable for the partial fulfillment of the degree of Doctor of Philosophy in Botany. To the best of my knowledge this thesis has not been submitted elsewhere.*

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DECLARATION

I, Farhana Islam Khan, hereby declare that the data presented in this thesis entitled **" Isolation and characterization of bacteria from domestic and industrial waste materials for biopolymer production"** which is submitted to the University of Dhaka, for the award of the Degree of Doctor of Philosophy in Botany is the result of research work carried out by me under the guidance and supervision of Dr. Mihir Lal Saha, Professor, Department of Botany during the period from December 01, 2014 to November 30, 2019 in the Laboratory of Microbiology, Department of Botany, University of Dhaka.

I further declare that the work presented in this thesis has not been submitted previously for the award of any other degree or diploma or any other similar titles.

Farhana Islam Khan

DEDICATED

TO

MY BELOVED PARENTS

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-Author

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbols	Details
%	Percent
&	And
ºC	Degree centigrade
α	Alpha
β	Beta
γ	Gamma
<	Less than
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cfu	Colony forming unit
cm	Centimeter
Co.	Company
Conc.	Concentration
Corp.	Corporation
DNS	3,5-dinitrosalisylic acid
Ed.	Editor
ed.	Edition
e.g.	Exempli grata (For example)
et al.	Et alliori (and others)
etc.	Etcetra
Fig.	Figure
FTIR	Fourier transform infrared spectroscopy
G or g	Gram
h	Hour
H_2O	Water
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulphide

Symbols	Details
HCl	Hydrochloric acid
H_2SO_4	Sulphuric acid
i.e.	Id est (that is)
Inc.	Incorporation
J.	Journal
K_2HPO_4	Dipotassium hydrogen phosphate
KCl	Potassium chloride
KOH	Potassium hydroxide
L or l	Liter
L	Levorotatory
Ltd.	Limited
M	Molar
Max	Maximum
mg	Milligram
$MgSO_4.H_2O$	Aqueous Magnesium sulphate
min	Minute
ml	Millilitre
mm	Millimeter
M.R.	Methyl red
µ	Micron
µg	Microgram
µl	Microlitre
µm	Micrometer
µM	Micromolar
N	Normal concentration
NA	Nutrient agar
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NH_3	Ammonia
$(NH_4)_2$ HPO_4	Diamonium hydrogen phosphate

Symbols	Details
No.	Number
nm	Nanometer
OD	Optical density
p	Page
pp	Pages
pH	Negative logarithm of hydrogen ion concentration
rpm	Revolutions per minute
SAB	Society of American Bacteriology
SAS	Statistical Analysis System
SD	Standard Deviation
Sec	Second
sp.	Species (Singular)
spp.	Species (Plural)
SPSS	Statistical Package for the Social Sciences
sq.	Square
St.	Saint
TTC	2,3,5-triphenyltetrazolium chloride
U	Unit
UV	Ultra violate
v.	Version
viz.	Videly (namely)
V.P.	Voges-Proskauer
v/v	Volume/volume
w/v	Weight/volume
(-)ve	Negative
(+)ve	Positive

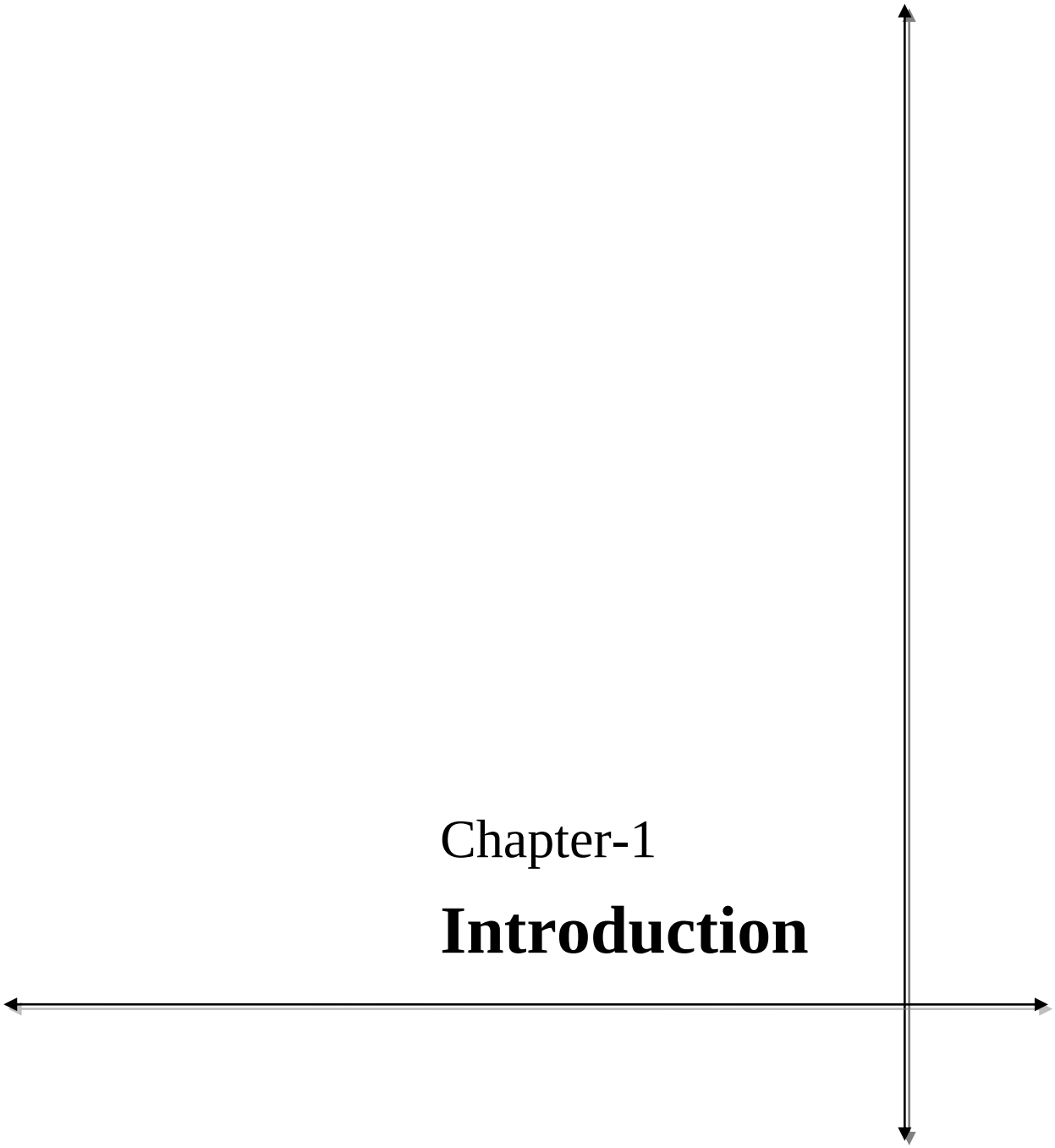
Abstract

Dependence on conventional plastics and their boundless usage have resulted in waste accumulation and greenhouse gas emissions. Recent technologies are directed towards the development of bio-green materials that exert negligible side effects on the environment. A biologically-synthesized plastic, polyhydroxybutyrate (PHB) has been attracting major interests due to its similar physical properties to synthetic plastics. Unlike synthetic plastics, PHB is produced from renewable resources and is degraded aerobically by microorganisms to CO_2 and H_2O upon disposal. PHB is a renowned biodegradable plastic that do not release any toxins or residues in the environment like petroleum based plastics. The present study was undertaken to isolate potential indigenous PHB producing bacteria from different waste materials. The isolated bacteria were tested for desired PHB production along with their biotechnological applications. The aerobic heterotrophic bacterial load of the collected samples ranged in between 5.50×10^3 and 2.52×10^7, 3.10×10^4 and 2.23×10^7, 7.50×10^3 and 1.01×10^7 cfu/g or cfu/ml in NAG (nutrient agar with 1% glucose), PYG and LB media, respectively. The maximum bacterial count (2.52×10^7cfu/g) was observed in soil of BDR market and minimum (5.5×10^3cfu/ml) in garments waste water. The highest number (33) of PHB producing bacteria was found in kitchen waste soil and the lowest number (9) was in the Turag River water.

Desired indigenous bacteria isolated from different waste materials were screened for maximum PHB production. On the basis of PHB activity, 30 isolates showed better performance among the isolates. Screening for PHB was done by Sudan black B and tested under microscope using sudan black staining. Both Gram positive and Gram negative PHB positive bacteria were found to be associated with studied samples. Among 30 isolates, 21 were Gram positive rods and 9 were Gram negative rods. Gram positive rods were provisionally identified as *Bacillus* spp. while Gram negative bacteria were identified as *Acetobacter, Enterobacter, Klebsiella, Neisseria, Pseudomonas, Rhizobium* and *Tatumella.* Potential 10 isolates were further studied for molecular identification. Among them, 9 genera were matched with their conventional identification except *Rhizobium leguminosarum.*

Molecularly identified 10 isolates were selected for quantitative PHB activity. The PHB producing capabilities of the isolated indigenous 10 isolates ranged in 3.43 ± 0.24 and 79.75 ± 6.10 µg/ml. All the isolates showed maximum PHB activity after 72 h of incubation except *Bacillus megaterium* LY6, which showed the highest PHB activity after 96 h of incubation. All the isolates showed decreased PHB production after 72 h of incubation with the increase of the incubation period. Among 10 isolates, *Bacillus cereus* HB45 showed the highest PHB activity (79.75 ± 6.10 µg/ml) at 72 h of incubation before optimization. Two isolates *viz. Bacillus cereus* HB45 and *Bacillus thuringiensis* B43 could produce PHB more than 70 µg/ml among the selected isolates and were optimized for PHB production. Before optimization these isolates could produce PHB 79.75 ± 6.10 and 72.72 ± 6.59 µg/ml, respectively. Through optimization, PHB production increased up to 867.17 ± 7.35 and 955.41 ± 7.35 µg/ml by *B. cereus* HB45 and *B. thuringiensis* B43. Before optimization *B. cereus* HB45 was better than *B. thuringiensis* B43 but after optimization *B. thuringiensis* performed better than *B. cereus.*

Characterization of extracted PHB was carried out by FT-IR, which later confirmed the presence of intracellular accumulated polymer and substantiated as PHB. PHB films were produced by *Bacillus cereus* HB45 and *Bacillus thuringiensis* B43 using best carbon and nitrogen source. The PHB films produced were very stiff and brittle in appearance and were found to be fragile. Use of bioplastics produced by these bacteria will be helpful to reduce environmental pollution. The result clearly reflected that biotechnologically these two isolates could be useful and have got potential for commercial applications.



Chapter-1

Introduction

Introduction

Synthetic polymers or plastics are viewed as a vital endowment of present day sciences and innovation to humankind (Luckachan and Pillai 2011). Plastics are utilized in almost every manufacturing industry in the world ranging from automobiles to medicine. Unfortunately, this great discovery gets buried in landfills everyday and takes up space because of its non-degradable nature (Shah 2014). Plastics are manmade synthetic polymers that are comprised of long chains of rehashing atomic units called monomers. Monomers are the building blocks of polymers. Monomers such as vinyl chloride, styrene and acrylonitrile are produced by the petrochemical industry through crude oil distillation at refineries. Plastics are incredibly different regarding compound creation, properties and potential applications, generally circulated in the general public and nature. The petroleum based plastics are irreplaceable to our day by day life (Alarfaj *et al.* 2015). Improper disposal of plastics has become threatened natural environment worldwide. Petrochemical plastics being xenobiotic or recalcitrant to microbial degradation pose a big threat to the ecosystem. Exorbitant sub-atomic size (50,000 to 1,000,000 Da.) is by all accounts for the most part answerable for the obstruction of these synthetic concoctions to biodegradation and their constancy in soil for quite a while. Approximately 140 million tons of plastic is consumed every year globally, which necessitate the processing of about 150 million tons of fossil fuel and directly causes massive environmental pollution. The waste generated by this pollution can take thousands of years to naturally deteriorate, if it degrades at all (Hempel *et al*. 2011).

Plastic is one of the major lethal poisons within recent memory. Being a non-biodegradable substance, composed of toxic chemicals, plastic pollutes earth, air and water. There is no way whatsoever you can safely dispose of plastic waste. Plastic makes genuine harm to environment both during its production and disposal (Shaaban *et al*. 2012). Tremendous measure of plastic is likewise disposed of into marine condition each year slaughtering many marine animals and affecting aquatic flora-fauna. Although plastics can be disposed of by incineration or recycling, but incineration is very difficult, dangerous and expensive, and recycling is a long process and not very efficient. If plastics were made biodegradable then these would no longer accumulate, and recycling and incineration troubles could be overcome (DeMarco 2005).

Plastics are minimal effort, solid and adaptable materials that can be considered valuable to the humanity in many ways as they improve life quality and economic activity. However, in the past decade plastics have been vilified by underlining their negative impact on the environment. The intrinsic qualities of durability and resistance to degradation, over the last two decades, have been increasingly regarded as a source of environmental and waste management problem mandating from plastic materials (Singh 2014). These days, the issue of plastic contamination (mainly plastics based on fossil fuels) is gaining much more attention by researchers as well as by common people as synthetic plastics are presenting a big threat to our environment. Considering the environmental and waste management problem, good alternative is the biopolymer (Mikkili *et al*. 2014). To overcome plastic related pollution problems attempts are being made to reduce plastic consumption and promote plastic recycling. The prominence of plastic pollution is correlated with plastics being inexpensive and durable, which lends to

high level of plastics used by humans. A large percentage of plastic produced each year is used to make single-use, disposable packaging items or products which will get permanently thrown out within one year. Often, consumers of the various types of plastics mainly use them for one purpose and then discard or recycle them. As per the United States Environmental Protection Agency, in 2011 plastics constituted over 12% of municipal solid waste (http://en.wikipedia.org/wiki/Plastic pollution). As plastics are produced from non-renewable resources such as petroleum and are not friendly with natural carbon cycle because of their non-degradable feature, so to overcome this problem, the production and applications of eco-friendly products such as bioplastics is mandatory.

Biopolymers could be a potential option in contrast to regular plastics. On the off chance that they are completely biodegradable their utilization permits the conservation of constrained assets, yet additionally suits the idea of sustainability (Jendrossek and Handrick 2002). The term “biopolymer” or “bioplastic” is not yet uniformly defined. Common definitions of the term “biopolymer” also include biodegradable plastics from fossil fuels and non-biodegradable plastics from renewable resources as shown in the Figure 1.1 (Pittmann 2015).

Biopolymers are made from renewable resources and biodegradable waste materials (e.g. waste water, sewage sludge, organic waste) and are fully biodegradable by naturally occurring microorganisms. This definition ensures that polymers from fossil resources and non-biodegradable polymers, which cause at least one of the mentioned problems, are excluded and that the term biopolymer is just used for polymers, which allow the preservation of limited resources and also suit the idea of sustainability. This type of biopolymers is shown in the upper right of the Figure1.1.

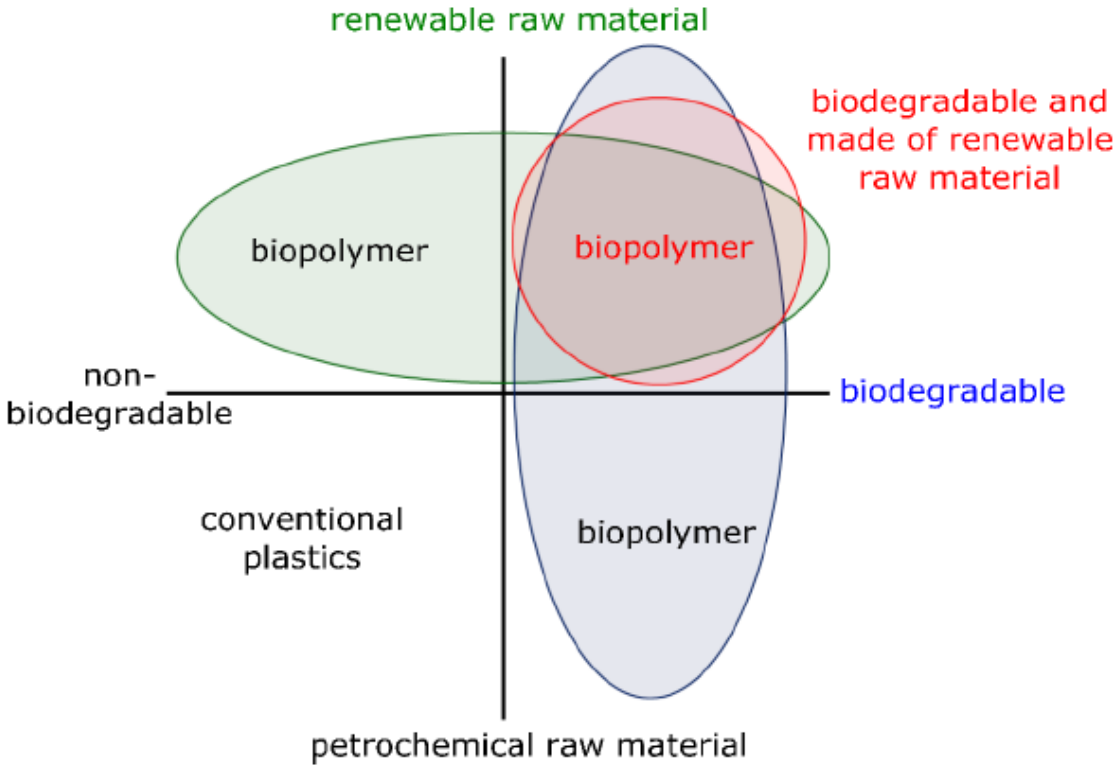


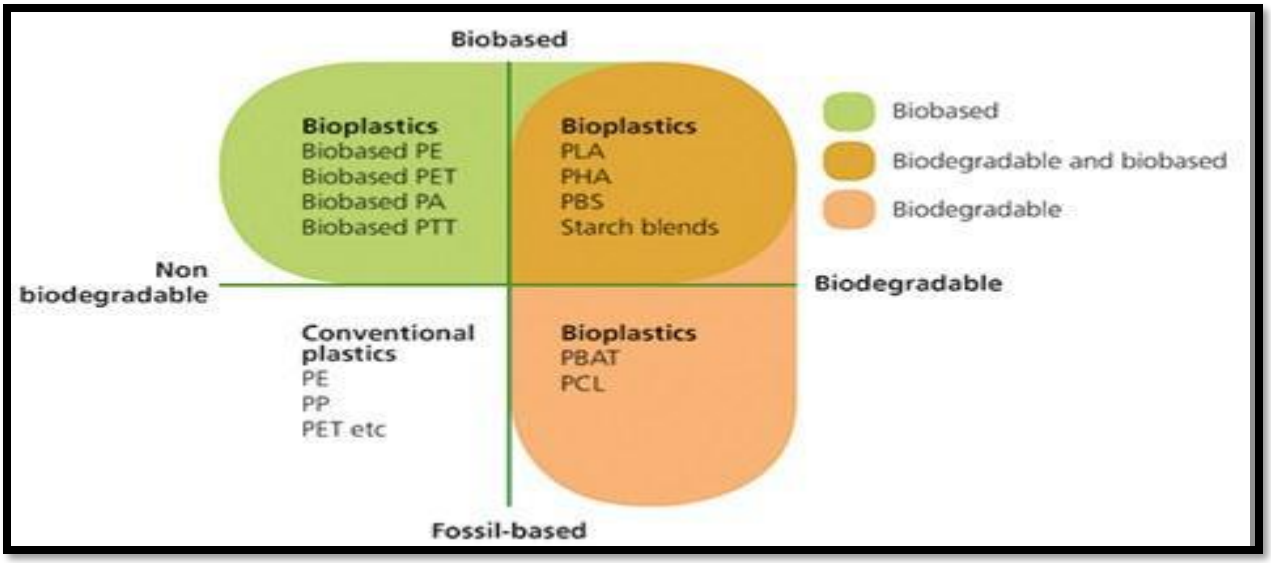
Fig. 1.1: Definition of biopolymers, including the stringent definition on the upper right.

Bioplastics have been researched for many years as a substitute for synthetic plastics. The manifestation of bio-feedstocks and bio-based commodity polymers production, in tandem with rising oil prices, growing consumer awareness and recuperating economics, has ushered a new and rousing era of bioplastic commercialization. Bioplastics are not just one solo substance, they comprise of a whole family of materials with differing properties and applications. The production of biodegradable polymers from renewable resources is the need of the modern day life, in the face of these ecological facts (Aarthi and Ramana 2011).

Bioplastic is a family of a new generation of biobased and biodegradable plastics with different applications (Gill 2014). It is eco-friendly alternative to plastics and biodegradable by the microbial enzymes into CO_2, methane, H_2O and biomass. Bioplastic production will prompt abatement utilization of fossil fuels and CO_2 emissions as well as reduce plastic waste generation (Azios 2007). The name biopolymer is presently used for polymers that are either synthesized by living organisms or formed from substrates obtained from living organisms. Bioplastics which also known as green plastics may be derived from renewable biomass sources, such as vegetable oil, corn starch, pea starch or microbiota and such bioplastics are supposed to be biodegradable. Examples of the first class of biopolymers are naturally occurring polymers such as cellulose, starch, and polyhydroxyalkanoates (PHAs).

Bioplastics are biopolymers with plastic characteristics. They act as imperative substitutes to petroleum-based plastics. Bioplastics/ biopolymers are derived from renewable biomass resources and these biopolymers can be fabricated in several different organisms e.g. plants and microbes. Bioplastics do not cause dangers since they are biocompatible to the hosts (e.g. polyhydroxyalkonates are synthesized in bacteria). Biopolymers synthesized in microbes are mostly lipid in nature and amassed in the form of mobile granules and help microbes to survive under stress conditions (Rasheed 2011).

Bioplastics are plastic materials which are either bio-based, biodegradable, or having both properties. In short, divergent to conventional fossil-based plastics, bioplastics are bio-based, biodegradable, or both (Fig.1.2).



*(Polypropylene (PP), polyethelyene (PE), polyethylene terepthalate (PET), polyamides (PA), polytrimethylene terephthalate (PTT), polylactic acid (PLA), polyhydroxyalkanoate (PHA), polybutylene succinate (PBS), polybutyrateadipateterephthalate (PBAT), polycaprolactone (PCL).

Fig. 1.2: Different types of bioplastics and their biodegradable behavior.

Biopolymers are increasing substantially more enthusiasm for industrial sectors around the world. The term biopolymers incorporate chemically unrelated products that are synthesized by microorganisms under different environmental conditions (Degeest *et al*. 2001). The principle advantage of this type of polymers is that since they are of biological origin, they degrade naturally and completely to CO_2 and water under natural environment by the enzymatic activities of microbes. Bioplastics which are lipid in nature are amassed as storage materials (in the form of mobile amorphous, liquid granules); allowing microbial survival under stress conditions (Sudesh *et al*. 2000).

Biodegradable polymers can be categorized according to source or method of preparation. On the basis of origin, biodegradable polymers are derived from renewable and petrochemical resources. Biodegradable plastics are normally high-priced than their petro-based synthetic competitors. However, systematic efforts been used to produce

these polymers having more competitive price. This would provide an opportunity to bio-based biodegradable polymers to be more competitive. Biodegradable polymers from renewable resources include: polylactide (PLA), polyhydroxyalkanoates (PHAs) such as poly-3-hydroxybutyrate (PHB), thermoplastic starch (TPS), cellulose, chitosan and proteins (Fig. 1.3). Biodegradable polymers from petroleum sources comprise: aliphatic polyesters and copolyesters (e.g. poly butylene succinate (PBS)); poly (butylene succinate/adipate) (PBSA), aromatic copolyesters (e.g. polybutylene adipate terephthalate (PBAT)), poly (ε-caprolactone) (PCL), poly (esteramide) (PEA) and polyvinyl alcohol (PVA).

There are three ways by which polymers are formed and/or recovered from renewable resources:

1. Polymers synthesized by nature and could be modified before their processing (e.g. starch, chitin, pectin, etc.);
2. Polymers that are created by chemical polymerization of monomers attained by fermentation of bio-based substrates (e.g. polylactic acid derived from starch).
3. Microorganisms synthesized polymers are stored within the cells in response to certain nutrient conditions (polyhydroxyalkanoates).

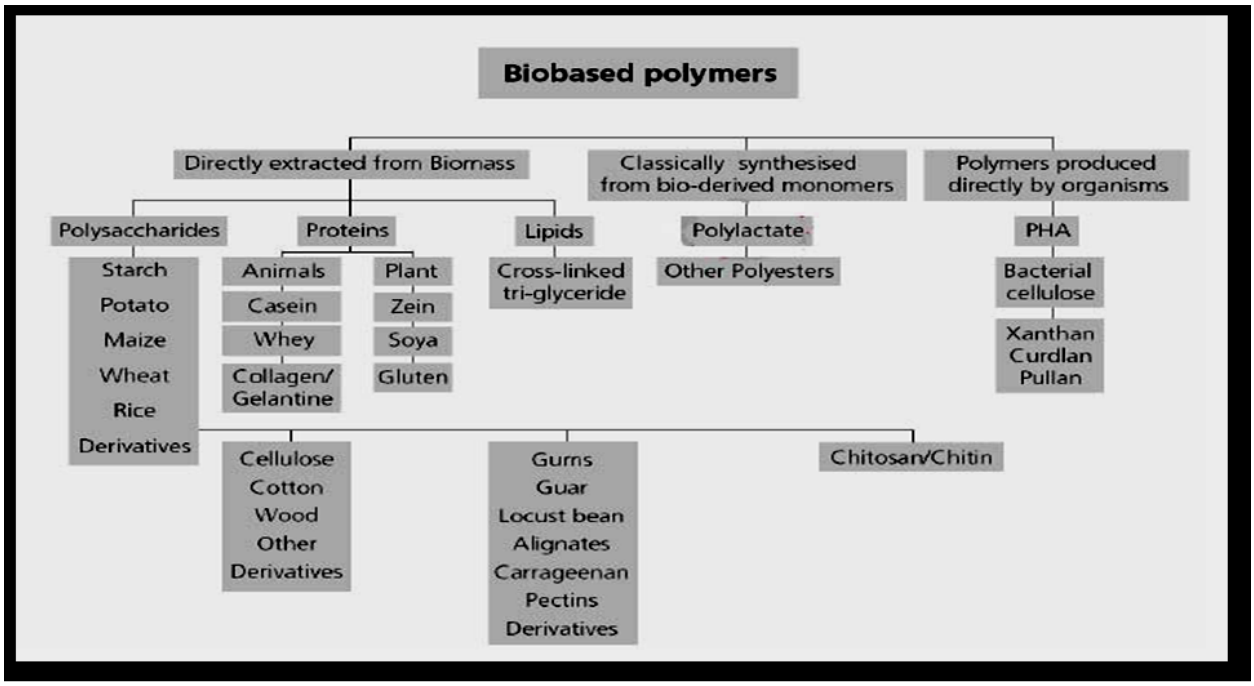


Fig. 1.3: Different categories of bio-based polymers.

Polyhydroxyalkanoates: The natural biopolymers

Among the entire bio-based and bio-degradable polymer, polyhydroxyalkanoates (PHAs) are well-known as they are produced from microorganisms. PHAs are the common name of a wide variety of β-hydroxyalkanoic acid polymers. PHAs are members of polyester of hydroxyalkanoates. These are bio-based and biodegradable without waste and also recycled to CO_2 and water. PHAs are aggregated in intracellular granules as carbon or reducing power storage material in microbial cells. PHAs are synthesized and stored by a wide assortment of Gram positive and Gram negative bacteria (Luengo *et al.* 2003). These are produced when carbon source is in plentiful but other nutrients such as nitrogen, phosphorus, sulfur etc. are constrained. They serve as energy storage molecules to be utilized when common energy sources are not available. They are thermoplastic polymers and totally biodegradable (Bhuwal *et al.* 2013).

All bacteria capable of PHAs synthesis and can accumulate during the stationary phase of growth and these PHAs granules facilitate cell survival during stressful conditions. PHAs production is usually a two stage process. In the first stage, initial balanced growth phase, high protein biomass is produced. In the second stage, nutrient limiting phase, the number of cells remains constant but cell size increases because of PHAs accumulation (Yuksekdag *et al*. 2004). The number and size of granules, the monomer composition, macro molecular structure and physio-chemical properties vary, depending upon the organism. Among the variety of biodegradable plastics a family of more than 40 PHAs and their co-polymeric derivatives has emerged as very attractive materials due to their complete biodegradability. Much effort has been spent in optimizing the poly-β-hydroxybutyrate (PHB) production using pure substrates and pure cultures. The expense of this PHB is still around multiple times higher than that of conventional plastics (Wang and Lee 1997).

PHAs have increased significant consideration due to their structural diversity and close analogy to plastics. Other types of bioplastics are formed directly or indirectly use food resources for their production but PHAs are produced legitimately by microorganisms, preferably bacteria under stringent conditions. PHAs in bacteria had been known since 1926, when Maurice Lemoigne observed that *Bacillus megaterium* produces an intracellular polymer of hydroxybutyrate monomers, later called polyhydroxybutyrate. This initial perception got intensified during 1970's when the world was facing acute petroleum crisis, and a scientific movement aimed at discovery of alternative sources of fossil fuel reserves was undertaken (Prieto 2007).

PHAs are polyesters of hydroxyalkanoic acids with the general structure shown in Fig. 1.4. PHAs are linear polyesters of β-hydroxyalkanoic acid monomers with a chiral β carbon making the polymer optically active and all monomer units occur in the R (-) configuration. The polyester is formed when carboxylic group of one monomer forms an ester bond with the hydroxyl group of an adjacent monomer, catalyzed by the PHA synthase enzyme whose stereo specificity renders chirality and the polymer can have a molecular weight in the range of 2 x 10^5 to 3 x 10^6 Da. In PHAs structure, R can be hydrogen or hydrocarbon chains up to around C13 in length, and x can range from 1 to 3 or more. Varying x and R provides a broad range of physical and mechanical properties, such as hydrophobicity, glass transition temperature (Tg), melting point (Tm)

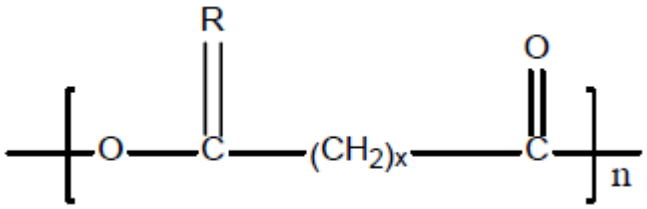


Fig. 1.4: General structure of PHAs, R= H or alkyl groups. (C_{1-13}, x = 1-4 and n = 100-30,000)

and level of crystallinity which can range from around 70% to very low, giving tremendous stiffness or elasticity as required (Zhang 2010). PHAs are the only water proof thermoplastic materials available that are fully biodegraded both in aerobic and anaerobic environments. PHAs are polyesters formed by condensation of carboxylic acids with hydroxyl alcohol.

Types of PHAs and their physical properties

PHAs are classified into two distinct groups based on the number of carbon atoms in the monomers. This includes short chain length (scl) polymers consisting of 3–5 carbon atoms containing monomers, synthesized by numerous bacteria, including *Cuprivadus necator* and *Alcaligenes latus*. The other group is the medium chain length (mcl) polymers, consisting of 6–14 carbon atom containing monomers, also synthesized by bacteria including *Pseudomonas putida and Pseudomonas mendocina.* Furthermore, copolymers of PHAs are found among the scl-PHAs, e.g. poly (3-hydroxybutyrate-co-3-hydroxyvalerate), P(3HB-co-3HV) and mcl-PHAs, e.g. poly (3-hydroxyhexanoateco-3-hydroxyoctanoate), P(3HHx-co-3HO). A comparison of physical properties of homopolymer and heteropolymer PHB and a common petroleum polymer can be seen in Table 1.1 (Akaraonye *et al.* 2010).

Table 1.1: Physical properties of PHAs and polypropylene.

Properties	Poly(3)HB	Poly(3HB-3HV)	Polypropylene
Melting temperature (°C)	180	145	176
Glass transition temperature (°C)	4	-1	-10
Tensile strength (Mpa)	40	20	34.5
Elongation at break (%)	5	50	400
Young's modulus (Gpa)	3.5	1.2	1.7

Scl-PHAs such as P(3HB) and P(3HB-co-3HV) are crystalline polymers which are quite brittle and stiff, with high melting points and low glass transition temperatures. However, P(4HB), another scl-PHA, is a strong, pliable thermoplastic polyester with a relatively simple structure, despite its biosynthetic route (Martin *et al.* 2007). Some scl-PHAs have

higher tensile strength than polypropylene and polystyrene. Mcl-PHAs on the other hand are thermoplastic elastomers with low crystallinity and tensile strength but high elongation to break. They have lower melting points and glass transition temperatures when compared with scl-PHAs and polypropylene, as shown in Table 1.2 (Zinn and Hany 2005). PHAs have attracted commercial interest as plastic materials because of their remarkable similarities in physical properties with synthetic polymers such as polypropylene. The major advantage of PHAs is that both the physical properties and the rate of degradation of PHAs can be altered by changing the bacterial source of the polymer and the corresponding fermentation conditions used. Special growth conditions

Table 1.2: Physical properties of scl-PHAs and mcl-PHAs with polypropylene.

Properties	scl-PHAs	mcl-PHAs	Polypropylene
Crystallinity	40-80	20-40	70
Melting temperature (°C)	53-80	30-80	176
Density (g cm^{-3})	1.25	1.05	0.91
Tensile strength (Mpa)	43-04	20	34
Glass transition temperature (°C)	-148-4	-40-150	-10
Extension to break (%)	6-1000	300-450	400
UV light resistant	Good	Good	Poor
Solvent resistant	Poor	Poor	Good

of microorganisms allow inclusion of additional chiral monomers and functional groups to suit particular applications. PHAs are optically active biological polyesters which are insoluble in water and exhibit a high degree of polymerization that ranges from 10^5 to 10^7. The mechanical properties and biocompatibility of PHAs can be further improved by blending with other polymers, modifying the

surface or combining PHAs with other inorganic materials, thus making them useful for a wider range of applications (Kang *et al*. 2001 and Scandola *et al*. 1997).

Applications of PHAs

PHAs can be are position for some regular petrochemical products in applications including molded goods, paper coatings, performance additives, foils, films and diaphragms (Lauzier *et al.* 1993 and Chen and Wu 2005). PHAs can likewise be utilized for manufacturing disposable everyday articles such as shampoo bottles and cosmetic materials (Hocking and Marchessault 1994). PHAs can be used as hot-melt adhesives and non-woven fabrics. PHAs can also be used as sources for the synthesis of enantiomerically pure chemicals such as hydroxyalkanoic acids and as raw materials for the production of latex paints (Steinbuchel 2001). PHAs have promising uses as a new source for small molecules that have potential applications as biodegradable solvents and as carriers for long term slow release of herbicides and insecticides (Lee 1996 and Galego *et al.* 2000). There are further applications of PHAs within the medical and pharmaceutical industries, primarily due to their biodegradability and biocompatibility. PHAs are also used in the production of repair patches, ligament and tendon grafts, bone marrow scaffolds, bone graft substitutes and bone dowels. PHAs can be used in the fabrication of three-dimensional, porous, biodegradable heart valve scaffolds (Martin and Williams 2003 and Sodian *et al.* 2000). Currently, EMPA is assessing the use of PHAs as a biopolymer for fiber production. Such fabric made from these fibers could be used in medical applications such as wound dressing. P(3HB) is also being considered for cartilage tissue engineering as scaffolds for the development of a three-dimensional articular cartilage tissue for implantation. Currently, the biomedical field is facing

challenges in the need for tissue adhesives, tissue sealants and soft tissue fillers as a replacement for collagen and tissue adhesion preventives for the treatment of severe burns. Hence, PHAs and modified PHAs are being considered as solutions for the ever increasing biomedical challenges. To this effect, PHA composites containing collagen sponges and gels, polyglycolide (PGA), hyaluronic acid matrices, polylactide (PLA), poly(lactideco-glycolide) (PLGA), poly(L-lactide-co-caprolactone) (L-PLCL), and poly(glycolide-co-trimethylene carbonate) (PGTMC) are currently being employed in regenerative medicine (Freed *et al.* 1998). Some of the major areas of application of PHAs in medicine are listed in Table 1.3 (Zinn *et al.* 2001).

Table 1.3: Potential applications of PHA in medicine.

Type of application	Products
Wound management	Sutures, skin substitutes, nerve cuffs, surgical meshes, staples, swabs etc.
Vascular system	Heart valves, cardiovascular fabrics, pericardial patches, vascular grafts etc.
Orthopaedy	Cartilage tissue engineering, spinal cages, bone graft substitutes, internal fixation devices (e.g. screws).
Drug delivery	Micro and nanospheres for anticancer therapy
Urology	Urological stents
Dental	Barrier material for guided tissue regeneration in periodontitis
Computer assisted tomography and ultrasound imaging	Contrast agents

Prologue of Polyhydroxybutyrate (PHB)

Of the big family of PHAs, a homopolymer of poly-3-hydroxybutyrate (PHB) is the most wide spread in nature and the best characterized. PHB represents one of the most studied PHAs, and could be one of the strong contenders for bioplastic production. A French microbiologist Maurice Lemoigne discovered the first polyester, P(3HB), in the form of granules within the cells of Gram positive bacterium *Bacillus megaterium*in 1926. They are linear polyesters composed of 3HB repeating units and possess the general formula (Fig. 1.5). PHB being the reserved food polymer produced during time of starvation is metabolized to provide carbon and energy when external carbon source is exhausted (Lopez *et al*. 2012).

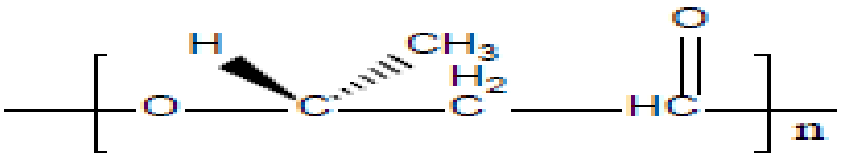


Fig. 1.5: Chemical structure of polyhydroxybutyrate.

PHBs are non-toxic, biocompatible, biodegradable, and recyclable thermoplastics and hence, can potentially replace synthetic plastics. However, the structure, biosynthetic pathways and applications of many bioplastics have now been established. PHB made much interest in industry and research, due to its biocompatible, biodegradable, thermoplastic and piezoelectric properties. Except for PHB which is a homopolymer, most PHA contains two or more different monomers and is referred to as heteropolymers. Oxidation of PHB to carbon dioxide and water yields a lot of vitality. For these reasons, PHB is a perfect carbon and energy reserve for bacteria (Verlinden *et al*.

2007). The material properties and features of poly(3-hydroxybutyrate) are summarized in Table 1.4.

Table 1.4: Properties and features of poly (3-hydroxybutyrate).

1.	Water-insoluble and relatively resistant to hydrolytic degradation.
2.	Good oxygen permeability and ultra-violet resistance.
3.	Poor resistance to acids and bases.
4.	Soluble in chloroform and other chlorinated hydrocarbons.
5.	Biocompatible, hence is suitable for medical applications.
6.	Melting point 175°C and glass transition temperature 2°C.
7.	Highly crystalline.
8.	Tensile strength 40 Mpa, close to that of polypropylene.
9.	Sinks in water.
10.	Nontoxic to mammalian cells (biocompatible to various cell lines including osteoblasts, epithelial, cells and chondrocytes.
11.	Strong and malleable thermoplastic (ability to be moulded, made into films and fibres).
12.	Biodegradable (degradable into CO_2 and H_2O, aerobic, CH_4 and H_2O, anaerobic).
13.	Can be produced from naturally sustainable and bio-renewable agricultural feed stock.
14.	Enantiomeric and optically active.
15.	High degree of polymerization.
16.	Possesses piezoelectric properties useful in osteoinduction.

Application spectrum of PHB

Bacteria derived PHBs can be used for various applications including agricultural, medical and industrial applications. Two primary properties of PHB like biodegradability and biocompatibility makes it a suitable alternative biopolymer in many applications. It provides a broad series of potential end-use applications. Former applications of PHA

were mostly in packaging but now its importance in medical industry has become noteworthy. In medical and pharmacology fields; the perspective area of PHB application is development of implanted medical devices for dental, craniomaxillofacial, orthopaedic, hernioplastic and skin surgery. A number of potential medical devices with PHB like biocompatible surgical sutures, biodegradable screws and plates for cartilage and bone fixation, surgical meshes with PHB coating for hernioplastic surgery wound coverings (Shivakumar 2012) had been developed. PHB have been established for numerous medical applications. Biodegradability without toxicity and thermo-process ability make PHBs as attractive biomaterials for applications in both conventional medical devices and tissue engineering (Jain *et al*. 2010). The slow evolution of bioplastics development in the medical field can be attributed to several unique challenges in developing clinical materials compared to developing traditional polymers. The biocompatibility and biodegradability character plays major role in its application in medical industry. PHA is also useful source for stereo regular compounds that can serve as chiral precursors for the chemical synthesis of optically active substances, particularly in the synthesis of certain drugs or insect pheromones (Zinn *et al.* 2001). Bioplastics had increased a great deal of ubiquity in clinical field because of its exceptional merits such as a drug carrier and scaffold material for tissue engineering, due to their biodegradability and biocompatibility with mammalian tissues; one of the most important applications of PHB is that they can be successfully implanted in the body without producing an immune response, which is the cause of 'rejection' of most foreign materials by the body and it is slowly degraded to 3-hydroxybutyric acid, which is a normal constituent of the bloodstream (Colin and Kristiansen 2004).

In addition to its application in medical field, PHB can be used to produce systems for sustained enzyme activators or inhibitors liberated for advance physiological models. Later on, pens, cups, and packaging elements made with PHAs also came in the market. PHAs are biocompatible and thus they have additionally stood out as crude material to be utilized in medical devices (Wu *et al*. 2009). In any case, the assembling of PHAs is done at little offices and, as a result; it does not have the beneficial preferred position of a large scale production (Chanprateep 2010).

PHAs can be used to produce dairy cream substitutes or flavor delivery agents in foods. PHAs have also been processed into fibers which then were used to construct materials such as nonwoven fabrics. Combinations of bioplastics are also used in electronic products, like mobile phones (NEC Corporation and UNITIKA Ltd. 2006). Most of the above mentioned products are made from p(3HB) and p(3HB-3HV) polymers. In addition to its range of material goods and resulting applications, PHAs promise to be a new source of small molecules. They can be hydrolyzed chemically, and the monomers can be converted to commercially attractive molecules such as β-hydroxyacids, 2-alkenoic acids, β-hydroxyalkanols, β-acyllactones, β-amino acids, and β-hydroxyacid esters (Williams and Peoples 1997). PHB have also conceivable agricultural applications including encapsulation of seeds, fertilizers encapsulation for slow release, biodegradable plastic films for crop safeguard and biodegradable containers for hot house facilities and as biodegradable carriers for long haul dose of bug sprays and herbicides (Philip *et al*. 2007).

PHB has more advantages because it is far less permeable than PE and PP, this known as a better material for food packaging needless to use antioxidant. However, industrial application of PHB has been hampered owing to its low thermal stability and excessive brittleness upon storage (Matsusaki *et al*. 2000). Due to the poor physical properties of PHB, the incorporation of a second monomer unit into PHB can significantly enhance its properties. This has led to an increased interest to produce hetero-polymers with improved qualities. The incorporation of 3-hydroxyvalerate (3HV) into the PHB has results in a poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] which is more flexible and tougher than PHB, and more easier to degrade when discarded into the natural environment.

Biosynthesis of PHB

PHB are produced by many bacteria as inclusion bodies to serve as carbon source and electron sink. PHB are synthesized from acetyl-CoA produced by the bacteria in sequential action of three enzymes. 3-ketothiolase (phbA gene) catalyzes the formation of a carbon-carbon bond by condensation of two acetyl-CoANADPH dependent acetoacetyl-CoA reductase (phbB gene) catalyzes the stereo selective reduction of acetoacetyl-CoA formed in the first reaction to R-3- hydroxybutyrylCoA. The third reaction of this pathway is catalyzed by the enzyme PHB synthase (phbC gene) that catalyzes the polymerization of R-3- hydroxybutyryl-CoA to form PHB. The EC number is yet to be assigned to PHA synthase (Galehdari 2009). Figure 1.6 (Wang *et al.* 2009) showed the biosynthetic pathway of PHB from acetyl-CoA. PHB is a partially crystalline polymer which has material properties similar to polypropylene (PP) and polyethylene (PE) (Holmes 1988 and Lee1996). Therefore, PHB has been considered as one of the

most promising biodegradable plastics and as an alternative to petrochemical plastics. This is due to their biocompatibility, biodegradability and versatile properties make it an eco-friendly substitute for synthetic polymers (Brandl *et al*. 1988).

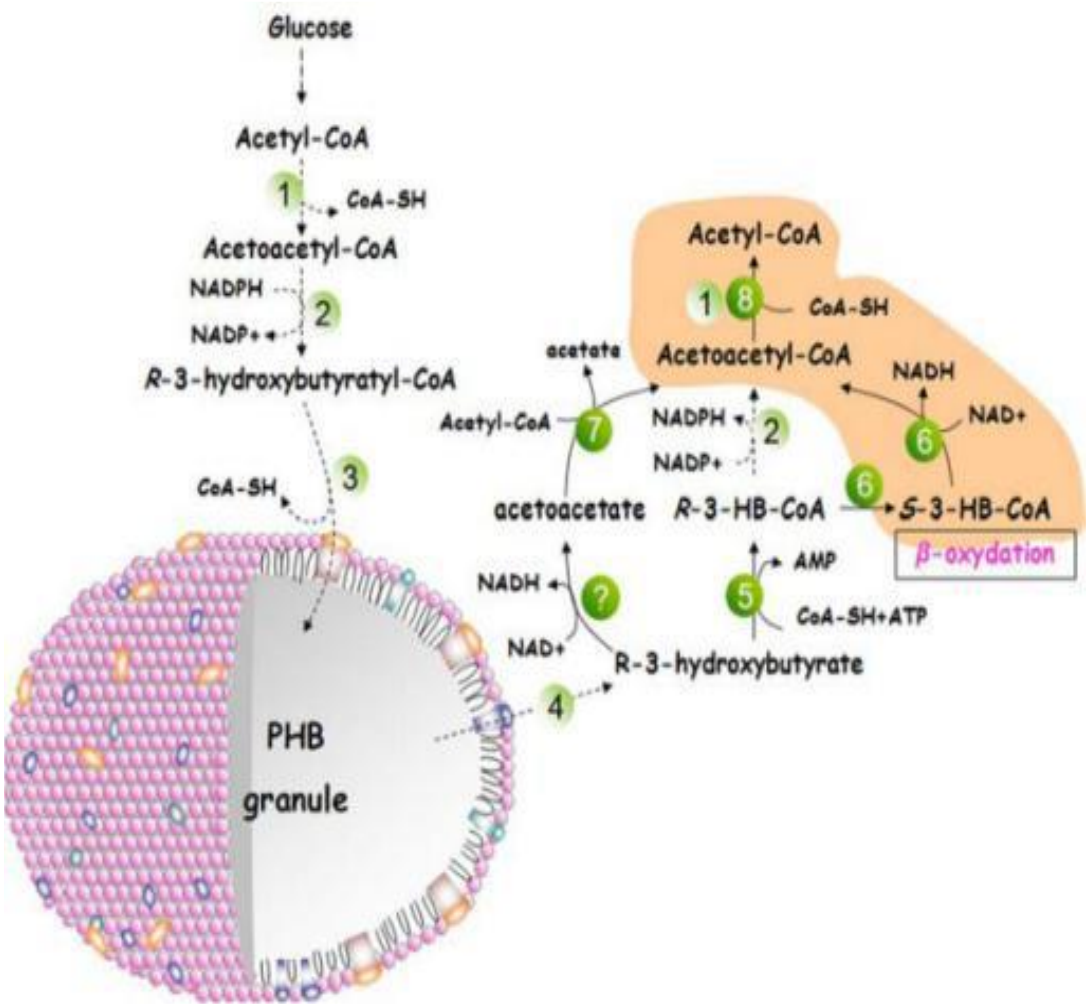


Fig. 1.6: Biosynthetic pathway of PHB.

PHB is accumulated or synthesized by microorganisms through different biosynthetic pathways. Depending on the organism and the kind (sugars, fatty acids etc.) of carbon source available, the quality and characteristics of the final polymer is determined (Divyashree *et al*. 2009). When glucose, a common simple sugar is the carbon source

available during PHB accumulating condition (un-balanced growth), it is catabolized through the Entner-Doudoroff pathway (Hong *et al*. 2003).

Bacterial PHB

Various bacterial strains among archaebacteria (Doi 1990), Gram positive and Gram negative bacteria and photosynthetic bacteria (Hassan *et al.* 1998) including cyanobacteria (Jau *et al.* 2005) have been identified to accumulate PHB both aerobically and anaerobically. Although more than 300 different bacterial species are known to synthesize PHBs, only a few bacteria have been employed for the production of PHBs viz. *Alcaligenes latus, Ralstonia eutropha, Azotobacter beijerinckii, Bacillus megaterium, Pseudomonas oleovorans* etc. (Bhuwal *et al.* 2013), *Halomonas campisalis* (Kulkarni *et al.* 2011), *Haloferax mediterranei* (Bhattacharyya *et al.* 2012), *Halomonas hydrothermalis* MTCC 5445, a halophilic bacterium (Bharadwaj *et al.* 2015). The β-proteobacterium *Ralstonia eutropha* is known as the model organism for PHB production, mainly because it can store high amounts of PHB i.e. up to 90% of its cell dry weight under nutrient limitation in the presence of sufficient carbon source (Riedel *et al.* 2012).

Gram-positive bacteria may be preferred when the intended application of PHB is in biomedicine. PHB production from Gram-negative bacteria may necessitate extra purification steps for eliminating potential contamination by endotoxins, while no such purification required when producing PHB from Gram-positive bacteria as these are devoid of outer membrane of lipopolysaccharide (LPS) (Lopez *et al.* 2012). Among several Gram-positive bacterial spp. viz. *Clostridium, Corynebacterium, Nocardia,*

Bacillus, *Rhodococcus*, *Streptomyces* and *Staphylococcus* have been reported for PHB production (Singh *et al.* 2009). *Bacillus* spp. offer several advantages and have been explored for wide range of industrial products including PHB (Narayan and Ramana 2012 and Singh *et al.* 2013). *Bacillus* spp. have relatively fast growth, and capability to utilize wide range of agro-industrial wastes as substrates (Masood *et al.* 2012) due to their ability to secrete variety of extracellular enzymes such as cellulases, xylanases, amylases and proteases (Bajaj and Singh 2010). Furthermore, *Bacillus* spp. represents a model system for the heterologous expression of foreign genes associated with PHA production and several other chemicals (Law *et al.* 2003 and Schallmey *et al.* 2004). Microalgae can also produce PHB under favorable conditions and have all the advantages of photosynthetically determined eukaryotic systems but lack many of the mentioned disadvantages i.e. they possess high growth rates, are easy to handle and do not need much more than light and water for cultivation (Prieto *et al*. 2014 and Rahman *et al.* 2014).

Pollution and bacterial PHB

The industrial and domestic waste contains various organic compounds such as protein, carbohydrate, fat, nucleic acid etc. Inexpensive substrate, renewable substrates and waste materials are used as nutrient sources for microorganisms for PHA production. Various types of waste products have been used for PHB production because it provides dual benefits of utilizing the waste and cost-effective production of biodegradable microbial bioplastic. Waste materials are also been considered as potential carbon sources for PHB production (Tripathi *et al.* 2013). There are a number of complex waste streams that can potentially act as carbon substrates for microbial PHA manufacture, such as waste streams from biodiesel

production (Kenny *et al.* 2012 and Escapa *et al.* 2013), municipal wastewater (Rahman *et al.* 2015), agricultural waste (Linton *et al.* 2012), syngas production (Drzyzga *et al.* 2015), traditional plastic waste (Wierckx *et al.* 2015) and others (Gomez *et al.* 2012). Food waste is a prime candidate for an inexpensive carbon source due to its wide spread availability and the potential to solve significant waste problems when used to produce PHAs.

Low cost agro-industrial residues like molasses, wheat bran, cassava powder, jackfruit seed powder, corn flour (Ramadas *et al.* 2009), rice bran (Shivakumar 2012) have been investigated for PHB production by various researchers. Bhattacharyya *et al.* (2012) used vinasse, a by-product from ethanol industry for production of PHB from *Haloferax mediterranei*.

Utilization of low-cost agro industrial residues like bakery waste hydrolysate (Pleissner *et al.* 2014), molasses (Tripathi *et al.* 2013), sugar industry waste water (Singh *et al.* 2013), soya flour, carboxymethyl cellulose (CMC), bagasse, wheat bran, wheat germ, rice bran, ragi bran (Shivakumar 2012), glycerol (Sindhu *et al.* 2011) etc. as carbon sources can reduce the production cost of PHB. Ramadas *et al.* (2009) used wheat bran, potato starch, sesame oil cake, groundnut oil cake, cassava powder, jackfruit seed powder to economize the production process however, corn flour starch (Halami 2008), waste activated sludge (Khardenavis *et al.* 2007), soy bean oil (Kahar *et al.* 2004) etc. are also good alternatives for the production of PHB. *Comamonas testosteroni* have ability to synthesize medium-chain length PHA (MCL-PHA) from vegetable oils such as castor seed oil, coconut oil, mustard oil, cotton seed oil, groundnut oil, olive oil and sesame oil (Thakor *et al.* 2005). PHB

synthesis and utilization are closely connected with the energy requirements of the cell including the nature of carbon utilized. It is desirable that PHB production be attempted by using inexpensive agricultural or other residues as carbon source to substantially reduce the cost of PHB production considering carbon source as the major cost determining factor for PHB production (Valappil *et al.* 2007).

Due to similarity in physical property with synthetic polymer, it is possible to use PHB in high scale. But the high production cost of PHB, restrict its wider application. Therefore optimization of temperature, time, pH, carbon and nitrogen sources and other growth conditions and easier downstream processing methods are required for reducing the cost. To make the PHB production financially more appealing, the use of proper fermentation process has been the subject of many investigations (Verlinden *et al*. 2011). Optimization has been vastly used for process improvement. Process optimization being an essential step in bioprocesses had been extensively used for various products commercial manufacturing. Several process parameters for PHB production were optimized viz., medium composition, carbon and nitrogen sources (Elsayed *et al.* 2013), pH (Shaaban *et al*. 2012), minerals, trace elements, temperature, aeration, inoculum age etc. (Singh *et al.* 2013). The method of 'one variable at a time' (OVAT) approach permits determining the specific requirements for growth and product formation by systematically adding or deleting components from the medium, with minimal complex medium interaction. The performance of multiple experiments by analyzing traditional OVAT approach is unpredictable, time consuming and lengthy process, and also interactions among the factors are ignored (Sathiyanarayanana *et al*. 2013). The use of organized statistical approach is more reliable than unplanned experiments.

The choice of operation strategy for production of bacterial PHAs depends on various factors including carbon source, culture type, mode of fermentation and bioreactor type. Batch fermentation for PHB production is a popular process due to its flexibility and low operation costs. However, it is associated with low PHB productivity since after utilization of the carbon source; bacterial cells degrade the accumulated PHB resulting in reduced PHB content. Kulpreecha *et al.* (2009) reported a higher PHB productivity under fed-batch mode compared to batch mode of fermentation. However, even though fed-batch fermentation, on its own, yields higher PHB productivity, the overall PHB production is still considered low in cases where nitrogen is the limited nutrient. Batch and fed-batch processes are thus combined as a result of low PHB content obtained by each process individually. The combined process is the most common fermentation strategy used for PHB production. Under this strategy, the process is divided into two stages: in the first stage the microorganism is grown under batch mode until the desired biomass is achieved and PHB accumulation has started. In the second stage the fermentation is shifted to fed-batch, where usually one or more essential nutrients are maintained in limited concentration and carbon source is continuously fed into the reactor to further produce and accumulate PHB in the cells.

Continuous culture, chemostat, is another option adopted as the third operation strategy for PHB production. In this method the culture broth is continuously replaced by sterile medium. In chemostat culture, the carbon source is continuously fed in excess, keeping one or more nutrients (e.g. phosphorous or nitrogen) in limitation. Chemostat is highly controllable as the specific growth-rate can be maintained by adjusting the dilution-rate. Therefore under appropriate growth conditions, continuous fermentation might have the

potential to give the highest PHB productivity levels. Nonetheless chemostat culture exhibits a higher risk of contamination (Zinn *et al.* 2001).

Biochemical characterization of PHB is a very crucial parameter not only to study the structure of PHB but also from application viewpoint. Fourier transform infra-red spectroscopy (FTIR) had been used for functional groups identification (Shah 2014). X-ray diffraction (XRD) analysis is a useful tool for determining the structure and crystallization of polymer matrices and to find that the polymer exists in crystalline or amorphous state (Khan *et al*. 2009). Nuclear magnetic resonance (NMR) had been used for structural elucidation of PHB extracted from bacterial isolate (Bhattacharyya *et al.* 2012).

Blending polymers is an economic process to develop new polymeric materials. PHB has several inherent deficiencies for use as a practical polymer material, such as its brittleness due to its high crystallinity. In the chase for new polymeric materials, either new monomers are polymerized or co-polymerization techniques are used to tailor made a new product (Khan *et al*. 2009). To overcome the drawbacks of PHB and to obtain some useful new material properties, physical blending and chemical modification have been adopted (Burlein and Rocha 2014). This polymer has poor mechanical properties. There are many attempts to blend PHB with other flexible polymers or low molecular weight plasticizers to transform PHB into materials with improved properties for impact strength, film formation, processing, mechanical strength, amphiphilicity, biodegradability, and biocompatibility (Singh *et al*. 2013). Biosynthesis of homopolymers, random copolymers and block copolymers could be achieved by changing bacterial synthesis pathways and mode of co-feeding of substrates, during the bacterial cultivation process

(Sindhu *et al.* 2011). At present, PHB and polyhydroxybutyrate-*co*-valerate P (3HB-*co*-3HV) are the only members of PHAs that are produced on a commercial spectrum. The first commercial plant for PHBV was built in the USA in a joint venture between Metabolix and Archer Daniels Midland. Currently, Tianan Biologic Material Co. in China is the largest producer of PHB and PHB copolymers (Ravenstijn 2010).

One of the main reasons for the sustainable research on PHB as eco-friendly plastic is because they are fully biodegradable in various environments such as compost, landfill and aquatic systems. Water content and temperature have been found to be important along with the microbial activity in a given environment influencing biodegradation, the enzymology of the process, and the importance of the polymer composition (Jain *et al*. 2010). Polymers in natural environment are degraded through hydrolysis, mechanical, thermal oxidative, and photochemical destruction, and biodegradation. One of the valuable properties of PHB is its biodegradability, and it was studied in simulated natural environment (in soil, compost, and industrial sludge) in laboratory (Saharan *et al.* 2014). PHB degrading enzymes are excreted by various bacteria and fungi in the environment. The degradation of biodegradable plastics is due to cell-mediated phenomena (microorganisms like fungi or bacteria; enzymes). When the degradation is the result of the action of microorganisms in a material and the material is eventually converted to water, carbon dioxide, methane and biomass, the material is considered biodegradable. The use of UV radiations has been found to accelerate the degradation process of PHB. The photo-degradability and biodegradability are two separate classes of degradation. Photodegradable plastics will breakdown into small-sized fragments when exposed to UV radiations.

The UV radiation activate such bonds to form free radicals, which then react further with oxygen in the environment, producing carbonyl groups in the main chain, although these fragments are unable to degrade any further. Biodegradable plastics (BDP) are capable of being fully degraded within the surroundings and have no eco-toxicological impact when degraded (Bagheriasl 2012).

Aims and objectives of the study

Bioplastics are industrially important biopolymers and are considered good substitutes for petroleum derived synthetic plastics because of their similar material properties to synthetic polymers and complete biodegradability after disposal. The main advantage of this type of polymers is that since they are of biological origin, they degrade naturally and completely to carbon dioxide and water under natural environment by the enzymatic activities of microbes. PHB is gaining increased attention due to its biodegradability, biocompatibility and negligible cytotoxicity to the cells.

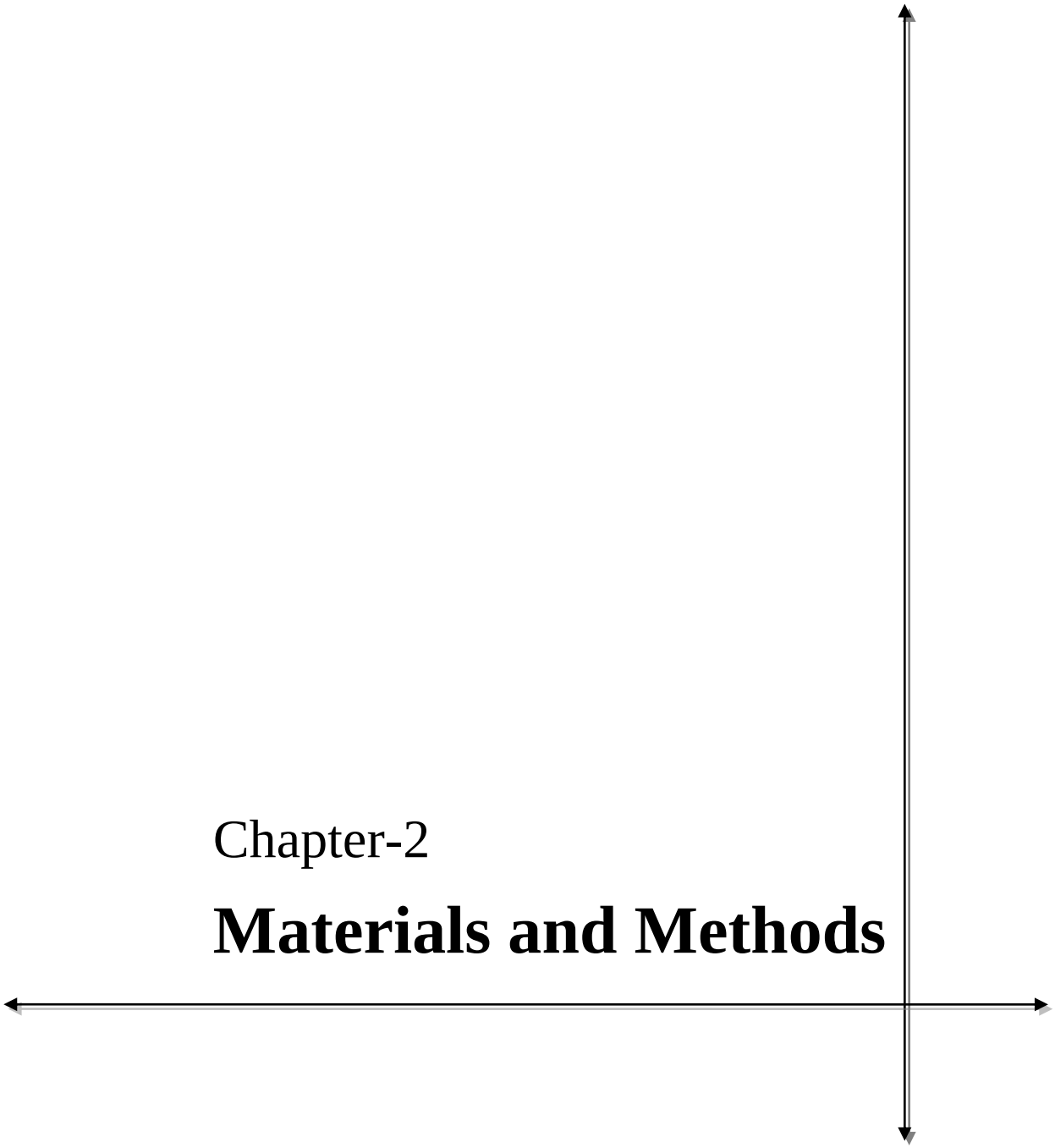
Considering numerous demerits of synthetic plastics i.e. non-degradability, utilization of fossil fuel for its production, and associated grave environmental concerns, there is need to develop fully biodegradable bioplastics. Furthermore, there is societal demand for development and usage of safer and eco-friendly products. PHB is the most proficient bioplastic with full-biodegradation property. PHB production from bacteria could be a suitable option as bacteria can be easily cultivated using different waste materials and this approach does not compete with the food resources. In addition, most of the studies on PHB production have been accomplished using Gram-negative bacteria. The use of Gram-positive bacteria could be a better alternative as they are completely free of

lipopolysaccharide (endotoxins). Conceding facts, importance and applications of biotechnologically and industrially valuable biopolymer the present study was undertaken with the following objectives:

1. Enumeration and isolation of indigenous PHB producing bacteria from different waste materials in and around Dhaka Metropolitan City.
2. Screening and characterization of local PHB positive bacterial isolates.
3. Identification of selected PHB positive bacterial isolates.
4. Optimization of PHB production of the characterized and identified bacterial isolates.
5. Lab scale PHB production from the selected potential bacterial isolates.

Chapter-2

Materials and Methods



Materials and Methods

2.1 Location of sampling

Soil and water are the good sources of potential indigenous bacteria. Therefore, soil and water were considered for the isolation of polyhydroxybutyrate (PHB) producing bacteria. Conceiving this view PHB producing bacteria enumerated and isolated from the soil and water of domestic and industrial waste in and around Dhaka Metropolitan City. Samples were collected from different habitats *viz*. Dairy Farm, The Bangshi River, The Turag River, The Buriganga River, Dumping waste, Navy Dockyard, Kitchen Waste and Garments waste etc. The map of the Dhaka city and the sampling sites are shown in Fig. 2.1, Fig. 2.2 and Fig. 2.3.

Fig. 2.1: Map of Dhaka city.

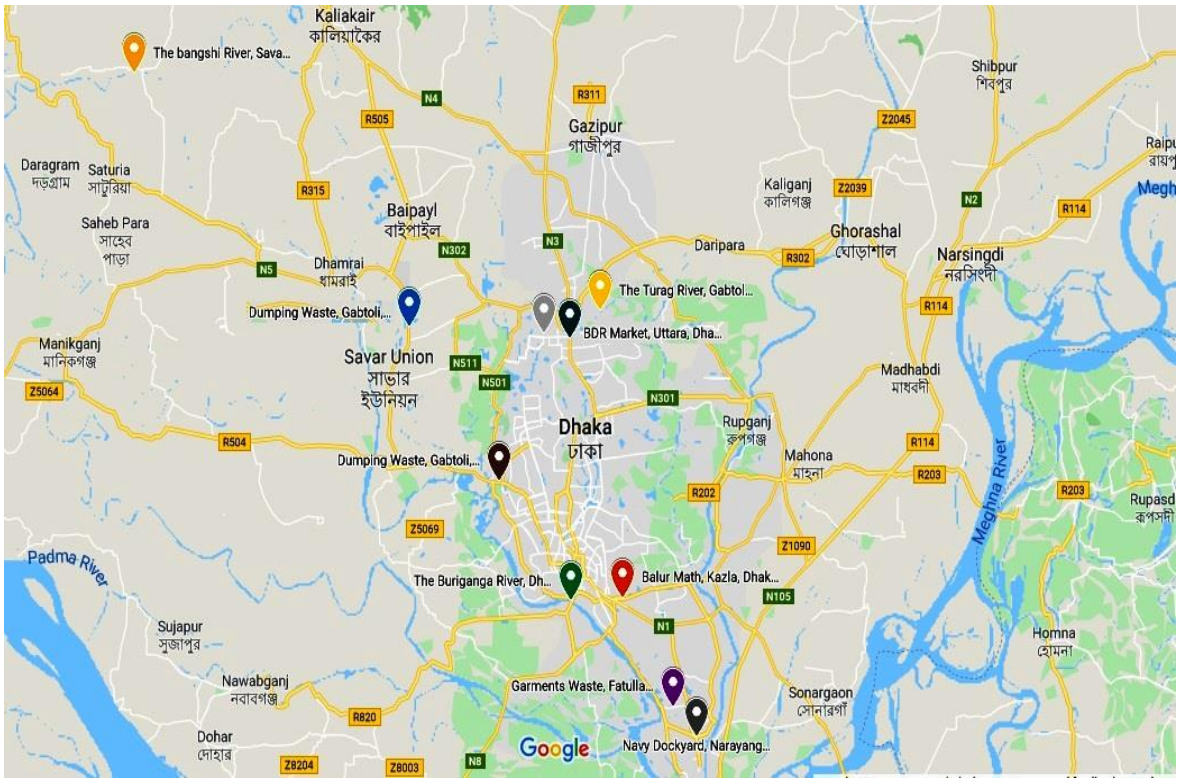


Fig. 2.2: Map of Dhaka Metropolitan City showing the location of sampling sites.

Fig. 2.3 (A-D): Photographs showing some of the sampling sites.

A. Dumping waste, Gabtoli, Dhaka B. Balur math, Kazla, Dhaka

C. The Turag River, Dhaka and D. Kitchen waste dumpsite, Uttara, Dhaka.

2.2 Collection of samples

During collection of samples, plastic bags, marker, pen, field notebook were taken to the sampling sites. Soil samples were collected aseptically in the sterilized plastic bags and water samples were collected in the sterilized plastic bottles. A total of 10 soil and water samples were collected. After collection, the samples were labeled properly and brought into the laboratory as soon as possible. Date of collection, sampling sites and types of sample are shown in Table 2.1.

2.3 Measurement of pH of the collected samples

pH of the collected samples were measured by a pH meter (ToA-DKK, HM-31P, Japan) immediately after the samples were brought into the laboratory. Samples were sieved before the measurement of pH. For measuring the pH of the soil sample, suspension was made at 1:2 ratio of sample and water.

2.4 Preservation of the samples

Collected samples were preserved in a refrigerator at 4°C before and after analysis.

2.5 Culture media and techniques used for the enumeration and isolation of bacteria

2.5.1 Culture media for enumeration and isolation of bacteria

Nutrient Agar with 1% glucose (NAG), Peptone Yeast Extract Glucose Agar (PYG) and Luria Bartani (LB) media were used for the enumeration and isolation of aerobic heterotrophic bacteria present in soil and water samples. The pH of the media was adjusted to 7.0 since pH of most of the sample was neutral.

Table 2.1: Date of collection, sampling sites and sample types.

Sample No.	Date	Sampling sites	Sample types
1	16.02.15	Dairy Farm, Savar, Dhaka	Soil
2	24.03.15	The Bangshi River, Savar, Dhaka	Water
3	31.03.15	The Turag River, Gabtoli, Dhaka	Water
4	12.04.15	The Buriganga River, Dhaka	Water
5	30.04.15	Navy Dockyard, Narayanganj	Water
6	11.05.15	Dumping waste, Gabtoli, Dhaka	Soil
7	01.06.15	Kitchen waste dumpsite, Uttara, Dhaka	Soil
8	09.06.15	BDR market, Uttara, Dhaka	Soil
9	25.06.15	Garments waste, Fotulla, Dhaka	Water
10	22.06.15	Balur Math, Kazla, Dhaka	Soil

2.5.2 Techniques employed for enumeration and isolation of bacteria

Serial dilution technique (Clesceri *et al*. 1998) was used for the isolation of bacteria. One gram of soil or one ml of water was diluted (1:100 or 1:99) with required amount of sterile waterin a sterile conical flask and shaken well. This suspension was transferred to 9 ml of sterile water in a test tube for ten-fold (1:10) dilution and further diluted up to 10^5 for the plating in four different media *viz*. NAG, PYG and LB.

One ml of each of the diluted samples was taken in a sterilized petri plate. Then desired molten agar medium was poured and mixed thoroughly by rotating the petri plate, first in one direction and then in the opposite direction. Plating in duplicated plates was made for each diluted samples. After setting the medium the plates were placed invertedly and incubated at 37°C for 24 h in an incubator (Memmert GmbH + Co Kg 8540 Sehwabach).

2.6 Enumeration of total viable bacteria

After 48 h of incubation the plates having well discrete colonies were selected for counting. The selected plates placed on colony counter (Digital colony counter, DC-8 OSK 100086, Kayagaki, Japan) and the colonies were counted. Lipase positive bacteria grown in TBA plates were also counted separately.

2.7 Isolation and primary screening of PHB producing bacteria

For the rapid detection and isolation of PHB producing bacteria, 0.02% alcoholic solution of Sudan black B was applied to stain bacterial colonies and the plates were kept undisturbed for 30 min. The excess dye was then decanted and plates were rinsed gently by adding ethanol. Colonies unable to incorporate the Sudan black B appeared white, while PHB producers appeared bluish black (Juan *et al.* 1998). Well discrete desired aerobic heterotrophic bacterial colonies were selected after counting. The selected colonies were isolated on NA slants for further studies.

2.8 Preliminary selection of the isolated bacterial isolates

Preliminary selection of the isolated possible PHB producing bacteria was made on the basis of their distinctive colony morphology of the inoculated and incubated plates.

2.9 Purification of the bacterial isolates

Although a few bacteria are so morphologically remarkable as to make them identifiable without isolation, pure cultures are nearly always a necessity before one can attempt identification of an organism. It is important to realize that the single selection of a

colony from a petri plate does not assure purity. Testing of pure cultures is essential to the success of any microbial identification system.

After preliminary selection, the selected PHB producing bacterial isolates were purified by streak plate method. When a plate yielded only one type of colony, the organism was considered to be pure.

2.10 Rapid screening of native bacterial isolates for PHB production

All the bacterial isolates were qualitatively tested for PHB production following the viable colony method of screening using Sudan Black B dye. For rapid screening of PHB producers, NA medium supplemented with 1 % glucose was sterilized by autoclaving at 121°C for 20 minutes and cooled to 45°C. The medium was poured into sterile Petri plates and allowed for solidification. Then the bacterial isolates were spotted on the Petri plates. The plates were incubated at 37°C for 24 hours. Ethanolic solution of (0.02%) Sudan Black B was spread over the colonies and the plates kept undisturbed for 30 minutes. Plates were washed with 96% ethanol to remove the excess stain from the colonies. The dark blue colored colonies were taken as positive for PHB production (Fig. 2.4). All the PHB positive isolates were marked with the code numbers.

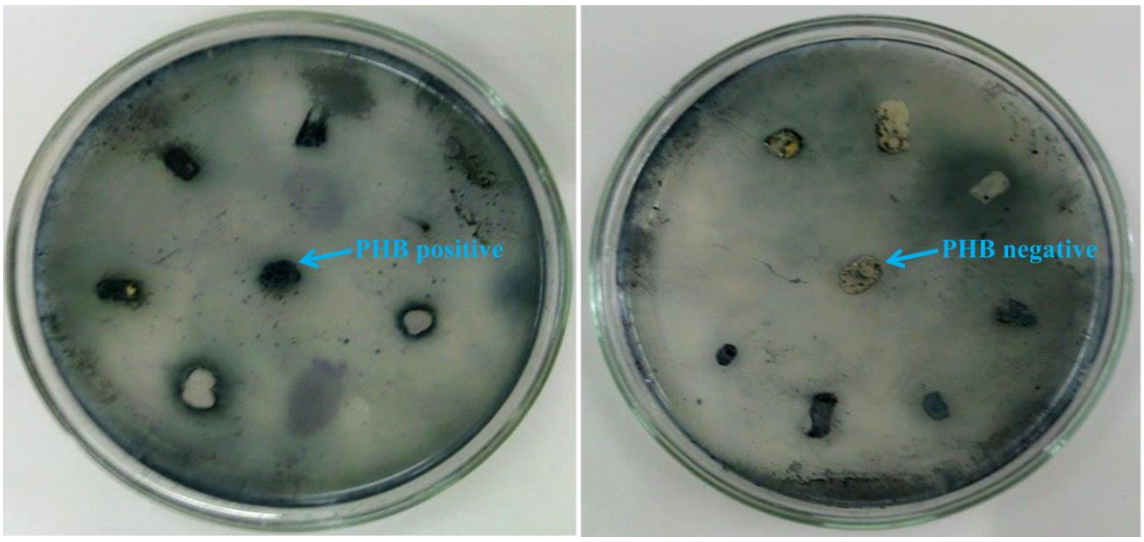


Fig. 2.4: PHB positive bacteria after purification.

2.11 Colonial morphology of the bacterial isolates

Bacteria grow on solid media as colonies. A colony is defined as a visible mass of microorganisms all originating from a single mother cell. Key features of these bacterial colonies serve as important criteria for their identification.

The isolated PHB producing bacterial colonies on plating medium were morphologically studied as their shape/form, pigmentation, surface elevation, margin, surface and opacity (Bryan 1950, Eklund and Lankford 1967).

2.12 Maintenance and preservation of bacterial isolates

The purified bacterial isolates were then transferred on NA slants. The slants were kept in polythene bags and preserved as stock cultures in refrigerator at 4°C for further studies. Periodical transfers of isolates on agar slants were done for maintaining viability of the organisms.

2.13 Morphological characteristics of bacterial isolates

For the identification of selected bacterial isolates, following morphological characters were studied and recorded accordingly.

2.13.1 Microscopic examination of bacterial isolates

Bacteria are microscopic single-celled prokaryotic organisms. Microscope is an essential tool for bacterial cell observation. Bacterial cell suspension made by using fresh culture with physiological saline. The prepared suspensions were used to make smear on glass slides. A good quality glass slide was used for this purpose. Thin smear was prepared on the clean and oil free slide. The smear was allowed to dry in air and get fixed. Two different staining methods *viz.* (i) Simple staining and (ii) Differential staining were employed to stain the fixed smears.

2.13.1.1 Simple staining

Manual of Microbiological Methods (SAB 1957) was followed for simple staining. Basic dyes *viz.* safranin was used. The fixed smear was flooded with safranin solution for one minute. The flooded smear was washed off with slow stream of tap water and dried in air.

2.13.1.2 Differential staining

Staining procedures that make visible differences between microbial cells or parts of cells termed as differential staining (Pelczar *et al.* 1993). In differential staining process, a combination of dyes is used that take advantage of chemical differences among cells (Claus 1995). The differential staining methods most frequently used are the Gram stain, Acid-fast stain and Spore stain (Tortora *et al*. 1998). For this purpose, fixed smear is exposed to more than one dye solution to differentiate cell and or its parts. In this study,

three differential staining techniques were used *viz.* (i) Gram staining, (ii) Spore staining and (iii) Intracellular lipid or fat or PHB staining.

2.13.1.2.1 Gram staining

Gram staining is one of the most important and widely used differential staining techniques which is considered as one of the important steps in identifying an unknown bacterium. For Gram staining, method described by Claus (1995) was followed. For this purpose, smear was made from 18-24 h old bacterial culture. Fixed smear was treated with the following solutions and after application of each solution; the slide was gently washed off with slow stream of tap water. Crystal violet solution 60 sec., Lugol's iodine solution 60 sec., 95% Ethyl alcohol 15 sec., Mercurochrome solution 60 sec. After staining and washing the slide was blotted dry and observed under advanced research microscope (Nikon Microphot, UFX-IIA, Japan).

The results were recorded as Gram positive (blue-violet) and Gram negative (light red) bacteria.

2.13.1.2.2 Spore staining

The method described by Claus (1995) was applied in spore staining. For this purpose, smear was made from 36-48 h old bacterial culture. The fixed smear was flooded with 5% aqueous solution of malachite green and heated over a brass plate for about 15 minutes taking care that the dye must not be dried off. Excess dye was then washed gently and safranin used as a counter stain for 1 minute. The slide was washed gently, dried and observed under microscope. The shape and position of the spores within

sporangia were observed. The swelling nature of the sporangium was also observed and properly recorded.

Spores were stained with green color of malachite green and vegetative cells or sporangia were stained with red color of safranin.

2.13.1.2.3 Intracellular lipid or fat or PHB staining

The presence of PHB as intracellular granules was confirmed by staining the cells with Sudan black B. The method described by SAB (1957) was applied in PHB staining. For this purpose, smear was made from 18-24 h old bacterial culture. Fixed smear was immersed in a filtered solution of 0.3% (w/v) Sudan black B for 15 min. Then, the slide was washed with distilled water and counter stain with safranin for 10 seconds. The slide was then rinsed with tap water and blot dried and examined under a microscope.

PHB were stained blue-black or blue-grey, while the bacterial cytoplasm is stained light pink.

2.13.2 Microscopic observation of the stained bacteria

The size and shape of vegetative cells of selected bacterial isolates were observed. The arrangement of cells whether single or in chains or clusters were carefully recorded. Gram reaction and spore formation of the isolates were also studied under microscope and recorded. Photomicrographs of the observed cells were also taken using advanced research microscope (Nikon Microphot, UFX-IIA, Japan).

2.14 Physiological and biochemical characteristics of the bacterial isolates

Physiological and biochemical characteristics e.g. carbohydrate utilization, amino acid degradation, enzyme production (catalase, amylase, protease, nitrate reductase, oxidase etc.), motility etc are used for identification of bacteria. Following Bergey's Manual (Krieg and Holt 1984, Sneath *et al.* 1986), Manual of Microbiological Methods (SAB 1957), Microbiological Methods (Collins and Lyne 1984) and Understanding Microbes (Claus 1995) the major important physiological and biochemical tests in relation to identification of the isolated bacteria were carried out and recorded accordingly.

2.14.1 Acid production from carbohydrate (Sneath *et al.* 1986)

The selected bacterial isolates were tested for their acid production from different major carbohydrates. For this purpose, inorganic nitrogen based agar medium was used. Bromothymol blue as an indicator and 1% carbon source (w/v) were added to this medium. D-glucose, D-Xylose, L-arabinose, D-mannitol, D-galactose, D-mannose, L-rhamnose (monosaccharides), sucrose, D-trehalose, D-cellobiose (disaccharides) and D-raffinose (oligosaccharides) were used as carbon sources. Sterilized medium was poured into the sterilized petri plates and allowed to solidify. Inoculation was done by point inoculation method and the plates were incubated for 48 h at 37°C.

Acid production from carbohydrate was determined by yellow color formation around the colony.

2.14.2 Gas production from glucose (SAB 1957)

Gas production during fermentation test from glucose is of considerable significance in the identification and classification of bacteria.

Fermentation tubes with glucose were made using bromothymol blue as an indicator. One Durham's tube was introduced in each of the test tubes. Then the tubes were inoculated in duplicates with 24 h old culture suspension with the help of sterilized pipette and incubated at 37°C for 48 h.

The change of color of the indicator from green to yellow indicated the production of acid. Presence of gas in the Durham's tube indicated the positive result. No gas indicates negative result.

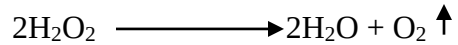
2.14.3 Potassium hydroxide solubility test (Schaad 1988)

Potassium hydroxide solubility test was done with 3% potassium hydroxide (KOH) solution. One to two drops of 3% KOH solution was placed on a clean and dry glass slide. A loop full of 24 h old culture of bacterial cells were transferred and mixed thoroughly with the inoculation loop and the slide was kept for 10 sec.

The organism was considered positive when KOH solution became viscous and showed a slimy thread and it was negative when there was no slime. The organisms showing positive reaction to KOH are supposed to be Gram negative bacteria.

2.14.4 Catalase test (Claus 1995)

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS).



To demonstrate catalase activity, a loop full of test organisms were taken by a sterilized loop on a glass slide and a drop of hydrogen peroxide was added to it.

The evolution of bubbles indicated the positive result.

2.14.5 Oxidase test (Claus 1995)

The enzyme oxidase, present in certain bacteria catalyzes the transport of electron from donor bacteria to the redox dye tetra-methyl-para-phenylene-diamine dihydrochloride. The dye in the reduced state has a deep purple color.

To perform this test, filter papers were soaked in 1% aqueous tetramethyl-phenylene-diamine dihydrochloride. Fresh young cultures were then rubbed on the filter paper with a clean glass rod. Results were recorded within 10 seconds.

Blue color indicated the positive result.

2.14.6 Motility test (Eklund and Lankford 1967)

Motile bacteria can move through semisolid, soft motility agar and their growth clouds the medium. Non-motile organisms will remain still and only grow near the site of inoculation. A chemical 2,3,5-triphenyltetrazolium chloride, which is reduced from the

colorless form to the insoluble red formazan by growing bacteria, was added to the medium at 0.001% (w/v). The color is developed only in that part of medium in which bacteria are present.

Color formation in the medium apart from inoculation site revealed the motility of bacteria.

2.14.7 Heat-shock test (Thiel *et al*. 1999)

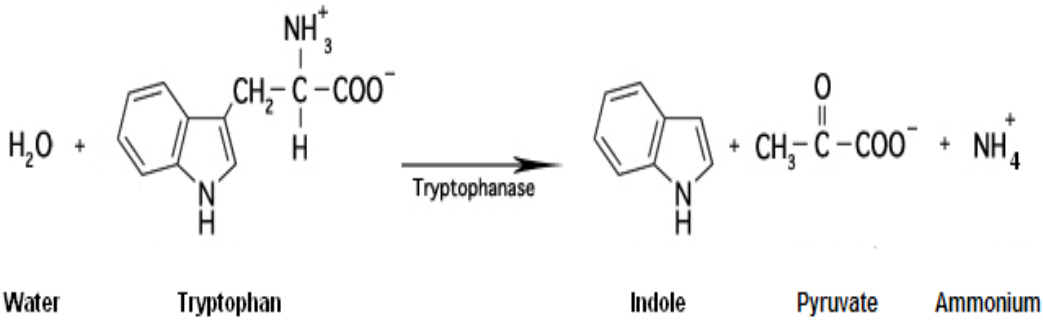
As the *Bacillus* sp. bacteria are mostly non-pathogenic and widely used for industrial enzyme production, heat-shock test was done for further confirmation to screen out the spore former bacteria representing *Bacillus* sp. In this test the non-spore forming bacteria are killed and only spore forming bacterial isolates survive.

For this test, a single colony of a pure isolate was taken in a test tube containing physiological saline. The tube was heated in water bath at 80ºC for 20 min. Then the test tubes were incubated at 37ºC for 24 h. About 300 µl suspensions were taken from the test tube and spread plate was done with NA medium. The plates were incubated at 37ºC for 24 h.

If any bacterial colonies developed on the plates, they were considered to be spore former.

2.14.8 Production of indole (Atlas 1997)

Indole is generated by reductive deamination from tryptophan via the intermediate molecule indole pyruvic acid. Tryptophanase catalyzes the deamination reaction, during which the amine ($-NH_2$) group of the tryptophan molecule is removed. Final products of the reaction are indole, pyruvic acid, ammonia (NH_3) and energy.



For this test, Kovac's modification method was followed. In this method 1% tryptone broth medium was used. The inoculated tubes were incubated at 37°C for 72 h. After incubation 2 ml of the test reagent (Kovac's reagent) was added.

A rose pink color indicated the formation of indole.

2.14.9 Nitrate reduction test (SAB 1957)

Nitrate reduction is evident by complete or partial disappearance of nitrate accompanied by appearance of nitrite, ammonia or free nitrogen. This test performed to observe the organisms' capability on the reduction of nitrate to nitrite. The formation of nitrite indicated the presence of the enzyme nitrate reductase in the organisms.

The following three reagents were required for this test:

Reagent A: Sulfanilic acid – acetic acid solution:

Sulfanilic acid	-	8.0 g
5N acetic acid	-	1000 ml

(1 part chemically pure acetic acid and 2.5 parts distilled water).

Sulfanilic acid was dissolved in acetic acid and stored in brown glass bottle.

Reagent B: Dimethyl-α-napthalamine solution:

Dimethyl-α-napthalamine	-	6.0 ml
5N acetic acid	-	1000 ml

(Stored in brown glass bottle)

Reagent C: Zinc dusts

The tubes of nitrate broth in duplicates were inoculated with test organisms and then incubated at 37°C for 72 h. After incubation, 1 ml of **reagent A** was added to the incubated tube and shaken. Then 1 ml of **reagent B** was also added to each tube and shaken well.

Formation of a distinct red or pink color indicated the reduction of nitrate to nitrite. Absence of nitrite may be due to complete conversion of nitrate as well as no reduction at all. A pinch of zinc dust then should be added to the tube showing absence of nitrite and it was allowed to stand for a few minutes. Any remaining nitrate (in case) would be reduced to nitrite by zinc and the characteristic pink or red color would appear and no color indicated complete reduction.

2.14.10 Production of hydrogen sulfide (SAB 1957)

To detect hydrogen sulfide (H_2S) production, peptone-iron agar deeps were inoculated with test bacteria by stabbing each bacterium with a straight inoculating needle. After incubation at 37°C for 48 h, culture tubes were examined for H_2S production.

Any blackening of the medium due to the formation of ferrous sulfide (FeS) shows positive reaction while the absence of black color demonstrates negative result.

2.14.11 Voges-Proskauer Test (Sneath *et al.* 1986)

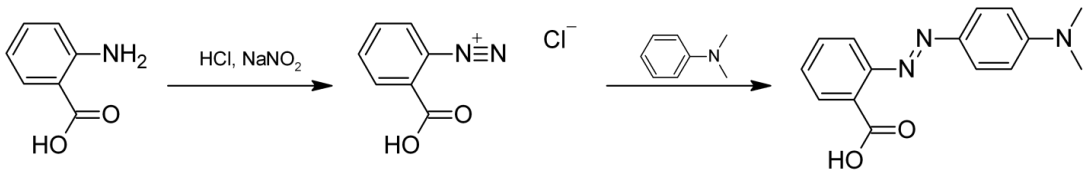
Voges-Proskauer (VP) test is a color reaction test for the production of a neutral product during glucose fermentation by microorganisms. Acetoin or acetyl-methyl carbinoloxidized to diacetyl, which reacts with creatine and forms a red complex.

For this test, VP broth tubes were inoculated with the test bacteria and incubated for 72 h at 37°C. When sufficient bacterial growth was observed, 3 ml of 5% alcoholic α-napthol solution added to each tube followed by 1 ml of 40% potassium hydroxide and 0.3% creatine solution. The tubes were then shaken vigorously and allowed to stand.

Development of crimson to ruby red color indicates a positive reaction that is the production of acetyl-methyl carbinol.

2.14.12 Methyl Red test (Sneath *et al.* 1986)

Methyl Red (MR) test is the test for mixed acid fermentation of glucose by microorganisms. Excreted acid contains large amount of formic, acetic, lactic and succinic acid and causes a major decrease in pH that can be detected by Methyl Red indicator.



For this test, tubes with VP broth was inoculated with the test bacteria and incubated at 37°C for 72 h.

After incubation 5 drops of methyl red indicator added to the culture broth. Red color throughout the broth indicated positive reaction whereas yellow or any yellowish red indicated negative reaction.

2.14.13 Hydrolysis of casein (Collins and Lyne 1984)

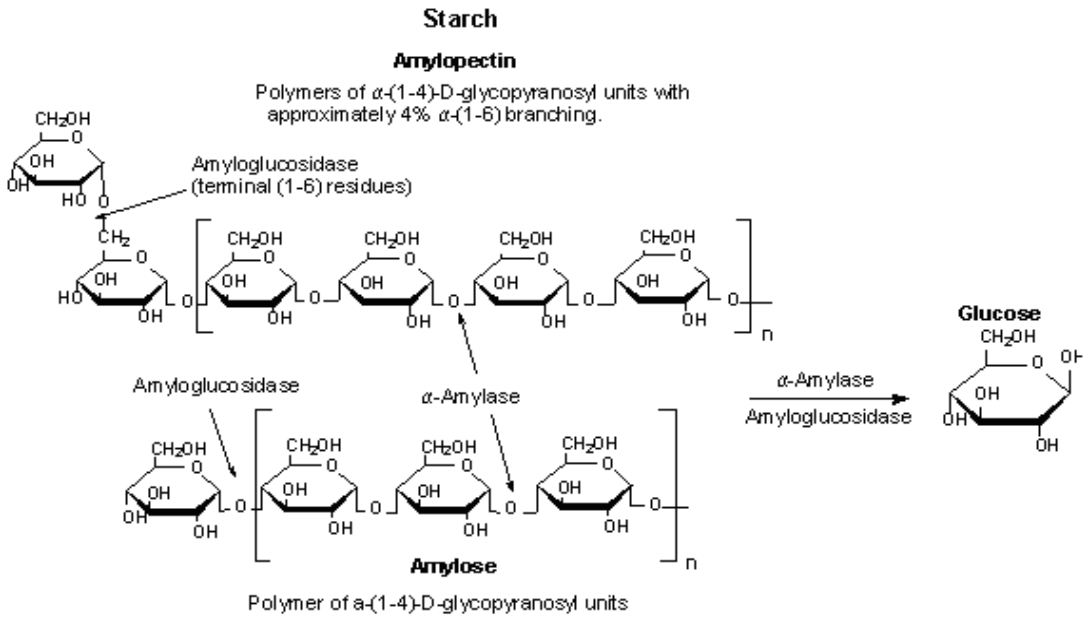
Casein is a milk protein. Casein comprises about 85% of the total protein in milk. Many microorganisms have the capacity to hydrolyze casein. This test demonstrates the ability of microbes to degrade casein into soluble peptides and amino acids by the enzyme caseiase.

One ml of sterilized milk was taken in a sterilized petri-plate and then melted agar medium was poured and mixed thoroughly. After solidifying, the plates were inoculated with fresh cultures and incubated at 37°C for 24 h.

Formation of a clear, transparent zone around the bacterial growth indicated hydrolysis of casein.

2.14.14 Hydrolysis of starch (Claus 1995)

Hydrolysis of starch test was used to identify and the potentiality of amylase production of the isolates. Organisms capable of hydrolyzing starch to form monosaccharide or disaccharide possess the enzyme amylase. As an extracellular enzyme, amylase diffuses outward from the bacterial cells and breakdown starch. This test reveals the presence or absence of the enzyme amylase in the organisms.



For this test, starch-agar plates were inoculated with test bacteria and the plates were incubated at 37°C for 48 h. After incubation, the surface of the plates was flooded with iodine solution.

Iodine reacts with starch and form starch iodide and gives the color deep blue. Development of a clear zone around the bacterial growth indicated starch hydrolysis and the presence of amylase.

2.14.15 Deep glucose agar test (SAB 1957)

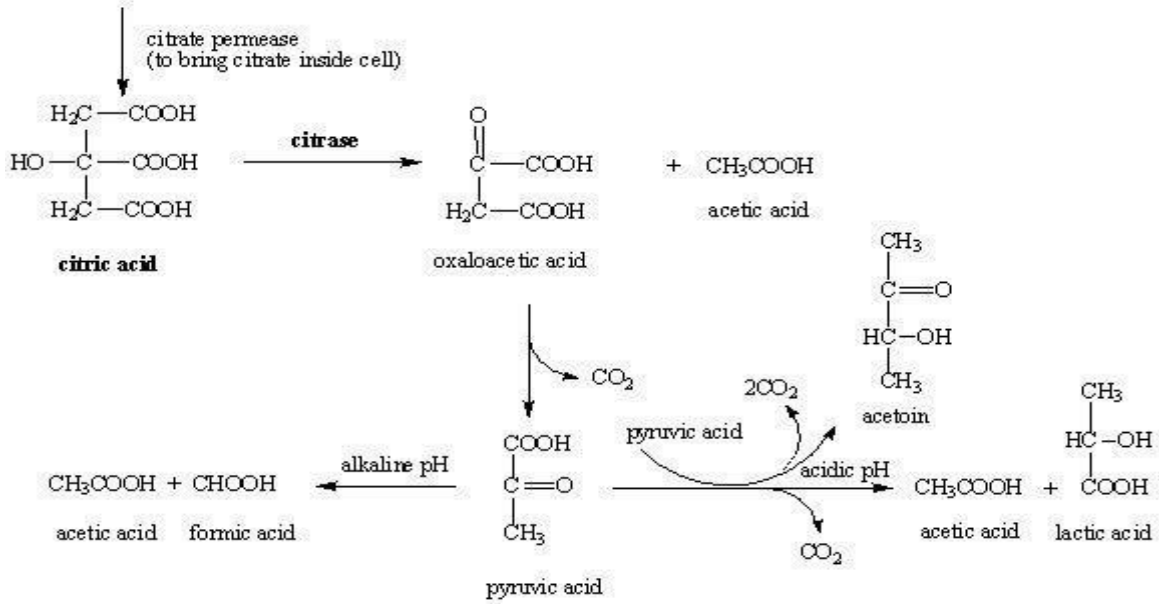
Microorganisms vary widely in their requirements for oxygen. The nature of microbial growth in agar deeps reflects the cells' relative need for oxygen or an oxygen free environment. In relation to free oxygen, organisms are generally classified as strict aerobes, microaerophiles, facultative anaerobes and strict anaerobes.

A tube of deep glucose agar medium was inoculated with the test bacteria in fluid condition approximately at 45°C. The tube was rotated to mix the inoculums with the medium and allowed to solidify. Inoculated test tubes were incubated at 37°C for 72 h.

Observation was made after incubation at 37°C for 72 h to find out whether the bacteria grew on the surface and in the upper layer of the medium (strict aerobes), or the organisms grew just a few millimeters below the surface (microaerophiles), or the organisms grew throughout the medium (facultative anaerobes), or the organisms grew deeper in the medium (strict anaerobes).

2.14.16 Utilization of citrate (Atlas 1997)

This test demonstrates the ability or inability of test organisms to use sodium citrate as sole source of carbon for metabolism and growth. The citrase enzyme hydrolyses the citrate to form oxaloacetic acid and acetic acid.



Tubes containing Simmon's citrate agar were inoculated with the test bacteria and incubated at 37°C for 48 h.

Utilization of citrate was determined by change of the color from green to blue.

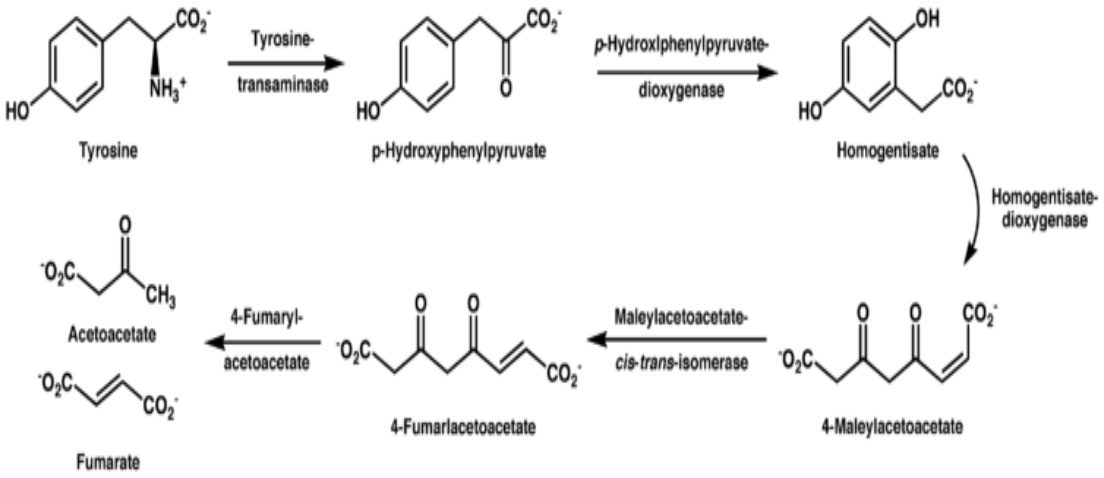
2.14.17 Utilization of propionate (Sneath *et al.* 1986)

Propionate is a short chain fatty acid and utilization of propionate is one of the key characters of bacterial identification. Propionate agar slants were inoculated with 24 h old bacterial culture and incubated at 37°C for 48 h.

Production of a pink color indicated the utilization of propionate by the isolates.

2.14.18 Degradation of tyrosine (Sneath *et al.* 1986)

Tyrosine is one of the 20 common amino acids found in all biological systems. It is catabolized as part of normal intermediary metabolism and in the breakdown of external proteins by microorganisms.



Tyrosine agar plates were inoculated with the test bacteria by point inoculation method and incubated for 72 h at 37°C.

Clearing of tyrosine crystals around and below the bacterial growth revealed degradation of tyrosine.

2.14.19 Egg-yolk lecithinase test (SAB 1957)

Bacterial lecithinases are of special interest because of the possible role of these enzymes

in pathogenicity. Lecithinases or phospholipases are enzymes released by bacteria that have the ability to destroy animal tissues. Phospholipid complexes are usually emulsifying agents occurring in tissues, serum and egg yolk. Lecithin is a normal component of the egg yolk. Bacterial lecithinases break down this lecithin to an insoluble diglycerides resulting in an opaque halo, surrounding the colony when grown on the egg yolk agar medium.

After autoclaving the medium, 1.5 ml egg-yolk aspirated aseptically was added to 100 ml of basal medium. After solidifying, the plates were inoculated with fresh bacterial cultures and incubated at 37°C for 48 h.

Bacteria that produce lecithinase appear as colonies surrounded by a zone of insoluble precipitate. Bacteria that produce lipase appear colonies with a pearly iridescent sheen.

2.14.20 Levan test (Schaad 1988)

Levan is a poly-fructose which is an extracellular capsular substance. Levan formation is detected on NA plate to which 5% sucrose (w/v) was added. Inoculated plates were incubated at 37°C for 72 h.

After 72 h incubation the presence of convex white mucoid bacterial colonies indicate levan formation.

2.14.21 Arginine dihydrolysis test (Schaad 1988)

The arginine dihydrolase catalyzes the hydrolysis of arginine into ornithine, NH_3 and CO_2 with a gain of ATP. Arginine dihydrolase is detected in routine laboratory test by the anaerobic tube method of Thornley (1960).The test medium of Thornley was stab

inoculated with the test bacteria at the base of the medium and each tube instantly sealed with 3 ml of the molten (3%) agar and incubated at 37°C for 24 h.

A positive alkaline reaction was indicated by the development of deep red color.

2.14.22 Kligler's Iron Agar test (Atlas 1997)

Kligler's Iron Agar (KIA) medium was used to differentiate Gram-negative enteric bacteria or their ability to ferment dextrose or lactose and their production of hydrogen sulfide. Tubes of KIA media were inoculated by stabbing the butt and streaking the slant with inoculum of 24 h. The inoculated media then incubated at 37ºC for 48 h.

Yellow color in the butt and slant indicated acid production while hydrogen sulfide production was indicated by blackening of slant. Break in the medium indicated gas formation. Red color in the butt and slant indicated alkaline reaction.

2.15 Identification of the isolates

2.15.1 Provisional identification

Provisional identification of selected bacterial isolates was carried out on the basis of their observed morphological and biochemical characteristics. Gram positive bacteria were provisionally identified following Bergey's Manual of Systematic Bacteriology (Sneath *et al.* 1986) while Gram negative bacteria were identified by using Bergey's Manual of Systematic Bacteriology (Krieg and Holt 1984).

2.15.2 Molecular identification

Molecular techniques have been used to identify bacteria more authentically than that of morphological and biochemical characteristics. Techniques utilizing the 16S rRNA gene

sequence data have been developed for use in the field of molecular level of identifications. The use of 16S rRNA gene sequences to study bacterial taxonomy has been by far the most common housekeeping genetic marker. For molecular identification following steps were followed.

2.15.2.1 PCR amplification

In order to identify the selected isolates based on sequence comparison, partial amplification of 16S rRNA was necessary. For the partial amplification of 16S rRNA gene the following primer pairs designed by Rudi *et al*. (1997) were used:

CC [F] 5'-16S rRNA: CCAGACTCCTACGGGAGGCAGC

CD [R] 3'-16S rRNA: CTTGTGCGGGCCCCCGTCAATTC

Preparation of Primer: Primers were dissolved in sterile miliQ water following the instruction product booklet to obtain 100 µM stock concentration of each primer. To make working concentration each primer was diluted 10 -fold further.

Preparation of Template: The bacterial isolates were cultured to grow as single colony and one colony for each isolate was suspended in 10 µl sterile water and subject to heat lysis by heating in a PCR machine for 10 minutes. The lysed cell suspension was centrifuges for 5 minute at 3,000 rpm in microcentrifuge (Mikro 200R) and then the supernatants were used as the source of template DNA for PCR amplification of 16S rRNA gene.

The following components (Table 2.2) were used to prepare PCR cocktail. The total volume of PCR cocktail was 573 µl for 10 samples.

Table 2.2 Component of PCR cocktail (for 10 reactions).

Sl. No.	Reagents	Amount for 1 sample	Amount for 10 samples
1	Sterile de-ionized distilled water	35.9 µl	359.0 µl
2	Taq Buffer B 10X	5.0 µl	50.0 µl
4	Primer Forward	2 µl	20.0 µl
5	Primer Reverse	2 µl	20.0 µl
6	dNTPs 10 mM	1 µl	10 µl
7	Taq DNA Polymerase 5 U/µl	0.1 µl	1 µl
8	Template DNA 25 ng/µl	4 µl	40.0 µl
	Total	50 µl	500 µl

During the experiment, PCR buffer, dNTPs, Primers and DNA sample solution were thawed in ice from frozen stocks. PCR master mix was prepared for each primer to be tested by adding the components of PCR in the following order (mentioned above in Table 2.2): ddH_2O, Buffer, primer, dNTPS and Taq DNA polymerase, mixed thoroughly and kept on ice. In the mean time the PCR tubes were marked compatibly with the thermocycler and the master mix was aliquoted to individual tubes marked for each DNA samples. Template DNA (25 ng/µl) were pipetted (4.0 µl) into PCR tubes containing PCR mix and mixed by tapering the tube following short spin of the tubes to recollect the total mixture. The tubes were then sealed and placed in a thermocycler and the cycling was started immediately. PCR amplification was done in an oil-free thermo cycler (UNO II, Biometra).

The reaction conditions for PCR are given below.

	Initial denaturation	94º C	For	5 minutes
30 cycles	Denaturation at	94º C	For	45 seconds
	Annealing at	65º C	For	30 seconds
	Extension at	72º C	For	40 seconds
	Final extension at	72º C	For	7 minutes

After completion of cycling program, the reactions were held at 4º C.

2.15.2.2 Electrophoresis of the amplified products and documentation

The amplified products were separated electrophoretically on 1% agarose gel. The gel was prepared using 1.0 g agarose powder containing ethidium bromide and 50 ml 1xTAE buffer. Agarose gel electrophoresis was conducted in 1xTAE buffer at 80 Volts and 300 mA for 30 min. 1kb marker was used alongside the amplified genomic DNA. DNA bands were observed on UV-transilluminator and photographed by a Gel Documentation system.

2.15.2.3 Automated sequencing

The bacterial isolates can be identified based on alignment of partial sequence of 16S rRNA gene with the existing sequences available in the database. In the present experiment, 10 different samples were used to amplify their 16S rRNA gene and PCR amplified DNA of the bacterial isolates were gel purified using phenol freeze method and sent for automated sequencing.

2.15.2.4 Sequence based identification

The sequence generated from automated sequencing of PCR amplified DNA was analyzed through BLAST (http://blast.ncbi.nlm.nih.gov/) and rRNA BLAST (http://bioinformatics.psb.ugent.be/cgi-bin/rRNA/blastform.cgi) programs to find out possible similar organism through alignment of homologous sequences.

2.15.2.5 Phylogenetic analysis of the bacterial isolates

A phylogenetic tree of the isolates identified by 16S rRNA sequencing was constructed. Sequences obtained from BLASTN (nucleotide blast) were in FASTA format and relation between each sequence could be known by multiple sequence alignment using a website CLUSTALW algorithm. The tree was generated using neighbor joining (NJ) distance- based algorithm of phylogenetic analysis.

2.16 Estimation of PHB activity of the selected bacterial isolates

2.16.1 Preparation of inoculum

For this experiment, inoculum prepared in Nutrient Broth medium and incubated at 37ºC on a rotary shaker (DAIHAN-LABTECH, Japan) at 150 rpm for 24 h. In order to keep the inoculum potential constant for each isolate, inoculums was prepared to obtain an initial cell density to adjust the turbidity of 0.5 McFarland standard (1.5×10^8 cfu/ml).

2.16.2 Preparation of 0.5 McFarland standards

McFarland turbidity standards are use to standardize the approximate number of bacteria in a liquid suspension by comparing the turbidity of a test suspension. Standard was prepared by adding barium chloride ($BaCl_2$) to H_2SO_4 to obtain a barium precipitate.

85 ml of 1% H_2SO_4 added to a 100 ml volumetric flask. 0.5 ml of 1.175% $BaCl_2$ added drop wise to the 1% H_2SO_4 while constantly swirling the flask. The volume was adjusted to 100 ml with 1% H_2SO_4. Then it was stirred for 5 minutes until the solution appears homogenous and free of clumps. Optical density was measured at 625 nm wavelength.

2.16.3 PHB production by submerged fermentation process

The PHB production was carried out in 100 ml plugged Erlenmeyer flask containing 50 ml of bacterial inoculum. The inoculated flasks were then incubated at 37ºC for five consecutive days with constant shaking at 150 rpm in a rotary shaker. Each experiment was performed in triplicates.

2.16.4 PHB extraction and quantification (Santhanam and Sasidharan 2010)

Incubated bacterial culture was centrifuged at 5000 rpm for 10 minutes. Cell pellet (fresh or frozen) is suspended in 3ml hypochlorite at 37°C for 1 hour to allow complete digestion of cell components except PHB. The suspension was centrifuged (10,000 rpm for 30 minutes) and the supernatant is discarded. Crude PHB can be observed as a white layer at the bottom. The crude PHB is washed twice with 10 ml distilled water by centrifugation (10,000 rpm for 30 minutes) to get rid of any remaining hypochlorite. After that plastic tubes were exchanged for glass tubes. Next the collected PHB was washed twice with acetone, to get rid of lipid contaminants. A final wash was given with pure methanol to get rid of any moisture that may still remain which will interfere with later steps. 10 ml chloroform was heated in a glass tube in a water bath (65 -70°C) and just when the chloroform starts to boil, it was transferred to the tube containing the extracted PHB. This suspension was moved to the water bath to ensure the chloroform is

hot enough to dissolve all the PHB. One ml of this suspension was transferred to another glass tube and the chloroform was allowed to completely evaporate. It can be left open at room temperature for 24 hours or the evaporation can be accelerated by gentle heating in a water bath at 40°C until all chloroform has vaporized. Once the chloroform was vaporized completely, 10 ml conc. H_2SO_4, was added to the tubes and capped with glass stoppers. The tube was heated in a boiling water bath (94-96°C) for 20 minutes for complete conversion of PHB to crotonic acid. Ensure no moisture remains into the tube, but also no pressure builds up inside. The sample was then cooled to room temperature, vortexed thoroughly. The solution was cooled and the absorbance measured at 235 nm against a sulfuric acid blank. The sample's absorbance was used in the equation from the standard curve to determine the amount of PHB in the cultivation sample. Based on the PHB yields, two potential bacterial isolates were selected for further studies.

2.16.5 Preparation of standard curve

A standard curve of crotonic acid (Law and Slepecky 1969) was prepared before the estimation of PHB activity of the bacterial isolates. Pure crotonic acid (Himedia, Japan) was used to prepare the standard curve. It (200 mg) was dissolved in 10 ml of concentrated H_2SO_4 and heated for 10 min which gave 20 mg/ml of crotonic acid. From the above stock, working standard solution was prepared by diluting 5 ml of the stock (containing 100 mg of crotonic acid) to 10 ml with H_2SO_4, which gives the final concentration of 10 mg/ml (0.01 g/ml). This was used for the preparation of the standard curve.

2.17 Optimization of PHB production of the bacterial isolates

Optimization of different parameters in PHB production was carried out for better PHB production showing bacterial isolates. To perform this experiment PHB production was carried out in 100 ml Erlenmeyer flask containing 50 ml of liquid medium on a rotary shaker with 150 rpm and incubated at 37°C for 72 h and the PHB activity was estimated. All experiments were carried out in triplicate.

2.18 Optimization of different parameters

Optimization of different parameters such as pH, temperature, carbon source, organic and inorganic nitrogen source and inoculum concentration were carried out.

2.18.1 Effects of pH on PHB production

Effects of pH on PHB production was performed at varying pH (5-9) of the medium at temperature 37°C. The bacterial isolates were inoculated in the Nutrient broth medium and incubated at 37°C for 72 h.

2.18.2 Effects of temperature on PHB production

To test the effect of temperature on PHB production, five different temperatures such as 20, 30, 37, 40 and 50°C were selected at pH 6. The bacterial isolates were inoculated in the Nutrient broth medium and incubated at selected different temperature for 72 h.

2.18.3 Effects of carbon sources on PHB production

Effects of carbon sources on the PHB production was analyzed by adding different carbon sources such as glucose, sucrose, starch, arabinose, xylose and mannitol at a

concentration of 1% (w/v) in the production medium. The inoculated production medium was incubated at 37ºC for 72 h.

2.18.4 Effects of organic nitrogen sources on PHB production

Effects of organic nitrogen sources on the PHB production was studied. The nitrogen sources *viz*. peptone, tryptone, yeast extract and beef extract at a concentration of 1% (w/v) was added to the PHB production medium and incubated at 37ºC for 72 h.

2.18.5 Effects of inorganic nitrogen sources on PHB production

Effects of inorganic nitrogen sources on PHB production was studied by adding three different nitrogen sources such as ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3) and ammonium ortho phosphate ($NH_4H_2PO_4$) at a concentration of 1% (w/v) to the medium. The inoculated PHB production medium was incubated at 37ºC for 72 h.

2.18.6 Optimization of better carbon and nitrogen sources on PHB production

Better carbon and nitrogen sources which support maximum PHB production were added at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% (w/v) in the production medium. For optimization the inoculated PHB production medium was incubated at 37ºC for 72 h.

2.18.7 Effects of inoculum concentration on PHB production

To evaluate the effect of inoculum concentration on PHB production varied cell concentrations 1.0 to 6.0% were added and the inoculated medium was incubated at 37ºC for 72 h.

2.19 PHB production using all optimum conditions

Considering all parameters for optimization of PHB production the best condition was employed. Using all possible optimum conditions, inoculated flasks were incubated at 37°C for 72 hat150 rpm on a rotary shaker. The experiment was performed in triplicates.

2.20 Characterization of PHB by FT-IR

Poly-3-hydroxybutyrate (PHB) was extracted with chloroform, dried and subjected to FT-IR (Fourier Transform Infrared Spectroscopy). Appropriate quantity of KBr and sample (in the ratio 100: 0.1) were mixed by grinding in an agate mortar. Pellets were made with about 100 mg mixture. FT-IR spectra were recorded with FT-IR 8400S Shimadzu spectrophotometer in the range of 4000-400 cm^{-1} (Resolution: 4 cm^{-1}; No. of scans: 30 times). The absorbance peak values obtained were compared with the available literature values and the sample was confirmed for the presence of PHB.

2.21 Preparation of biofilm

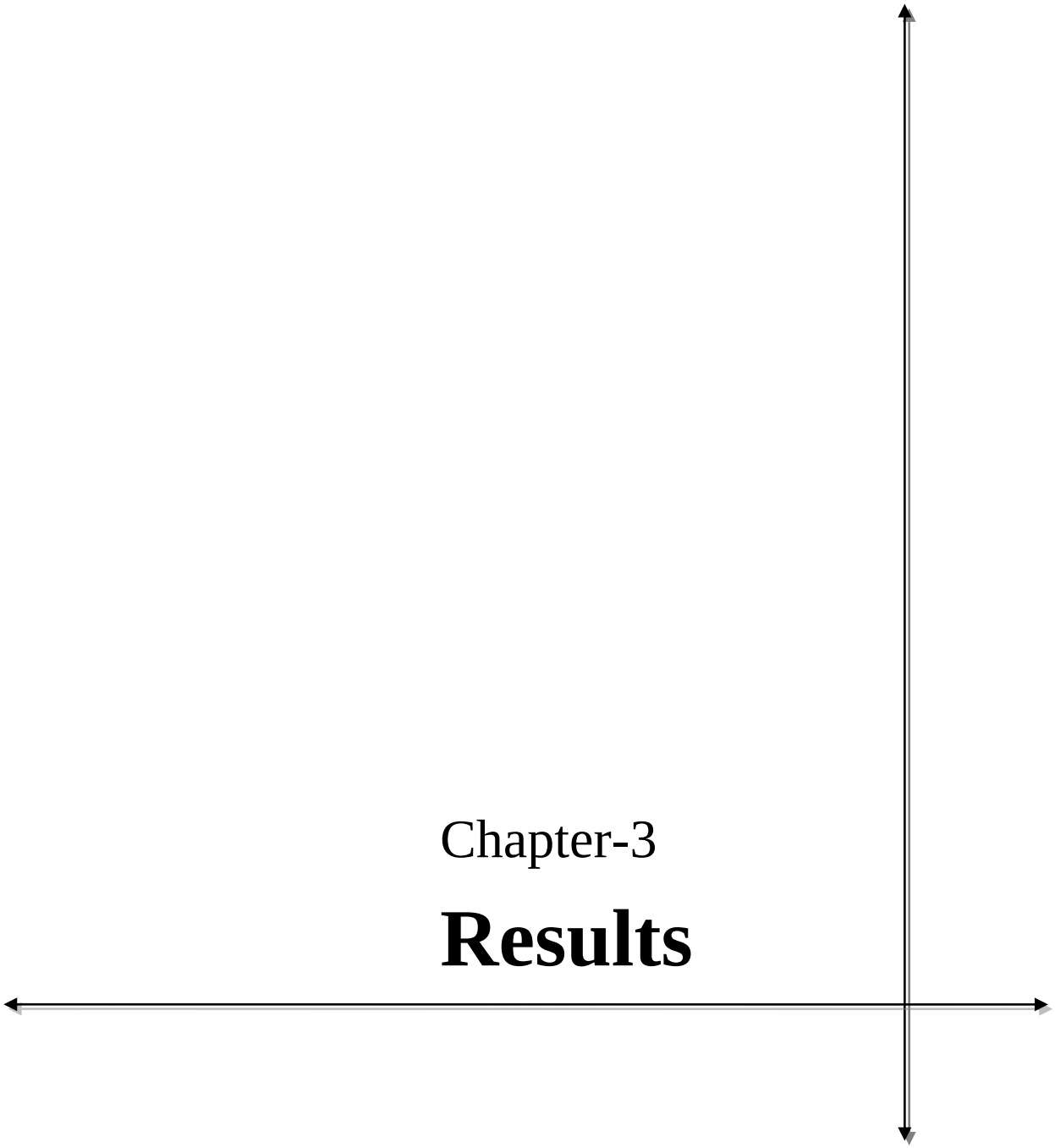
The PHB positive two selected bacterial isolates were inoculated in Nutrient broth supplemented with best carbon and nitrogen source at optimum concentrations and incubated under shaker for 72 h. After incubation, the bacterial cells containing the polymer were pelleted at 5,000 rpm for 10 min and resuspended in equal volume of 4% sodium hypochlorite and incubated at 37°C for 1 h. The mixture was again centrifuged and the supernatant discarded. The cell pellet containing PHB was again washed with acetone and ethanol. The polymer granules were then dissolved in chloroform. The chloroform was filtered and the filtrate was poured as a thin layer in a glass petri dish evaporated at 4°C, forming a film.

2.22 Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) v.20.0 for windows (SPSS, SAS Institute Inc. Cary, USA). The data were analyzed to determine the descriptive statistics such as Mean and Standard Deviation. Evaluation on differences between groups was based on the oneway ANOVA test at 95% confidence interval. Value of $p < 0.05$ was regarded as statistically significant.

Chapter-3

Results



Results

Poly-3-hydroxybutyrate (PHB) is the simplest biopolyester of the PHA family and accumulated as intracellular carbon and energy storage compound in many bacteria. PHB is a biodegradable thermoplastic polyester and analogous to many conventional petro-derived plastics currently in use. The production of PHB by microorganisms has achieved much attention in recent years due to enormous applications of PHB in medicine, agriculture and marine fields. In the present investigation, PHB producing bacteria were isolated from different waste materials in and around Dhaka Metropolitan City. Better PHB producing bacterial isolates were used for detailed study of PHB production, optimization and characterization of PHB.

3.1 Sampling sites and sample types

Considering potential indigenous bacteria ten different sampling sites were selected for the enumeration and isolation of indigenous PHB producing bacteria. Sampling sites were mainly domestic and industrial area river receiving untreated industrial influents. Five water and five soil samples were collected from the selected sites.

3.2 Bacterial load of the collected samples

A good number of aerobic heterotrophic bacteria were found to be associated with the collected samples (Table 3.1 and Fig. 3.1). The aerobic heterotrophic bacterial load ranged in between 5.55×10^3 and 2.52×10^7, 3.10×10^4 and 2.23×10^7, 7.50×10^3 and 1.01×10^7 cfu/g or cfu/ml in NAG, PYG and LB media, respectively. The maximum bacterial count (2.52×10^7cfu/g) was observed in NAG medium from soil sample of BDR

market. On the other hand the lowest bacterial load (5.5×10^3 cfu/ml) was observed in NAG medium from the garments waste water of Fatulla. The highest number (33) of PHB producing bacteria was found in kitchen waste soil and the lowest number (9) was found from the Turag River water sample. Higher bacterial count in the garbage soil samples may be due to availability of diversified and better nutrients in the waste substances. The results revealed that PHB producing bacteria are widely distributed in soil and water samples.

3.3 Isolation and primary screening of PHB producing bacteria

For the rapid detection and isolation of PHB producing bacteria, 0.02% alcoholic solution of Sudan black B was applied to stain bacterial colonies and the plates were kept undisturbed for 30 min. The excess dye was then decanted and plates were rinsed gently by adding ethanol. Colonies unable to incorporate the Sudan black B appeared white, while PHB producers appeared bluish black (Fig. 3.2). Well discrete desired aerobic heterotrophic bacterial colonies were selected after counting. The selected colonies were kept on Nutrient Agar slants for further studies.

3.4 Selection of bacteria

Based on colony morphology, a total of 160 PHB positive bacterial colonies were primarily screened from different collected samples. Colonies were found to be different form, shape and pigmentation. Selected colonies comprised of all aerobic heterotrophic bacteria. Fig. 3.1 and 3.2 showed bacterial colonies developed in different steps of enumeration and isolation respectively.

3.5 Purification of the selected bacteria

Purification was done by streak plate method. Well discrete single colony was considered as pure culture. Fig.3.3 showed purification of some isolates as purified on NA plates. The selected pure cultures were studied for final screening of PHB positive isolates.

3.6 Final screening of PHB positive bacteria

All the pure bacterial isolates were qualitatively tested for PHB production following the viable colony method of screening using Sudan Black B dye. For rapid screening of PHB producers, nutrient agar medium supplemented with 1% glucose was sterilized by autoclaving at 121°C for 20 minutes and cooled to 45°C. The medium was poured into sterile Petri plates and allowed for solidification. Then the 160 pure bacterial isolates were spotted on the Petri plates. The plates were incubated at 30°C for 24 hours. Ethanolic solution of (0.02%) Sudan Black B was spread over the colonies and the plates kept undisturbed for 30 minutes. They are washed with ethanol (96%) to remove the excess stain from the colonies. The dark blue colored colonies were taken as positive for PHB production. Finally there were about 148 isolates were found to be PHB positive. Among them 30 showed better PHB activity. These 30 isolates were selected for detailed study for morphological, physiological and biochemical characteristics.

Table 3.1: Aerobic heterotrophic bacterial load of collected samples.

Sample No.	Sampling sites	Samples	Bacterial load (cfu/ml of water and cfu/g of soil)			No. of PHB positive Bacteria
			NAG	PYG	LB	
1	Dairy farm, Savar, Dhaka.	Soil	4.35×10^6	5.30×10^6	ND	18
2	The Bangshi River, Savar, Dhaka.	Water	2.33×10^7	5.10×10^6	ND	10
3	The Turag River, Dhaka.	Water	2.76×10^4	1.15×10^5	4.40×10^4	**9**
4	The Buriganga River, Dhaka.	Water	1.90×10^4	3.40×10^4	1.99×10^4	10
5	Dumping waste, Gabtoli, Dhaka.	Soil	2.23×10^5	2.92×10^5	1.23×10^6	14
6	Navy dockyard, Narayanganj	Water	3.55×10^5	3.10×10^4	7.50×10^3	12
7	Kitchen waste dumpsite, Uttara, Dhaka.	Soil	1.35×10^6	8.75×10^6	3.80×10^5	**33**
8	**BDR market, Uttara, Dhaka.**	**Soil**	$\mathbf{2.52 \times 10^7}$	2.23×10^7	1.01×10^7	19
9	**Garments waste, Fotulla, Dhaka.**	**Water**	$\mathbf{5.55 \times 10^3}$	5.60×10^5	2.40×10^5	13
10	Balur Math, Kazla, Dhaka.	Soil	4.10×10^5	1.58×10^5	8.45×10^4	22

NB: NAG=Nutrient Agar with 1% Glucose, PYG=Peptone Yeast Extract Glucose, LB= Luria Bartani and ND=Not done.

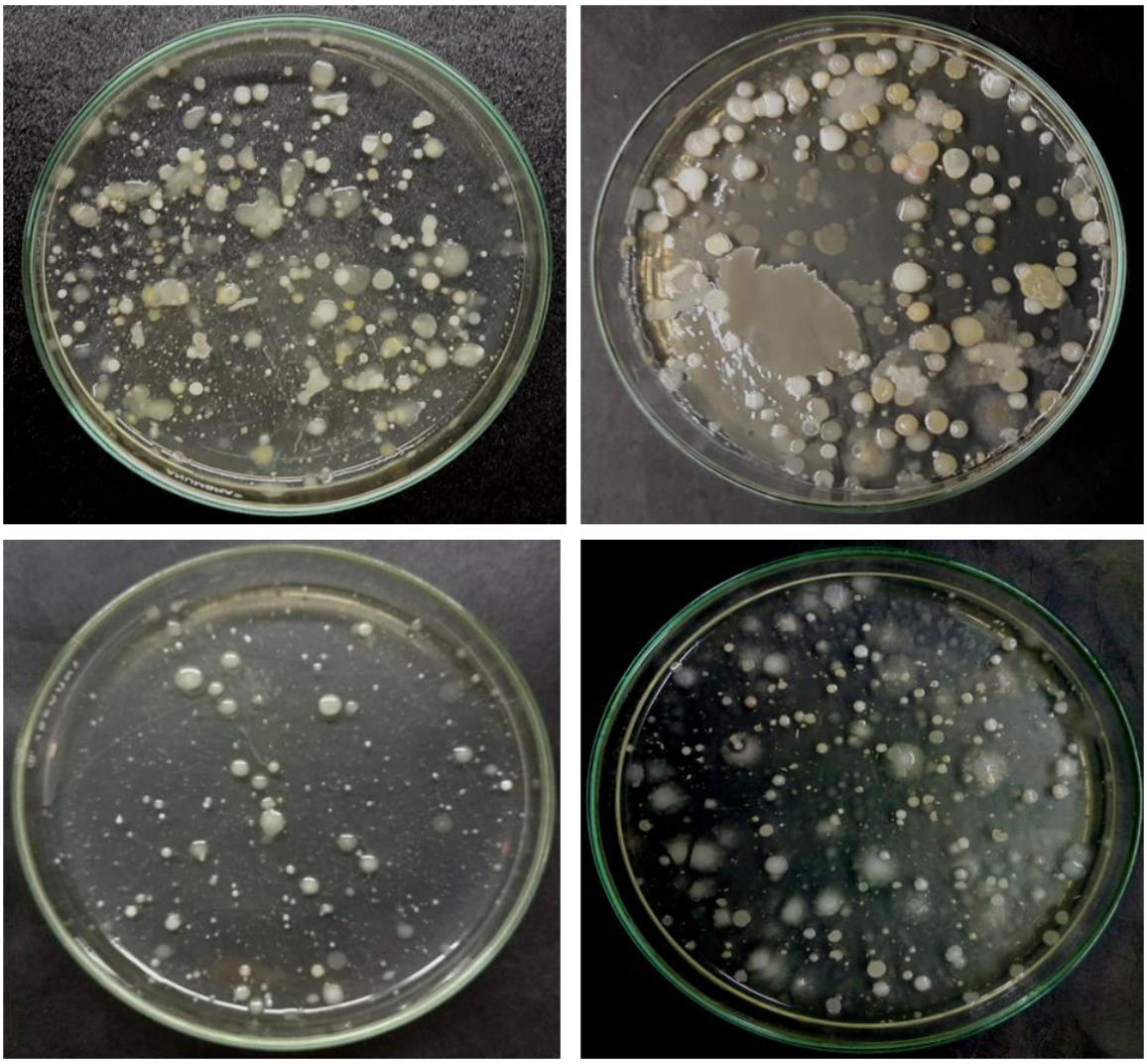


Fig. 3.1: Aerobic heterotrophic bacterial colonies developed during isolation.

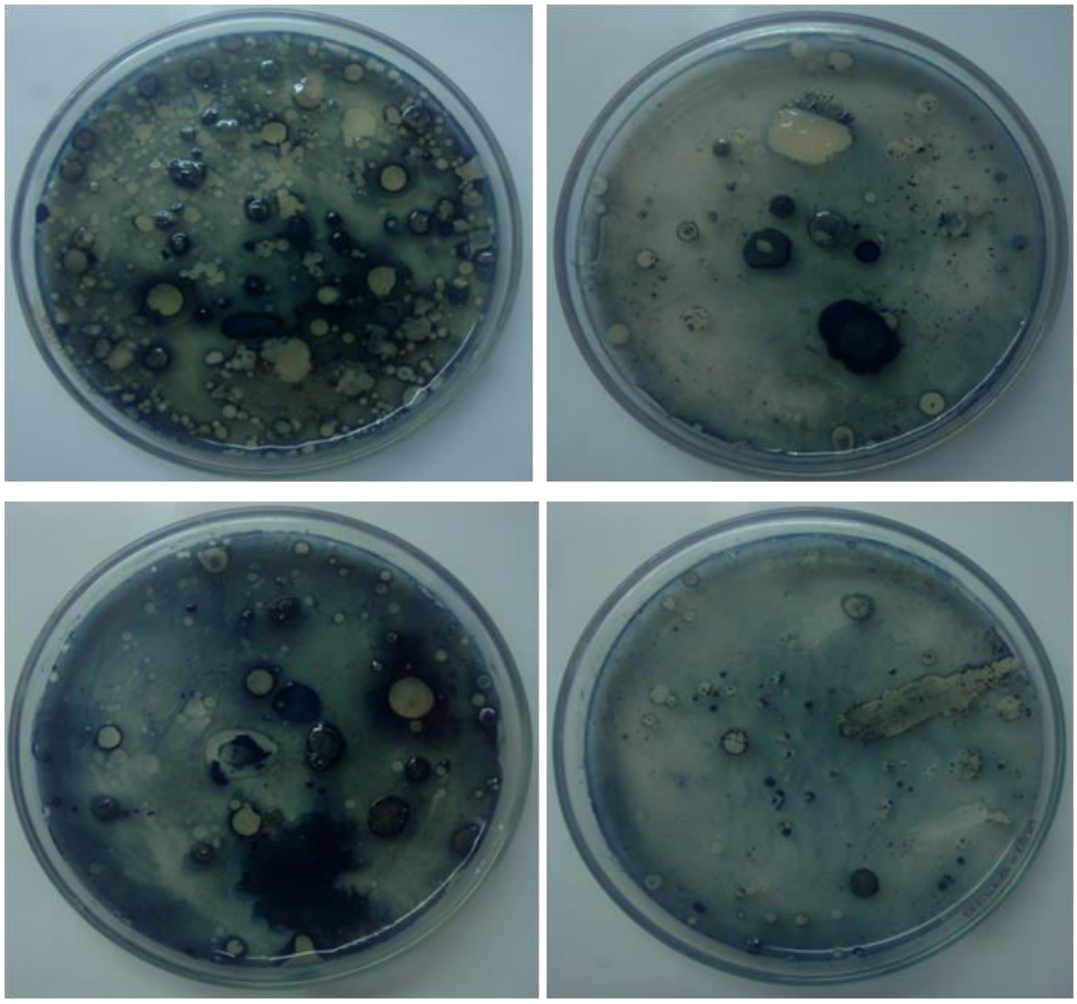


Fig. 3.2: PHB positive bacterial colonies during isolation.
Bluish black colonies indicate PHB producers.

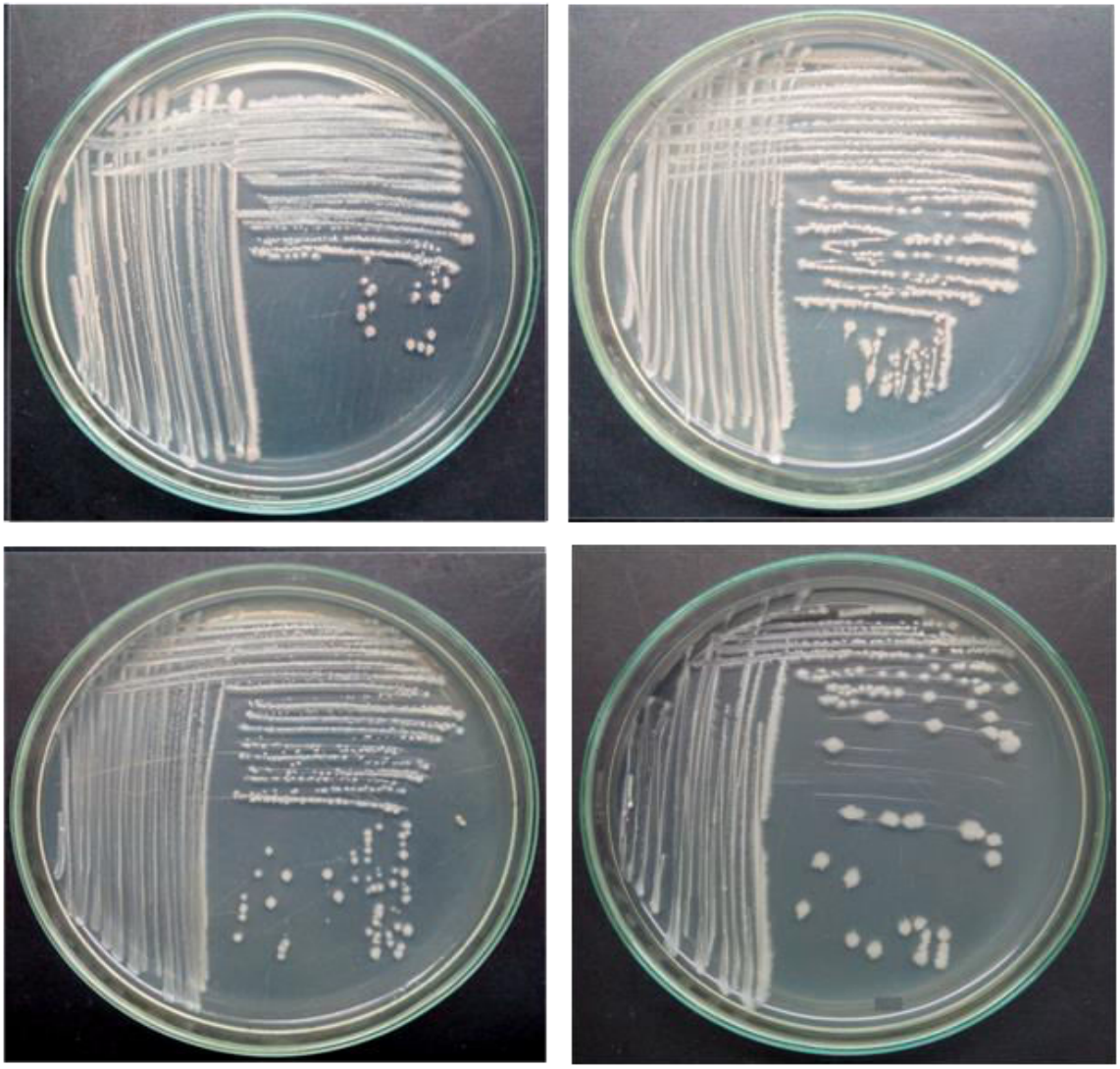


Fig. 3.3: Photographs showing streak plate method.

3.7 Colony morphology of the selected bacterial isolates

Colony morphology of the selected bacterial isolates were recorded on the basis of their shape, pigmentation, surface, surface elevation, margin and optical characteristics (Table 3.2). Most of the colonies of the selected isolates were found to be circular, flat, entire, smooth, moist, opaque and off-white. Fig. 3.4 showed the percentage of colony morphology of the selected isolates with special reference to colony shapes. Circular type of colony was found to be dominate (57%) over the punctiform (20%), irregular (13%) and filamentous (10%).

3.8 Microscopic observation of the selected bacterial isolates

Staining properties and microscopic observation of the selected bacterial isolates revealed that 21 isolates were Gram positive rod shaped and spore former and 9 were Gram negative short rod shaped (Table 3.3). Among 21 spore former bacteria most of the spores were ellipsoidal and non-swollen type of sporangia. Photomicrographs of some of the selected isolates are shown in Fig. 3.5 and Fig. 3.6. Rod shaped bacteria were found to be single, in pair and in chain.

Table 3.2: Colony morphology of the selected bacterial isolates.

Bacterial Isolates	Shape	Elevation	Margin	Surface	Dryness	Optical character	Color
S_1P-7	Irregular	Raised	Filamentous	Smooth	Moist	Opaque	Cream
S_2P-2	Circular	Convex	Entire	Smooth	Moist	Translucent	Off white
S_2P-3	Circular	Convex	Entire	Smooth	Moist	Translucent	Gray
S_2N-4	Circular	Flat	Erose	Rugose	Moist	Translucent	Gray
S_3P-1	Circular	Convex	Entire	Smooth	Dry	Opaque	Off white
S_3N-1	Punctiform	Convex	Erose	Smooth	Moist	Translucent	Cream
S_4N-2	Punctiform	Convex	Undulate	Smooth	Moist	Translucent	Gray
S_4P-2	Irregular	Flat	Undulate	Smooth	Moist	Opaque	Off white
S_4L-3	Filamentous	Raised	Filamentous	Smooth	Moist	Opaque	Off white
S_4L-10	Punctiform	Convex	Entire	Smooth	Moist	Translucent	Gray
S_6N-1	Circular	Raised	Entire	Smooth	Moist	Translucent	Off white
S_6P-3	Circular	Convex	Entire	Smooth	Moist	Translucent	Off white
S_6P-4	Circular	Raised	Erose	Smooth	Moist	Translucent	Off white
S_6P-7	Circular	Raised	Entire	Smooth	Moist	Translucent	Off white
S_6N-8	Punctiform	Raised	Undulate	Smooth	Moist	Translucent	Cream
S_7N-5	Filamentous	Convex	Filamentous	Smooth	Moist	Opaque	Cream
S_7P-19	Circular	Convex	Entire	Smooth	Moist	Translucent	Greenish
S_7P-27	Circular	Convex	Entire	Smooth	Moist	Opaque	Off white
S_7P-29	Circular	Raised	Filamentous	Radiate	Moist	Opaque	White
S_7P-32	Circular	Raised	Undulate	Smooth	Moist	Opaque	Off white
S_7L-25	Irregular	Flat	Erose	Smooth	Moist	Opaque	Off white
S_7L-26	Circular	Raised	Entire	Smooth	Moist	Translucent	Cream
S_8P-2	Punctiform	Convex	Entire	Smooth	Moist	Translucent	Brown
S_8N-5	Punctiform	Flat	Entire	Rugose	Moist	Translucent	Off white
S_8N-6	Filamentous	Raised	Erose	Contoured	Moist	Opaque	Redish
S_8P-11	Irregular	Raised	Lobate	Smooth	Moist	Opaque	Off white
S_8L-18	Circular	Raised	Erose	Cncentric	Moist	Opaque	Off white
S_9P-2	Circular	Convex	Entire	Smooth	Moist	Opaque	Yellow
S_{10}P-3	Circular	Convex	Entire	Smooth	Moist	Translucent	White
S_{10}L-16	Circular	Raised	Entire	Smooth	Moist	Opaque	Off white

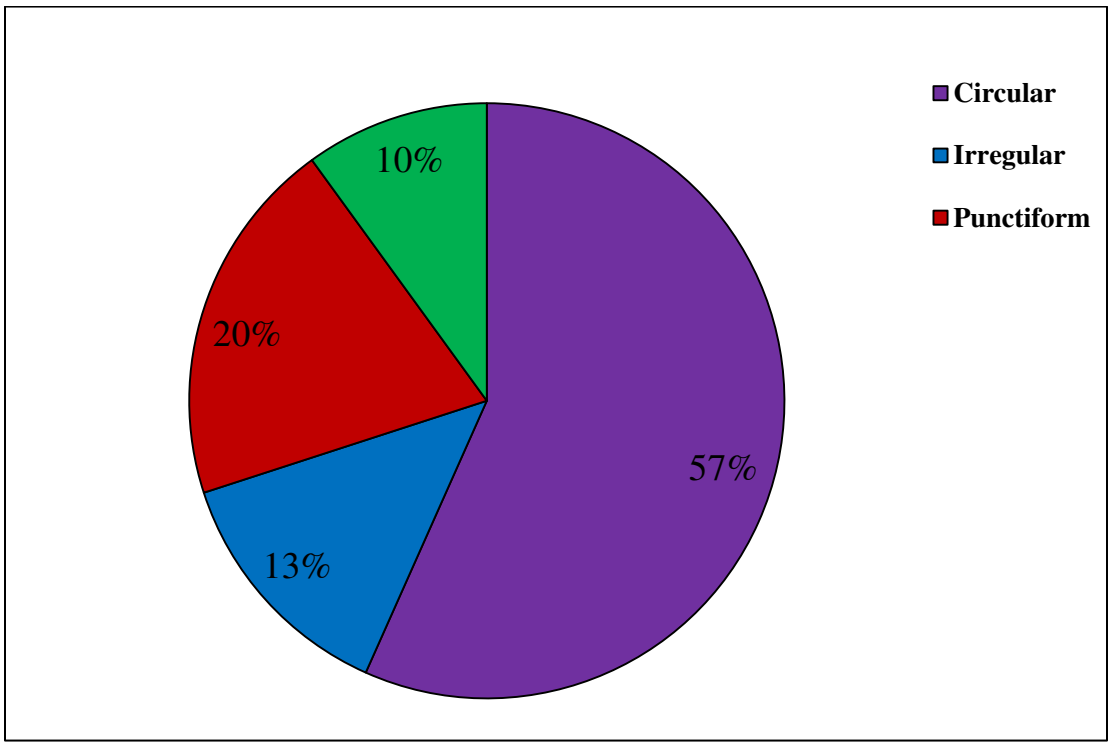


Fig. 3.4: Colony shape of the isolated bacteria.

Table 3.3: Microscopic observation of the selected bacterial isolates.

Bacterial Isolates	Vegetative cell	Spore	Sporangia	Gram reaction
S_1P-7	Rod, occur singly, pair and in chain	Ellipsoidal	Not Swollen	+
S_2P-2	Rod, occur singly, pair and in chain	Ellipsoidal	Not Swollen	+
S_2N-4	Rod, occur singly and in pair	Ellipsoidal	Not Swollen	+
S_3P-1	Rod, occur singly, pair and in chain	Ellipsoidal	Not Swollen	+
S_4P-2	Rod, occur singly, pair and in chain	Ellipsoidal	Not Swollen	+
S_4L-3	Rod, occur singly and in pair	Ellipsoidal	Not Swollen	+
S_6P-4	Rod, occur singly, pair and in chain	Ellipsoidal	Not Swollen	+
S_6P-7	Rod, occur singly, pair and in chain	Ellipsoidal	Not Swollen	+
S_6N-8	Rod, occur singly and in pair	Ellipsoidal	Not Swollen	+
S_7N-5	Rod, occur singly, pair and in chain	Ellipsoidal	Not Swollen	+
S_7P-27	Rod, occur singly, pair and in chain	Ellipsoidal	Not Swollen	+
S_7P-29	Rod, occur singly, pair and in chain	Ellipsoidal	Not Swollen	+
S_7P-32	Rod, occur singly and in pair	Ellipsoidal	Swollen	+
S_7L-25	Rod, occur singly, pair and in chain	Ellipsoidal	Not Swollen	+
S_7L-26	Rod, occur singly, pair and in chain	Ellipsoidal	Not Swollen	+
S_8N-5	Rod, occur singly and in pair	Ellipsoidal	Not Swollen	+
S_8N-6	Rod, occur singly and in pair	Ellipsoidal	Not Swollen	+
S_8P-11	Rod, occur singly, pair and in chain	Ellipsoidal	Not Swollen	+
S_8L-18	Rod, occur singly, pair and in chain	Ellipsoidal	Not Swollen	+
S_9P-2	Rod, occur singly and in pair	Ellipsoidal	Swollen	+
S_{10}L-16	Rod, occur singly	Ellipsoidal	Not Swollen	+
S_2P-3	Short rod, occur singly and in pair	Non spore former		-
S_3N-1	Short rod, occur singly and in pair	Non spore former		-
S_4N-2	Short rod, occur singly	Non spore former		-
S_4L-10	Short rod, occur singly and in pair	Non spore former		-
S_6N-1	Short rod, occur singly and in pair	Non spore former		-
S_6P-3	Short rod, occur singly	Non spore former		-
S_7P-19	Short rod, occur singly and in pair	Non spore former		-
S_8P-2	Short rod, occur singly and in pair	Non spore former		-
S_{10}P-3	Short rod, occur singly and in pair	Non spore former		-

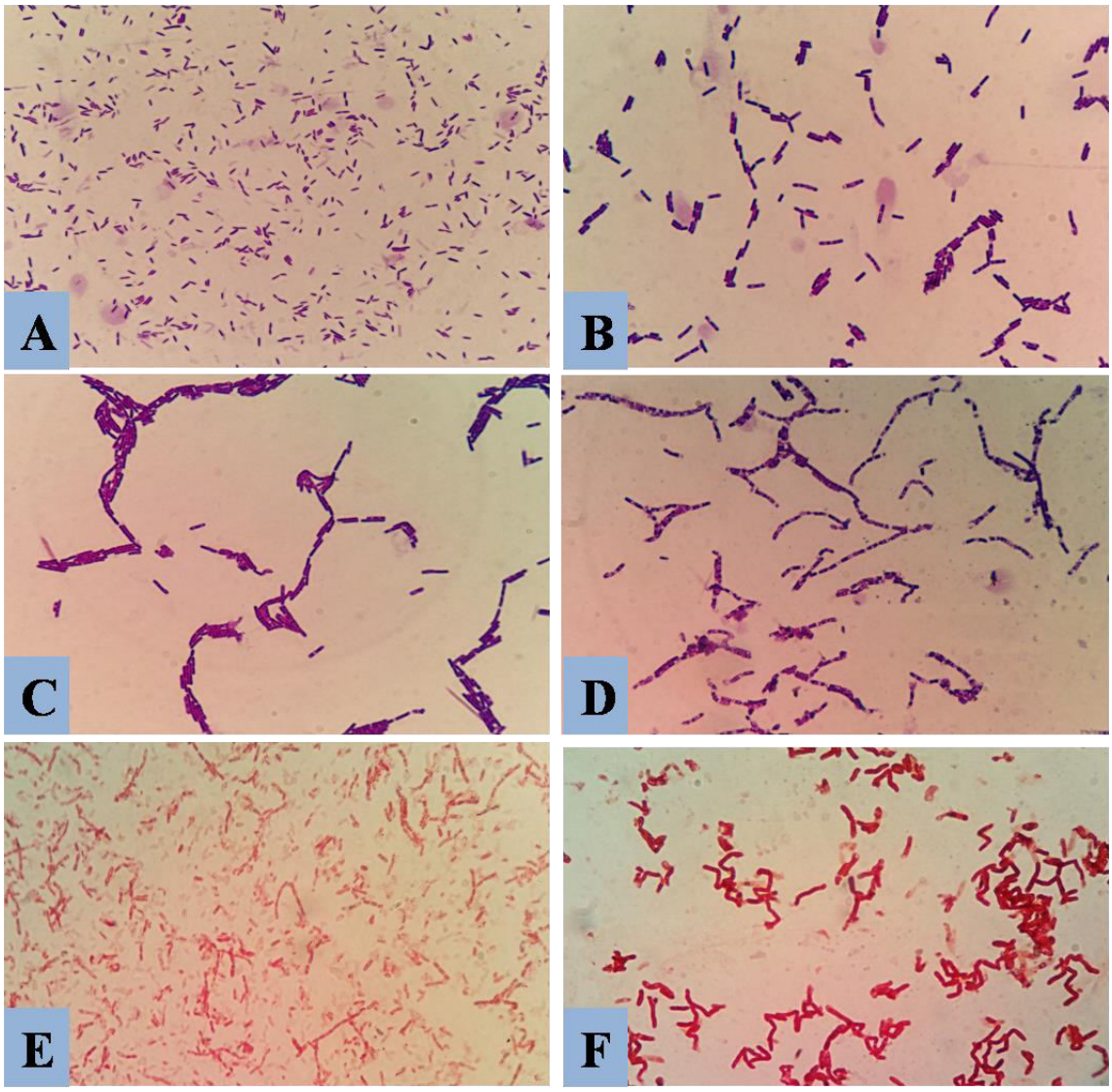


Fig. 3.5: Photomicrographs of some selected Gram stained bacterial isolates. (A-D Gram positive and E-F Gram negative)

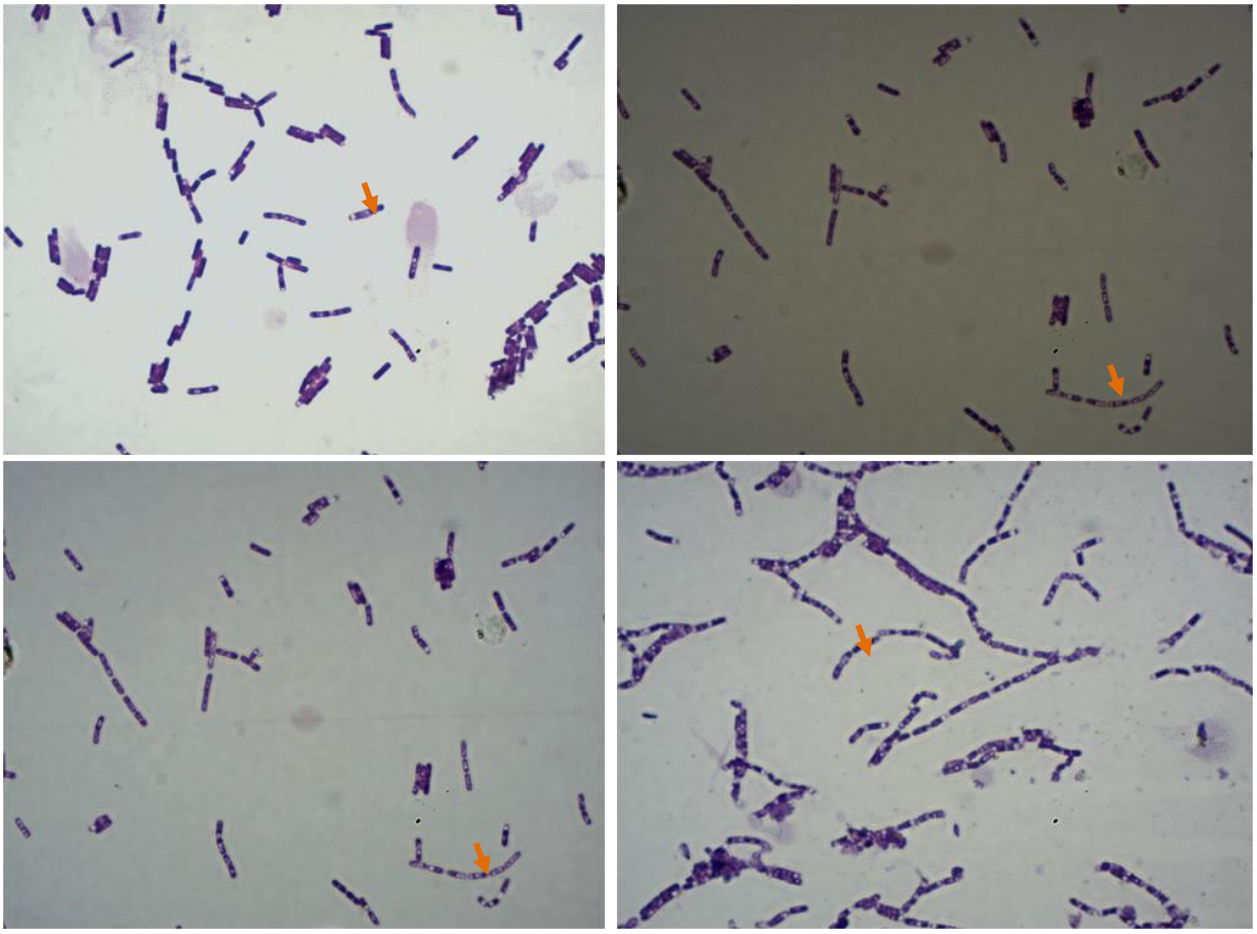


Fig. 3.6: Photomicrographs showing PHB granules in bacterial cell. Arrows indicate PHB granules inside the cell.

3.9 Physiological and biochemical characteristics of the selected isolates

Selected 30 bacterial isolates were studied for their major and important physiological and biochemical characteristics. The results of the fermentation tests with selected carbohydrates were shown in Table 3.4 and Fig.3.7. Among the 30 isolates, ten isolates *viz.* S_2P-2, S_3P-1, S_4N-2, S_4P-2, S_4L-10, S_6P-3, S_6P-7, S_6N-8, S_{10}P-3 and S_{10}L-16 could ferment all the tested carbohydrates. On the other hand the isolates S_8P-11 and S_8L-18 could not ferment any of the tested carbohydrates. Only three isolates S_2P-3, S_8N-5 and S_{10}P-3 could produce gas during glucose fermentation.

The results of some major physiological and biochemical tests were given in Table 3.5-3.7. Among 30 isolates, most were catalase positive and were negative in case of indole except S_2P-3. There are 5 isolates showed positive in oxidase and 17 were positive in nitrate reduction. All showed positive reaction in case of H_2S production except the isolate S_7P-19. Motility test showed that 20 isolates were non motile and 10 were motile.

Table 3.6 showed VP, MR, hydrolysis of casein and starch, and deep glucose agar test. Among 30 bacterial isolates, 14 isolates showed positive result in VP and 17 isolates showed positive result in MR test. Out of 30 isolates, 17 were found to be starch hydrolysis positive and 24 isolates were positive for casein hydrolysis. Most of the isolates showed growth only at the surface of the deep glucose agar medium and thus considered as aerobes. Only three isolates (S_6P-4, S_6P-7 and S_6N-8) were microaerophilic.

Table 3.4: Fermentation tests of the selected carbohydrates.

Bacterial Isolates	Carbohydrates			
	D-Glucose	L-Arabinose	D-Xylose	D-Mannitol
S_1P-7	A	-	-	-
S_2P-2	A	+	+	+
S_2P-3	-	+	+	+
S_2N-4	A	-	-	-
S_3P-1	A	+	+	+
S_3N-1	-	+	+	-
S_4N-2	A	+	+	+
S_4P-2	A	+	+	+
S_4L-3	A	-	+	-
S_4L-10	A	+	+	+
S_6N-1	-	+	+	+
S_6P-3	A	+	+	+
S_6P-4	A	+	+	+
S_6P-7	A	+	+	+
S_6N-8	A	+	+	+
S_7N-5	-	-	+	-
S_7P-19	A	-	-	+
S_7P-27	A	+	+	-
S_7P-29	A	+	-	-
S_7P-32	A	+	-	-
S_7L-25	A	-	+	+
S_7L-26	A	-	+	+
S_8P-2	-	+	+	+
S_8N-5	A	+	-	-
S_8N-6	A	-	-	-
S_8P-11	A	-	-	-
S_8L-18	-	-	-	-
S_9P-2	A	-	+	-
S_{10}P-3	A	+	+	+
S_{10}L-16	A	+	+	+

'+'= positive result, '-'= negative result and A= Acid

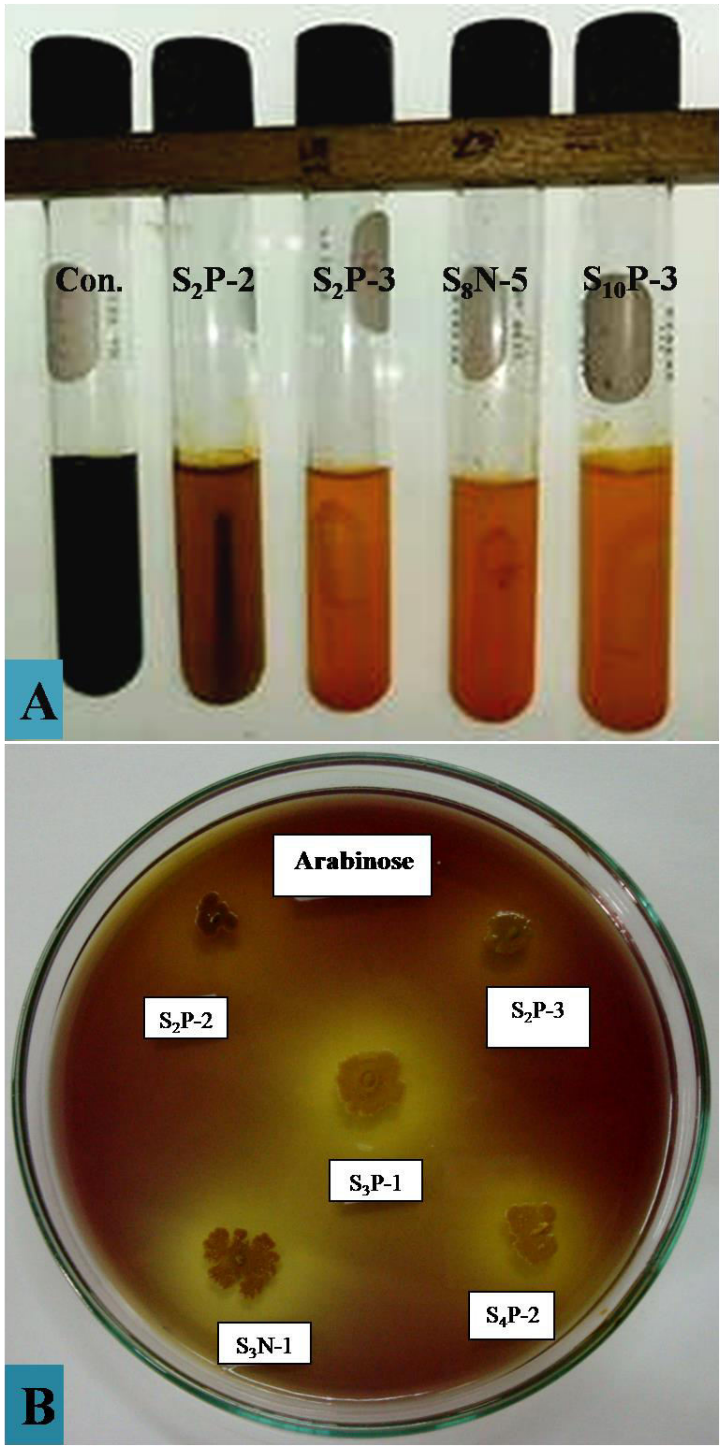


Fig. 3.7: Photographs showing the fermentation test of carbohydrates (A) D-glucose and (B) L-Arabinose.

Table 3.5: Major physiological and biochemical characteristics of the bacterial isolates.

Bacterial Isolates	Catalase	Oxidase	Indole	Nitrate reduction	H_2S production	Motility
S_1P-7	+	-	-	+	-	Non motile
S_2P-2	+	-	-	-	-	Non motile
S_2P-3	+	-	+	+	-	Non motile
S_2N-4	+	-	-	+	-	Motile
S_3P-1	-	-	-	+	-	Motile
S_3N-1	+	+	-	+	-	Non motile
S_4N-2	+	+	-	+	-	Non motile
S_4P-2	+	-	-	+	-	Non motile
S_4L-3	+	+	-	+	-	Non motile
S_4L-10	+	-	-	-	-	Non motile
S_6N-1	+	-	-	-	-	Motile
S_6P-3	+	-	-	-	-	Non motile
S_6P-4	+	-	-	-	-	Non motile
S_6P-7	+	-	-	-	-	Non motile
S_6N-8	+	-	-	-	-	Non motile
S_7N-5	-	-	-	+	-	Motile
S_7P-19	+	-	-	+	+	Non motile
S_7P-27	+	-	-	-	-	Non motile
S_7P-29	+	-	-	+	-	Non motile
S_7P-32	+	-	-	+	-	Motile
S_7L-25	+	-	-	+	-	Motile
S_7L-26	+	+	-	+	-	Non motile
S_8P-2	+	-	-	-	-	Motile
S_8N-5	+	-	-	-	-	Non motile
S_8N-6	+	-	-	-	-	Non motile
S_8P-11	+	-	-	+	-	Motile
S_8L-18	+	+	-	-	-	Motile
S_9P-2	-	-	-	+	-	Non motile
S_{10}P-3	+	-	-	+	-	Motile
S_{10}L-16	+	-	-	-	-	Non motile

'+'= positive result, '-'= negative result

Table 3.6: Some major physiological and biochemical characteristics of the bacterial isolates.

Bacterial Isolates	VP test	MR test	Hydrolysis of Casein	Hydrolysis of Starch	Deep glucose agar test
S_1P-7	-	+	+	+	Facultative anaerobe
S_2P-2	-	+	+	-	Obligate aerobe
S_2P-3	+	-	+	+	Facultative anaerobe
S_2N-4	+	+	+	-	Facultative anaerobe
S_3P-1	+	-	-	+	Facultative anaerobe
S_3N-1	-	+	-	+	Obligate aerobe
S_4N-2	-	-	+	-	Obligate aerobe
S_4P-2	+	+	+	+	Obligate aerobe
S_4L-3	-	+	+	+	Facultative anaerobe
S_4L-10	-	-	-	-	Facultative anaerobe
S_6N-1	-	-	-	-	Obligate aerobe
S_6P-3	-	-	-	-	Facultative anaerobe
S_6P-4	+	+	+	-	Microaerobic
S_6P-7	+	+	+	-	Microaerobic
S_6N-8	+	+	+	-	Microaerobic
S_7N-5	-	+	+	+	Facultative anaerobe
S_7P-19	-	-	+	-	Obligate aerobe
S_7P-27	+	-	+	+	Obligate aerobe
S_7P-29	-	+	+	+	Facultative anaerobe
S_7P-32	+	+	+	+	Obligate aerobe
S_7L-25	+	-	+	+	Obligate aerobe
S_7L-26	+	-	+	+	Obligate aerobe
S_8P-2	-	-	+	-	Obligate aerobe
S_8N-5	-	+	+	-	Facultative anaerobe
S_8N-6	+	+	+	+	Facultative anaerobe
S_8P-11	-	+	+	+	Facultative anaerobe
S_8L-18	+	+	+	+	Facultative anaerobe
S_9P-2	-	-	+	+	Facultative anaerobe
S_{10}P-3	+	-	-	-	Facultative anaerobe
S_{10}L-16	-	+	+	+	Obligate aerobe

'+'= positive result, '-'= negative result

Some of the major physiological tests of the selected isolates are shown in the Table 3.7. Among the isolates 10 showed positive in citrate and 2 showed positive in propionate utilization. Eight isolates showed positive results in tyrosine degradation and 13 positive in lecithinase production. Among 9 Gram negative isolates, 3 isolates (S_4N-2, S_7P-19 and S_{10}P-3) showed positive results in arginine dihydrolysis. Fig. 3.8 and 3.9 showed different physiological and biochemical test of the isolated bacteria. A comparative observation between different physiological and biochemical tests results of the selected isolates was shown in Fig. 3.10.

3.10 Identification of the isolates

After thorough characterization bacterial isolates were considered for both provisional and molecular identification.

3.10.1 Provisional identification

Consulting all morphological, biochemical and physiological characters of the isolates, provisional identification was done with the help of Bergey's Manual of Systematic Bacteriology Vol.1 (Krieg and Holt 1984) and Bergey's Manual of Systematic Bacteriology Vol.2 (Sneath *et al*. 1986). Provisionally identified Gram positive bacteria are shown in Table 3.8. All the Gram positive isolates belonged to the single genus *Bacillus*. The genus *Bacillus* comprises of various species matched with the standard species described in the Bergey's Manual of Systematic Bacteriology and the selected isolates were provisionally identified on the basis of resemblance to that of standard strains. The genus *Bacillus* comprises many species and the selected isolates were provisionally identified on the basis of resemblance to that of standard strains.

Table 3.7: Some major physiological and biochemical characteristics of the bacterial isolates.

Bacterial Isolates	Citrate utilization	Propionate utilization	Tyrosine degradation	Lecithinase production	Arginine dihydrolysis
S_1P-7	-	-	-	-	ND
S_2P-2	-	-	-	-	ND
S_2P-3	+	-	+	-	-
S_2N-4	-	-	-	+	ND
S_3P-1	+	-	-	-	ND
S_3N-1	+	-	+	+	-
S_4N-2	+	+	+	+	+
S_4P-2	-	-	-	+	ND
S_4L-3	-	-	-	-	ND
S_4L-10	+	-	+	-	-
S_6N-1	+	-	+	-	-
S_6P-3	+	-	+	-	-
S_6P-4	-	-	-	+	ND
S_6P-7	-	-	-	+	ND
S_6N-8	-	-	-	+	ND
S_7N-5	-	-	-	-	ND
S_7P-19	+	+	+	+	+
S_7P-27	-	-	-	-	ND
S_7P-29	-	-	-	-	ND
S_7P-32	-	-	-	+	ND
S_7L-25	-	-	-	+	ND
S_7L-26	-	-	-	+	ND
S_8P-2	+	-	+	-	+
S_8N-5	-	-	-	-	ND
S_8N-6	-	-	-	-	ND
S_8P-11	-	-	-	-	ND
S_8L-18	-	-	-	+	ND
S_9P-2	-	-	-	-	ND
S_{10}P-3	+	-	-	-	+
S_{10}L-16	-	-	-	+	ND

'+'= positive result, '-'= negative result

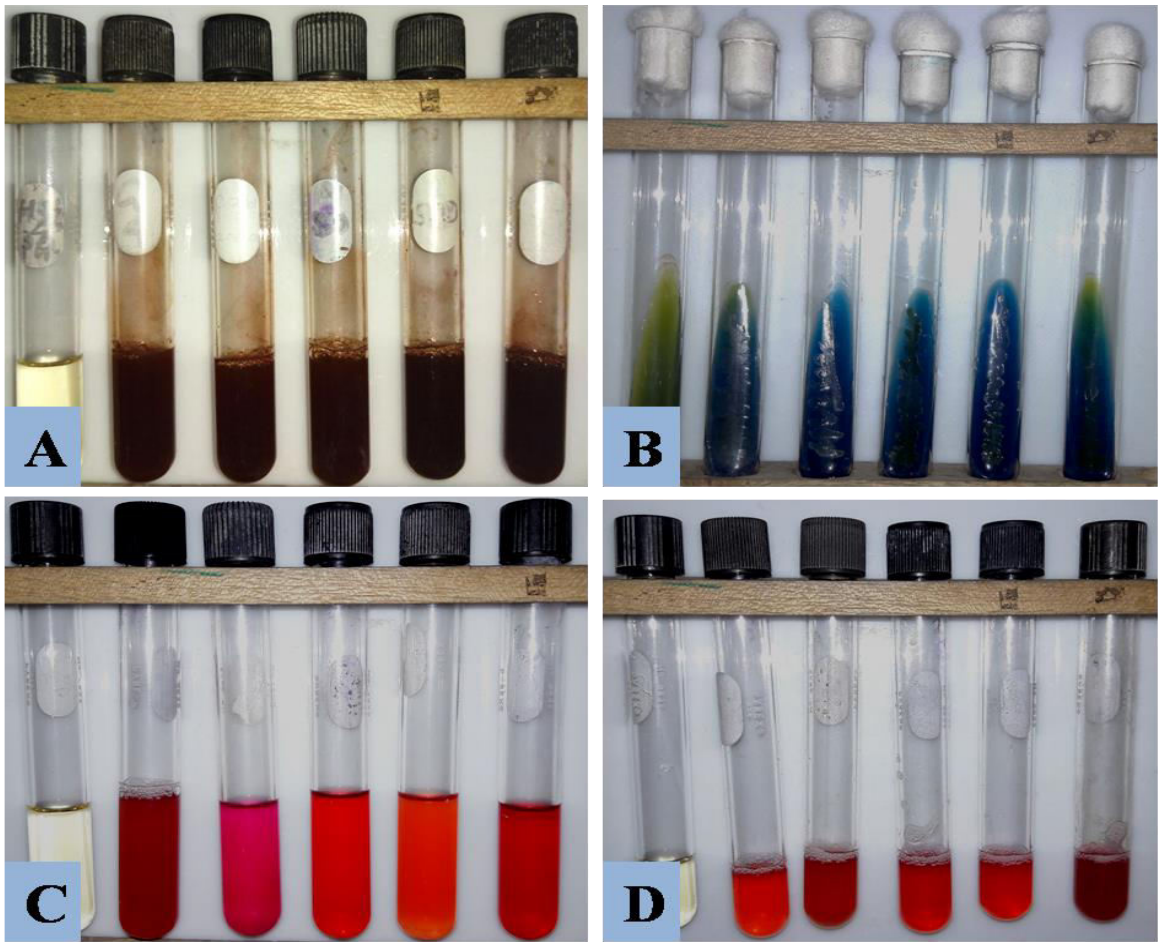


Fig. 3.8 (A-D): Photographs showing some physiological and biochemical tests. (A) Nitrate Reduction test, (B) Citrate test, (C) MR test and (D) VP test.

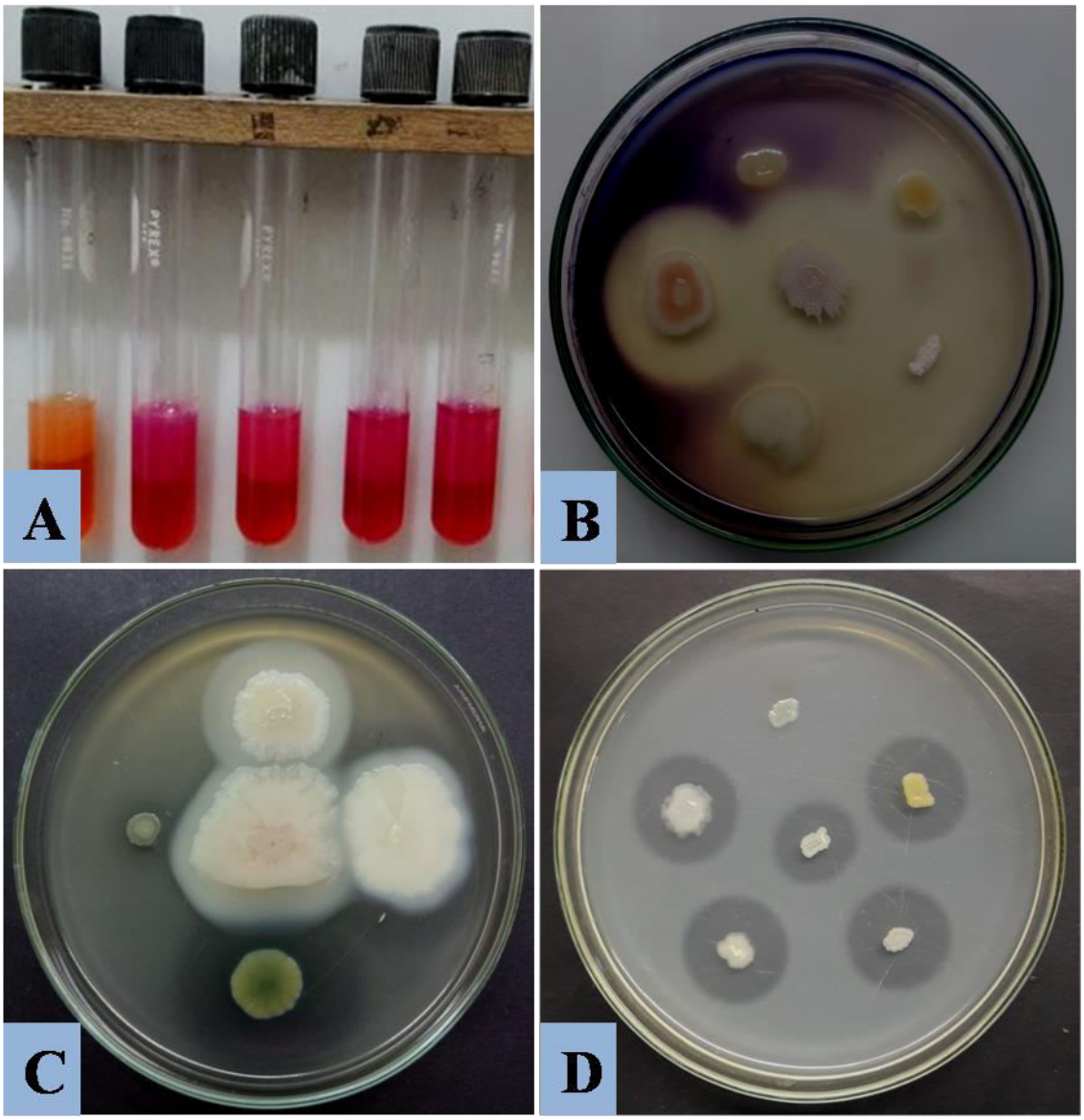


Fig. 3.9 (A-D): Photographs showing some physiological and biochemical tests. (A) Arginine dihydrolysis, (B) Hydrolysis of Starch, (C) Egg yolk lecithinaseand (D) Hydrolysis of Casein.

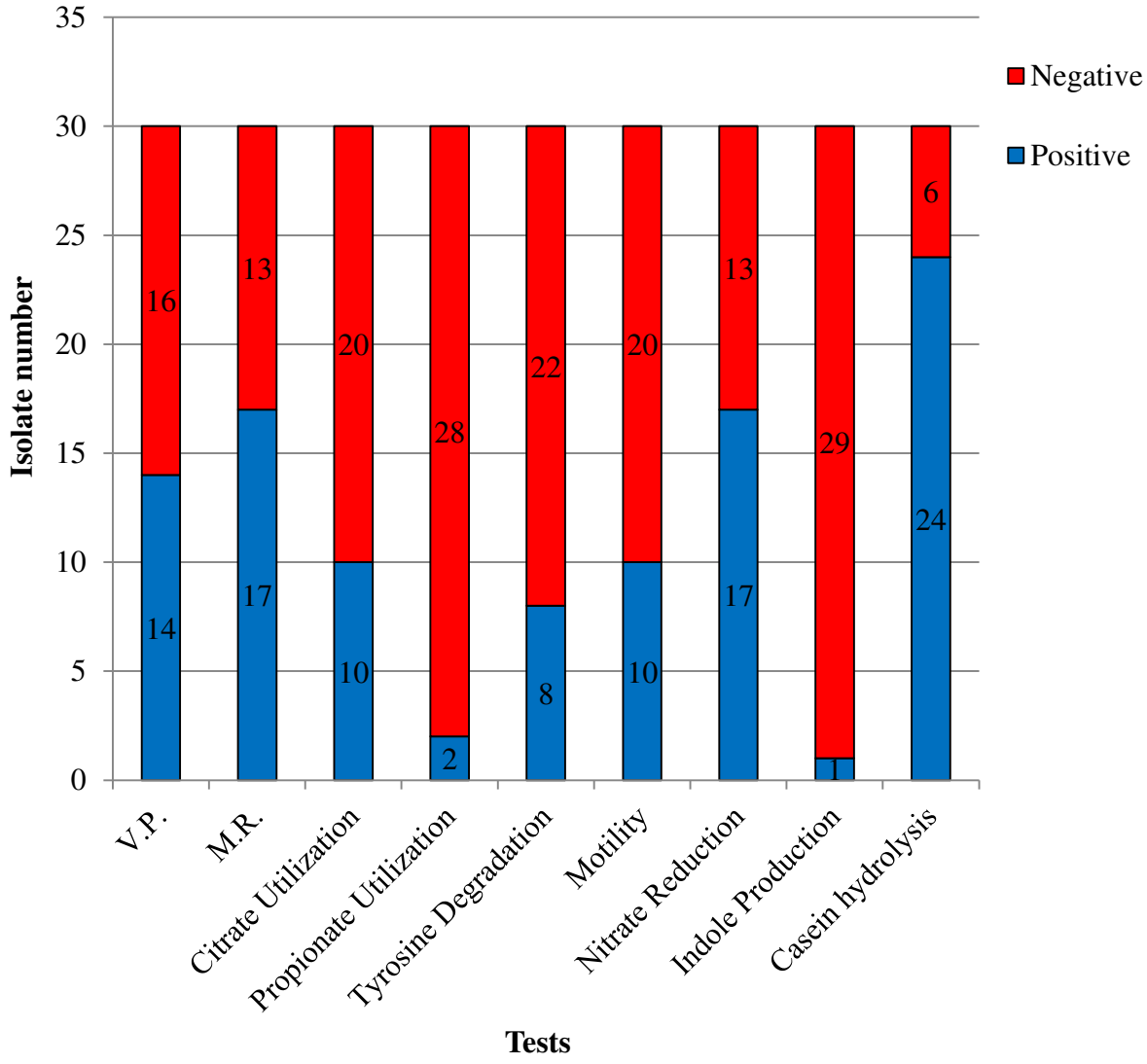


Fig. 3.10: Physiological and biochemical test results of the selected isolates.

Table 3.8: Provisionally identified Gram positive bacterial isolates.

Bacterial Isolates	Source of isolates	Sample type	Provisionally identified name
S_1P-7	Dairy Farm, Savar, Dhaka	Soil	*Bacillus anthracis*
S_2P-2	The Bangshi River, Savar, Dhaka	Water	*B. mycoides*
S_2N-4	The Bangshi River, Savar, Dhaka	Water	*B. cereus*
S_3P-1	The Turag River, Gabtoli, Dhaka	Water	*B. pumilus*
S_4P-2	The Buriganga River, Dhaka	Water	*B. subtilis*
S_4L-3	The Buriganga River, Dhaka	Water	*B. thuringiensis*
S_6P-4	Dumping waste, Gabtoli, Dhaka	Soil	*B. megaterium*
S_6P-7	Dumping waste, Gabtoli, Dhaka	Soil	*B. megaterium*
S_6N-8	Dumping waste, Gabtoli, Dhaka	Soil	*B. lentus*
S_7N-5	Kitchen waste dumpsite, Uttara, Dhaka	Soil	*B. cereus*
S_7P-27	Kitchen waste dumpsite, Uttara, Dhaka	Soil	*B. mycoides*
S_7P-29	Kitchen waste dumpsite, Uttara, Dhaka	Soil	*B. anthracis*
S_7P-32	Kitchen waste dumpsite, Uttara, Dhaka	Soil	*B. schlegelii*
S_7L-25	Kitchen waste dumpsite, Uttara, Dhaka	Soil	*B. pumilus*
S_7L-26	Kitchen waste dumpsite, Uttara, Dhaka	Soil	*B. subtilis*
S_8N-5	BDR market, Uttara, Dhaka	Soil	*B. anthracis*
S_8N-6	BDR market, Uttara, Dhaka	Soil	*B. mycoides*
S_8P-11	BDR market, Uttara, Dhaka	Soil	*B. thuringiensis*
S_8L-18	BDR market, Uttara, Dhaka	Soil	*B. cereus*
S_9P-2	Garments waste, Fotulla, Dhaka	Water	*B. schlegelii*
S_{10}L-16	Balur Math, Kazla, Dhaka	Soil	*B. megaterium*

They belonged to *Bacillus anthracis, B. cereus, B. mycoides, B. megaterium, B. schlegelii, B. subtilis, B. pumilus, B. thuringiensis and B. lentus*. Isolated seven Gram negative bacteria were *Acetobacter, Enterobacter, Klebsiella, Neisseria, Pseudomonas, Rhizobium* and *Tatumella* (Table 3.9). Among Gram negative bacteria *Acetobacter* and *Tatumella* were the dominating genus. Fig. 3.11 shows the percentage frequency of the identified bacterial isolates.

3.10.2 Molecular Identification of the selected bacterial isolates

Among 30 bacterial isolates, potential 10 PHB producers were selected for molecular identification. Using a pair of bacterial universal primer the 16S rRNA gene was amplified from ten better PHB production activity showing bacterial isolates. The PCR amplified DNA of the ten isolates (S_7N-5= lane 1, S_7P-29= lane 2, S_2N-4= lane 3 and S_{10}L-16= lane 4, S_8P-2= lane 5, S_8N-6= lane 6, S_8P-11= lane 7, S_4P-2= lane 8, S_4L-3= lane 9 and S_8L-18= lane 10) and 100 bp ladder (lane M) were gel purified (Fig. 3.12) and automatically sequenced. The sequences obtained (Fig. 3.13 – 3.22) were used for BLAST and rRNA BLAST search to find out possible correct match present in the data mentioned before.

Followed by genomic DNA extraction from 10 isolates, primers CC (F) and CD(R) were used to amplify 16S rRNA sequences from bacterial genomic DNA. PCR products were sequenced and BLAST (NCBI) searches were conducted on the DNA sequence to compare the different 16S rRNA sequences and determine the most closely related species. Molecular identification of 10 bacterial isolates is shown in Table 3.10.

Table 3.9: Provisionally identified Gram negative bacterial isolates.

Bacterial Isolates	Source of isolates	Sample type	Provisionally identified name
S_2P-3	The Bangshi River, Savar, Dhaka	Water	*Klebsiella oxytoca*
S_3N-1	The Turag River, Gabtoli, Dhaka	Water	*Neisseria elongata*
S_4N-2	The Buriganga River, Dhaka	Water	*Acetobacter liquefaciens*
S_4L-10	The Buriganga River, Dhaka	Water	*Tatumella ptyseos*
S_6N-1	Dumping waste, Gabtoli, Dhaka	Soil	*Acetobacter aceti*
S_6P-3	Dumping waste, Gabtoli, Dhaka	Soil	*Tatumella ptyseos*
S_7P-19	Kitchen waste dumpsite, Uttara, Dhaka	Soil	*Pseudomonas aeruginosa*
S_8P-2	BDR market, Uttara, Dhaka	Soil	*Rhizobium leguminosarum*
S_{10}P-3	Balur Math, Kazla, Dhaka	Soil	*Enterobacter cloaceae*

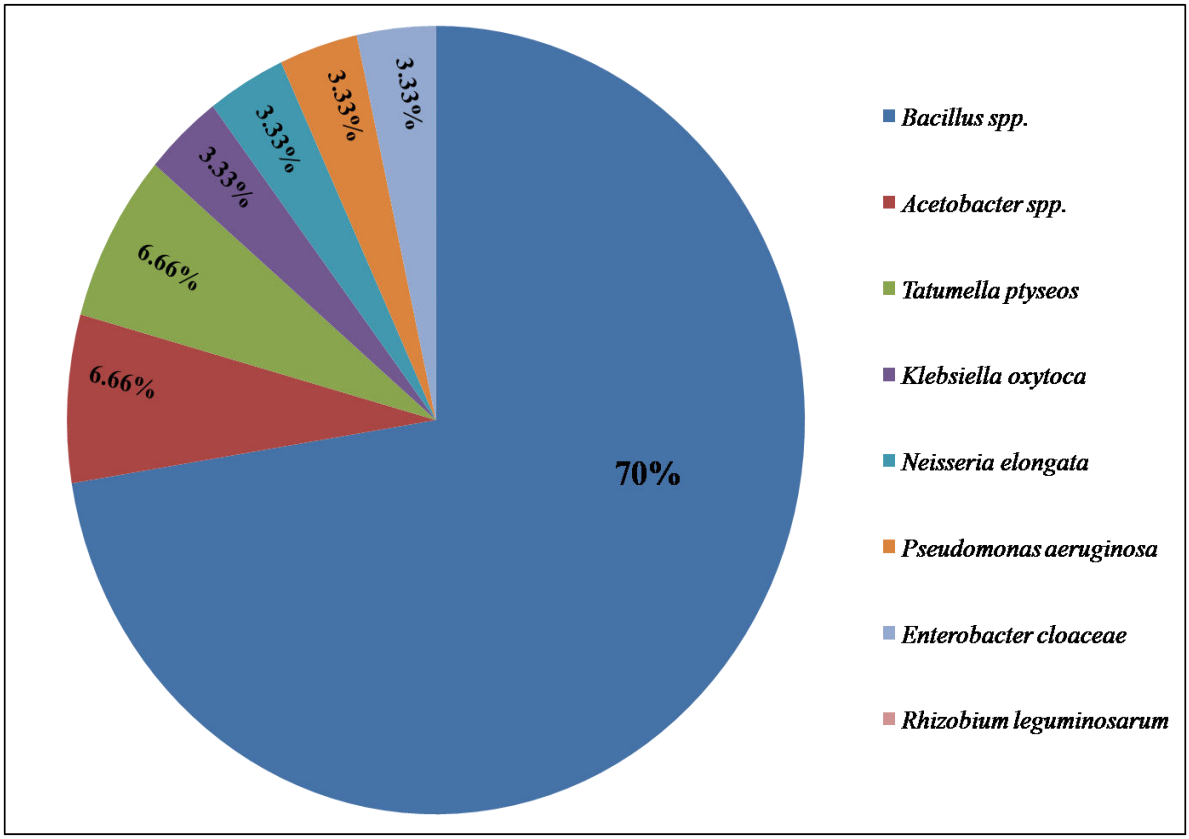


Fig. 3.11: Percentage frequency of the identified bacterial isolates.

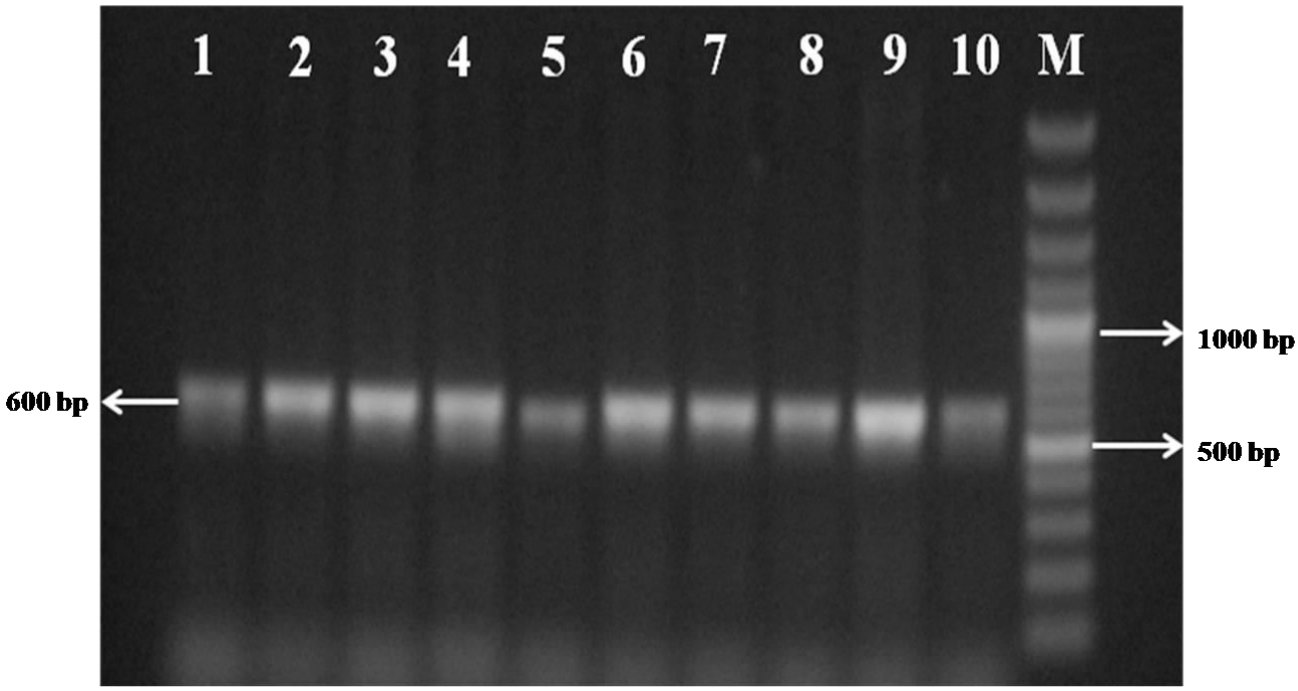


Fig. 3.12: PCR amplification of part of the 16S rRNA gene.

Lanes 1-10 are representing 10 different bacterial isolates *viz.*S_2N-4, S_4P-2, S_4L-3, S_7N-5, S_7P-29, S_8P-2, S_8N-6, S_8P-11, S_8L-18, S_{10}L-16 and M=100 bp ladder. In the gel size of the amplified DNA bands were approximately 600 bp.

TCTCTAGCTCTCTCGGAGCAGCGAAGGAATCTTCCCCAAGGACAAAATTCGC
GGAGCCACGCCCGTTGTTATTAAGCCTTTGGGTTGTAAAACCCTGTTGTTAGG
GAAGAACAAAGTCCAGTTGATATAAGCGGCCACCTTGACGGGTACCTAAAC
AAGAAGGCACGGGCTAAATACTGTCCCAGAGGCCCGCGTTATACTAAGTGTG
CCAACCGTAACCCGGAAATATTGGCCGTAAACCGCGCGCAGGTTGGTTTTTT
AAGTTCTGATGGGAAAGCCCCACCGCTCAACCGGTGGAGGGTCATTGGAAA
CTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTG
AAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCT
GTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAAAAGAGTTTCCGC
CCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGCTAGCACGTCCGTC
ATGGATTAG

Fig. 3.13: 16S rRNA partial sequence of isolate S_2N-4.

TCTGAGCTCTCTCGGAAGCACCATAGGGAATCTTCCGCAAGGACGAAAGTCT
GACGGAGCAACGCCGCGGAGTGAGAAAGTTTTCGGATCGTAAGCTTCGTTGT
TAGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTACCA
GAAAGCCACGGGCTAATTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCA
AGCGTTGTCCGGAATTATTGGGCGAAAGGGCTCGCAGGCGGTTTCTTAAGTC
TGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAAC
TTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAG
AGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACG
CTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCT
GCAGCTAACGCATTAAGCATCCGCCTGGTTAGTAGAGTCTCACAACATTGCG

Fig. 3.14: 16S rRNA partial sequence of isolate S_4P-2.

TCCAGACTTCTTCGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAG
TCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTC
TGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTA
CCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTT
TCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAA
CTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTG
AAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCT
GTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGTGGTTTCCGCCC
TTTAGTGCTGAAGTTAACGCATAAGCACTCCGCCTGGGAGCACGTCCGTAAG
TATGATG

Fig. 3.15: 16S rRNA partial sequence of isolate S_4L-3.

TCCAATCTCTCGAGCACGTTGGATTCTCCCAATGGCGAATTCGACGAGCACG
CCGCGTGATGATGAAGCTTTCGGGTCGTAAACTCTGTGTTAGGGAAAAACAA
TGCTAGTGGATAAAGTGGCACCTTGCCGGACCTTAACCGAAAAGCCACGGCT
AATTCTGTCCACCAGCCCGCGGTATACGTAGTGGCCAACCGTAATCGGAATT
ATTGGGGCGTAAAAGGCCGCGCCAGGTGGTTTCTTAAATTCTGATGTGAAAG
CCCCACGGCTCCAACCCGTGGAGGGTCATTGAAACTGGGAGACTTGAGGTGC
AGAAGAGGAAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATAT
GGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGG
CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCAGGTAGTCCACGCCG
TAAACGATGAGTGCTACTGTGTTAGGAAAGGTCCGCCCTTTAGTGCTGAAGT
AACGCATAAGCACTCCGCCTGGGATACTTCTTTATGTCTCTTTGG

Fig. 3.16: 16S rRNA partial sequence of isolate S_7N-5.

TCCTCACATCTCTCTCGACTAGTGAGAACTTCCCATGGCACAAATTCGCCGAG
CACGCCGCTGATGATGAAGGCTTTCGGGTCGAAAATTCTGTTGTTAGGGAGG
ACCAGGGCTAGTGGATTAACTGGCCACCCTTGACGGAACTAACCCGAAAGGC
CACGGCCTAACTTCGGGGCAAGAAGCCGCGGTAAATAACGTAGGTGGCAAA
CGTTATCCCGGAAATTATTGGGCGTAAAGCGCGCCGCAGGTTGGTTTCTAAA
TTCTGATGTGAAAAGCCCACCGGCTCAACCGGGGGAGGGGTCATTGGAAACT
GGGAGGACTTGAGTGCAGAAGAGGAAAGGGGAATTCCATGTGTAGCGGTGA
AATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTG
TAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTCCGCCCT
TTAGTGCTGAAGTAACGCATAAGCATCCGCCTGGTACTACACCGTAAACGGT
TTAGA

Fig. 3.17: 16S rRNA partial sequence of isolate S_7P-29.

CCCAGACTTCCCTCGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAA
GCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCT
CTTTCACCGATGAAGATAATGACGGTAGTCGGAGAAGAAGCCCCGGCTAACT
TCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTAC
TGGGCGTAAAGCGCACGTAGGCGGATATTTAAGTCAGGGGTGAAATCCCAGA
GCTCAACTCTGGAACTGCCTTTGATACTGGGTATCTCGAGTATGGAAGAGGT
GAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACC
AGTGGCGAAGGCGGCTCACTGGTCCATTACTGACGCTGAGGTGCGAAAGCGT
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAAT
GTTAGCCGTCGGGCAGCTTGCTGTTCGGTGGCGCAGCTAACGCATAAACATC
CGCCTGGGGAGTAGCGTCGCAAGTTAGTTAGG

Fig. 3.18: 16S rRNA partial sequence of isolate S_8P-2.

TCCAAGACTTTCTACGGGAGGCAGCAGTTAGGGAATTCTCCGCAAATGGACG
AAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAA
ACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACG
GTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGG
TTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAA
ACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTG
AAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCT
GTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCT
TTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGAGCACGGCCGGAAA
GGGGTGATA

Fig. 3.19: 16S rRNA partial sequence of isolate S_8N-6.

TCCAGACTCCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAA
GTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACT
CTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTA
CCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA
GGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTC
TTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTG
GGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAAT
GCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAA
CTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGTGTTTTCCGCCCTTTAGT
GCTGAAGTTAACGCATTAAGCATCCGCCTGGGAGCACGGCCTCAGGGGATTG
ACGG

Fig. 3.20: 16S rRNA partial sequence of isolate S_8P-11.

TCCAGACTCCCTCGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAG
TCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCT
GTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACC
TAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
TGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTT
AAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGG
GAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATG
CGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAAC
TGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGTGTTTTCCGCCCTTTAGTG
CTGAAGTTAACGCATTAAGCACTCCGCCTGGGAGCACGTCCGTCACGTGATTC
CGG

Fig. 3.21: 16S rRNA partial sequence of isolate S_8L-18.

TCCCACCATAGAATTTTCCCATGGAAAATTTAGGGAAACCCCTGAGTATAAAC
CTTTCGGTTTTAAAATTTTTTTTGGGAGACCAATCCCGAGGTAATTGTTTTACC
TTACCGTACTAACCCGAAAACCCCCGCGTAATTTCGTGCCACCAGCCCCGGTA
ATTCTATGTGTCAAAGCTTATTCCGAATTTATTGGGCTTAAACGCGCGGCCGG
CGGTTTTCTTAAGTCTAATGAAAACCCCACGGCTCCACCCTTGGAGGGTCCAT
TGAAAACTGGGGAACTTGAGTGCAAAAGAGAAAAAGCGGAATTCCACGTGTA
GCGGTGAAATGCGTAGAGATGTGGAGGAACACCCAGTGGCGAAGGCGGCTTT
TTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGAGTTCTAAGTGTTAGAGGGTTC
CGCCCTTTTAGTGCTGCAGCTAACGCATAAGCATCCGCCTGGGACCAGCTTCG
CCAAGGGTTGGGC

Fig. 3.22: 16S rRNA partial sequence of isolate S_{10}L-16.

Table 3.10: Molecular identification of selected bacterial isolates.

Bacterial isolates	Molecular identification			
	Scientific name	Strain	Max score	Identity match (%)
S_2N-4	*Bacillus cereus*	HB45	680	93
S_4P-2	*Bacillus subtilis*	NXUSASNFB008	913	97
S_4L-3	*Bacillus thuringiensis*	B43	1007	99
S_7N-5	*Bacillus cereus*	AK9	784	95
S_7P-29	*Bacillus anthracis*	S2CB42	691	91
S_8P-2	*Sinorhizobium* sp.	R25067	961	98
S_8N-6	*Bacillus mycoides*	TCCC11292	1007	98
S_8P-11	*Bacillus cereus*	WCF2	1013	99
S_8L-18	*Bacillus cereus*	PU	1007	99
S_{10}L-16	*Bacillus megaterium*	LY6	913	97

3.11 Comparative analysis between provisional and molecular identification

A comparative analysis in between provisional and molecular identification of 10 isolates was shown in Table 3.11. Among 10 isolates, 9 genera were matched with their provisional identification. Molecularly identified *Sinorhizobium* sp. R25067 differs with its provisional identification. It was provisionally identified as *Rhizobium leguminosarum.* The result revealed that the provisional identification based on morphological, physiological and biochemical characters were still found to be valid to some extent.

Table 3.11: Comparative analysis of provisional and molecular identification of selected bacterial isolates.

Bacterial isolates	Provisional identification	Molecular identification
S_2N-4	*Bacillus cereus*	*Bacillus cereus*
S_4P-2	*Bacillus subtilis*	*Bacillus subtilis*
S_4L-3	*Bacillus thuringiensis*	*Bacillus thuringiensis*
S_7N-5	*Bacillus cereus*	*Bacillus cereus*
S_7P-29	*Bacillus anthracis*	*Bacillus anthracis*
S_8P-2	*Rhizobium leguminosarum*	*Sinorhizobium* sp.
S_8N-6	*Bacillus mycoides*	*Bacillus mycoides*
S_8P-11	*Bacillus thuringiensis*	*Bacillus cereus*
S_8L-18	*Bacillus cereus*	*Bacillus cereus*
S_{10}L-16	*Bacillus megaterium*	*Bacillus megaterium*

3.12 Phylogenetic tree

A phylogenetic tree among ten isolates (Fig. 3.23) were generated using neighbor joining (NJ) distance based algorithm of phylogenetic analysis based on 16S rRNA sequences previously acquired. In the phylogenetic tree it could be seen that the closely related bacterial strains were grouped together while strains having distant relationships were placed separately.

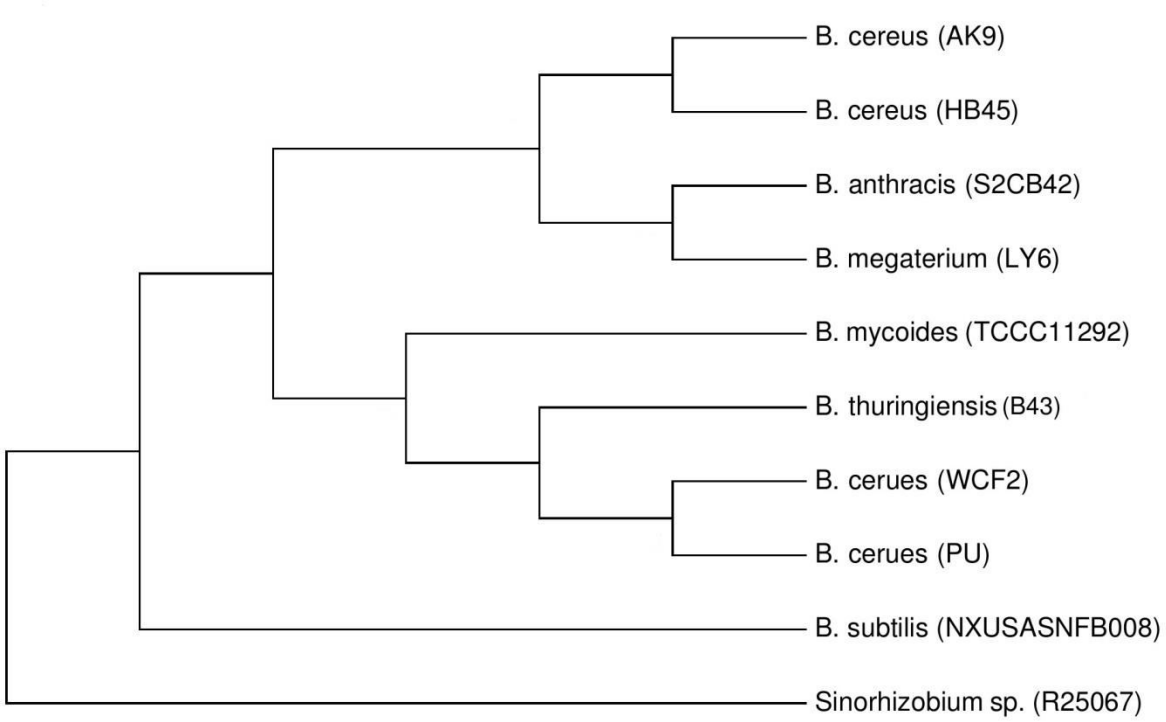


Fig. 3.23: Neighbor-joining tree of 10 bacterial isolates.

3.13 Evaluation of PHB activity

The intracellular PHB was extracted using sodium hypochlorite. This reagent digests non PHB biomass (i.e., all the cellular materials except for PHB) which sediments PHB as white powder. The extracted PHB was purified. PHB was insoluble in the solvents used for purification and precipitated as white opaque powder. The samples from production medium for PHB accumulation were extracted. The extracted PHB samples were quantified as per protocol.

3.13.1 Quantification of PHB

A standard curve was developed with absorbance from commercially available crotonic acid (Fig. 3.24). There was a linear between the amount of crotonic acid and the corresponding absorbance which resulted in a straight line with an equation $y = mx+c$ where "y" is the absorbance plotted in the y axis, m is the slope of the line, "x" is the amount of crotonic acid (which will be equivalent to PHB) in μg plotted in the x axis and "c" is a constant. This was used as a standard to determine the concentration of the unknown PHB from samples.

3.13.2 Estimation of PHB activity of the selected isolates

The potentiality of 10 better PHB activity showing isolates was examined for PHB producing capability. The comparative study of the isolates for PHB production was carried out in NA medium at 37°C. PHB production and estimation of PHB activity was shown in Fig. 3.25 and Fig. 3.26. The potentiality of 10 better PHB activity showing bacterial isolates (9 Gram positive and 1 Gram negative) was examined for

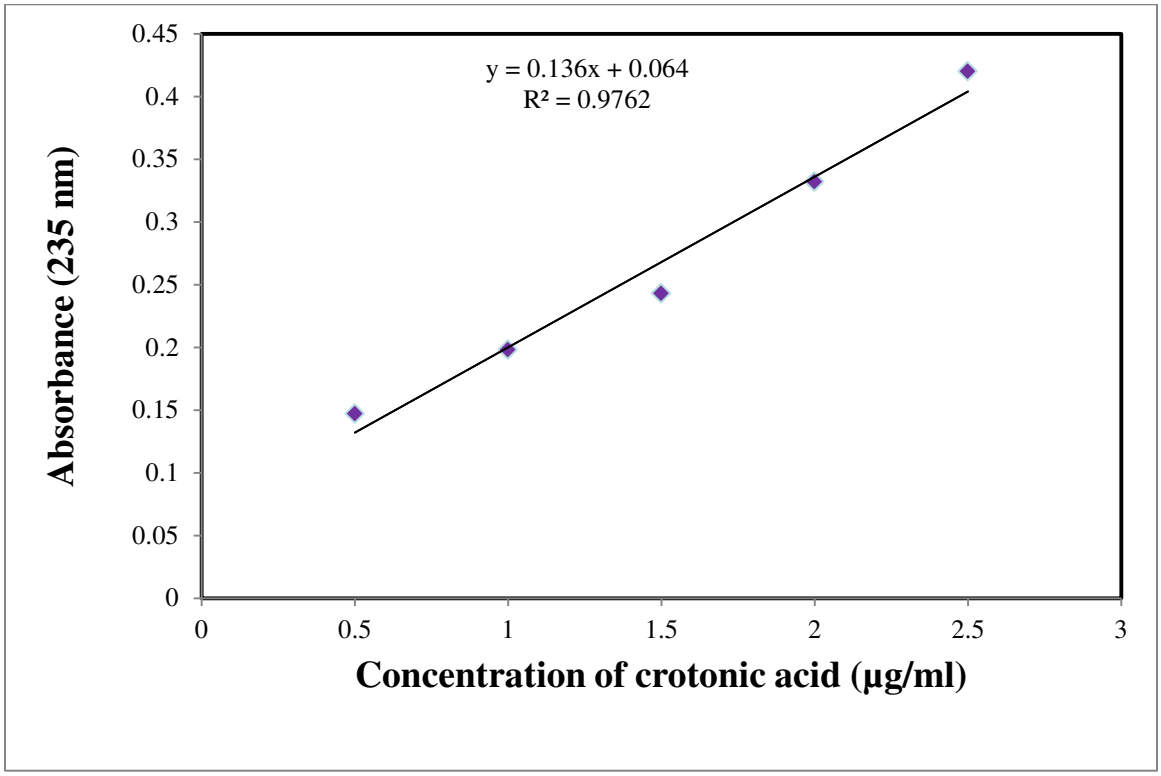


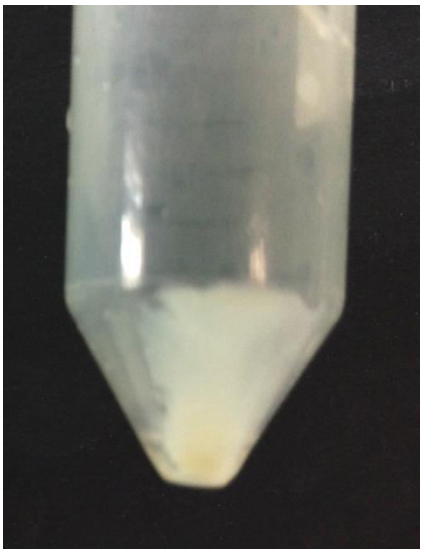
Fig. 3.24: Standard curve of crotonic acid.

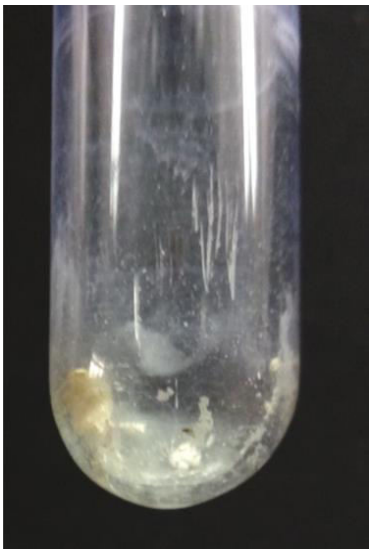
PHB production. The PHB producing capabilities of Gram positive isolates ranged in between 1.94 ± 0.86 and79.75 ± 6.10 µg/ml and Gram negative isolate *Sinorhizobium* sp. R25067 could produce 5.88 ± 0.83 µg/ml of PHB after 72 h of incubation at pH 7. The Gram positive *Bacillus cereus* strain HB45 was found to be the most potent isolate for PHB production which could produce PHB up to 79.75 ± 6.10 µg/ml at 72 h of incubation.

PHB activity of 10 bacterial isolates is shown in Fig. 3.27. The PHB yield analysis showed that there was the highest accumulation of PHB at 72 h of incubation by all the isolates except *Bacillus megaterium* LY6. *Bacillus cereus* HB45, *Bacillus thuringiensis* B43, *Bacillus anthracis* S2CB42 and *Bacillus mycoides* TCCC11292 showed maximum PHB activity at 72 h of incubation and then PHB activity gradually decreases. The time of incubation showed sharp decrease in PHB activity after 72 h. This reduction in PHB production after 72 h might be due to lack of micronutrients as well as increase in metabolites that might have negative effect on the PHB production.

Among the 10 bacterial isolates, 2 isolates *viz. Bacillus cereus* HB45 and *Bacillus thuringiensis* B43 showed PHB activity higher than 70 µg/ml and were chosen for further study towards optimization of PHB production, FTIR analysis and bioplastic production.

(A) Production of Polyhydroxybutyrate (PHB)





(B) Cell pellet after centrifugation **(C) Crude PHB**

Fig. 3.25 (A-C): Polyhydroxybutyrate (PHB) production.

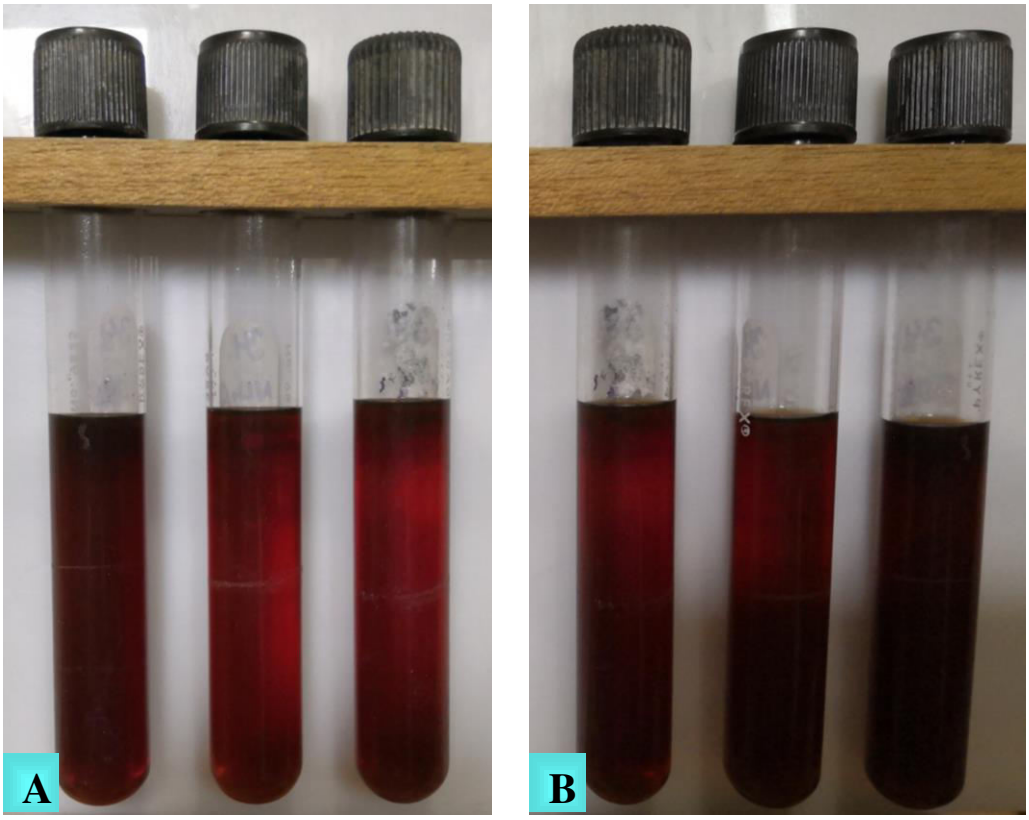


Fig. 3.26: Estimation of PHB.

(A) *Bacillus cereus* HB45 and (B) *Bacillus thuringiensis* B43.

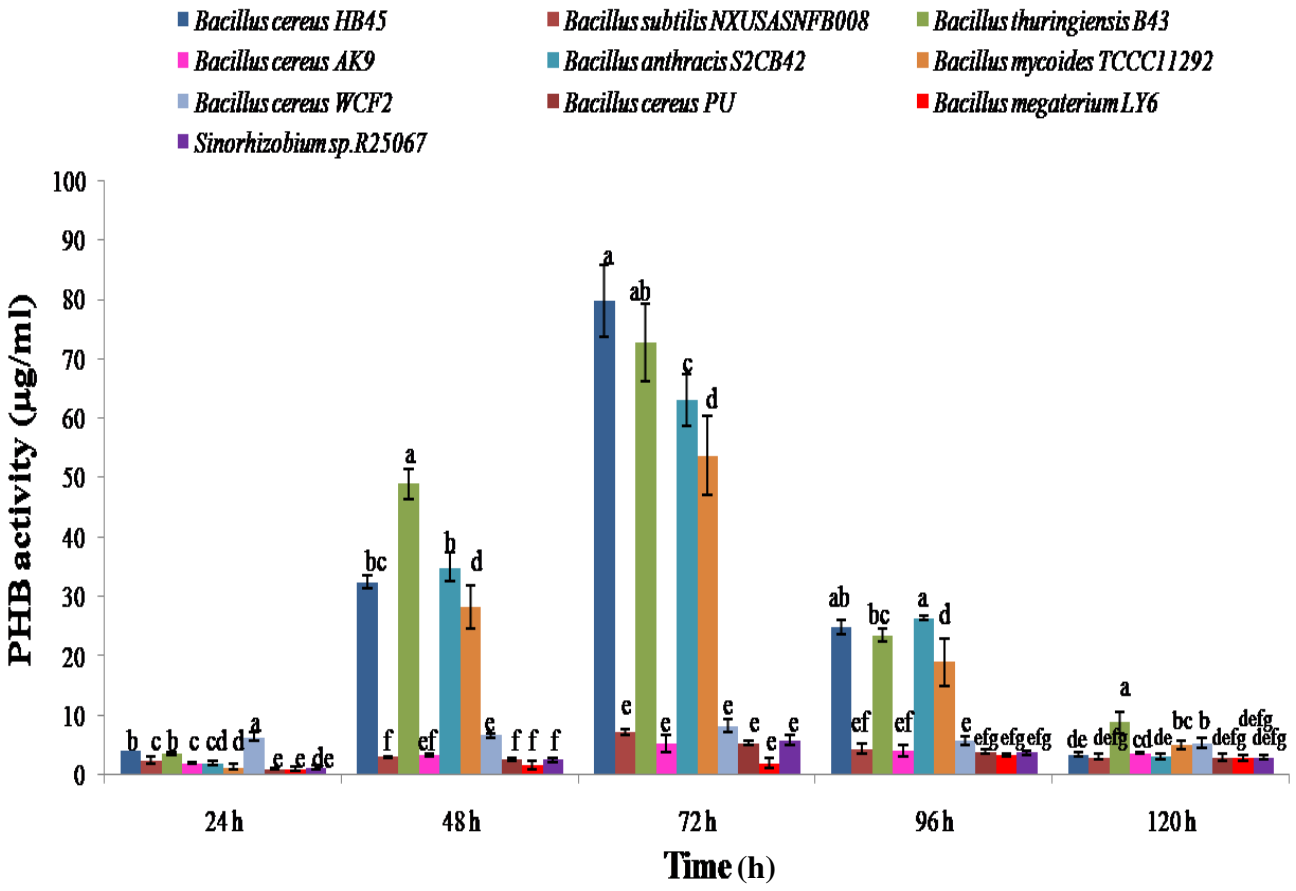


Fig. 3.27: PHB activity of bacterial isolates.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

3.14 Optimization of PHB production

Optimization of PHB production was carried out at 72 h of incubation using different parameters (pH, temperature, carbon, nitrogen source and inoculum concentration). Two isolates *viz. Bacillus cereus* HB45 and *Bacillus thuringiensis* B43 were selected for optimization.

3.14.1 Effects of pH on PHB production

Initial pH of the production medium is one of the most critical parameters affecting both growth and PHB production. PHB production was tested against different pH ranging from 5 to 9. The result clearly indicated that the selected two isolates showed the highest activity at pH 6 (Fig. 3.28 and Fig. 3.29).

Maximum PHB activity was 161.24 ± 3.94 and 253.16 ± 20.41µg/ml was shown by *Bacillus cereus* HB45 and *Bacillus thuringiensis* B43, respectively at pH 6 while minimum activity 36.44 ±1.93 and 16.99 ±0.75 µg/ml at pH 9. Therefore, it could be concluded that the pH 6 would be optimum for the PHB production for the isolated bacteria.

3.14.2 Effects of temperature on PHB production

Temperature would be one of the important parameters for better PHB production. The effect of temperature was carried out against different temperatures (20, 30, 37, 40 and 50°C). Fig. 3.30 and Fig. 3.31 showed the effect of temperature on PHB production. Results revealed that both the isolates showed the maximum production of PHB at temperature of 37°C.

The incubation temperature revealed that increasing the incubation temperature up to 37°C resulted an increasing PHB activity up to 207.29 ±10.35 and 280.09 ±10.35 µg/ml, respectively. *Bacillus cereus* HB45 and *Bacillus thuringiensis* B43 showed the lowest activity (65.88 ±3.41 and 84.28 ±3.40 µg/ml) at 20°C. The PHB production activity drops rapidly above 40°C in both isolates.

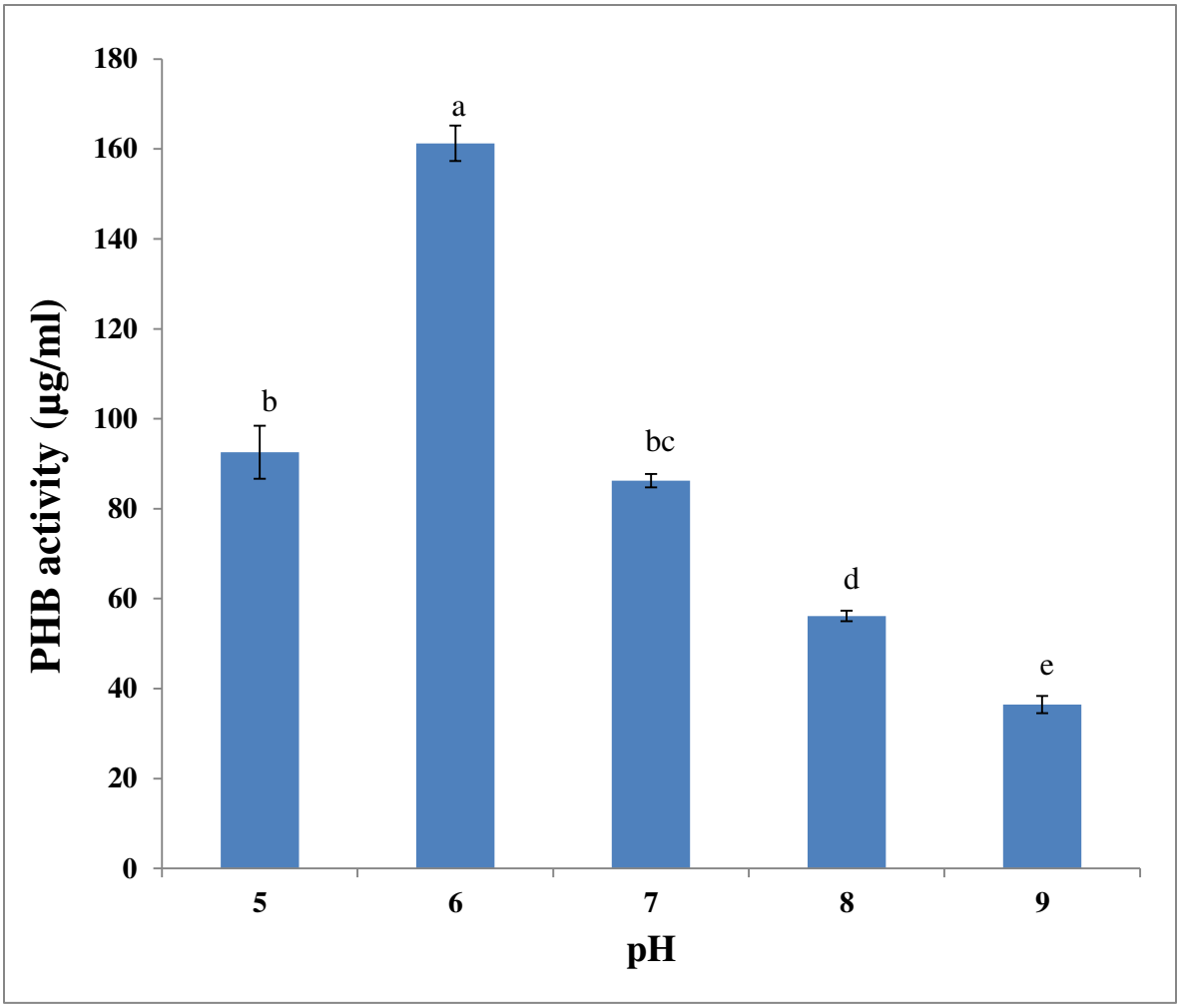


Fig. 3.28: Effects of pH on PHB production by *Bacillus cereus* HB45.
Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

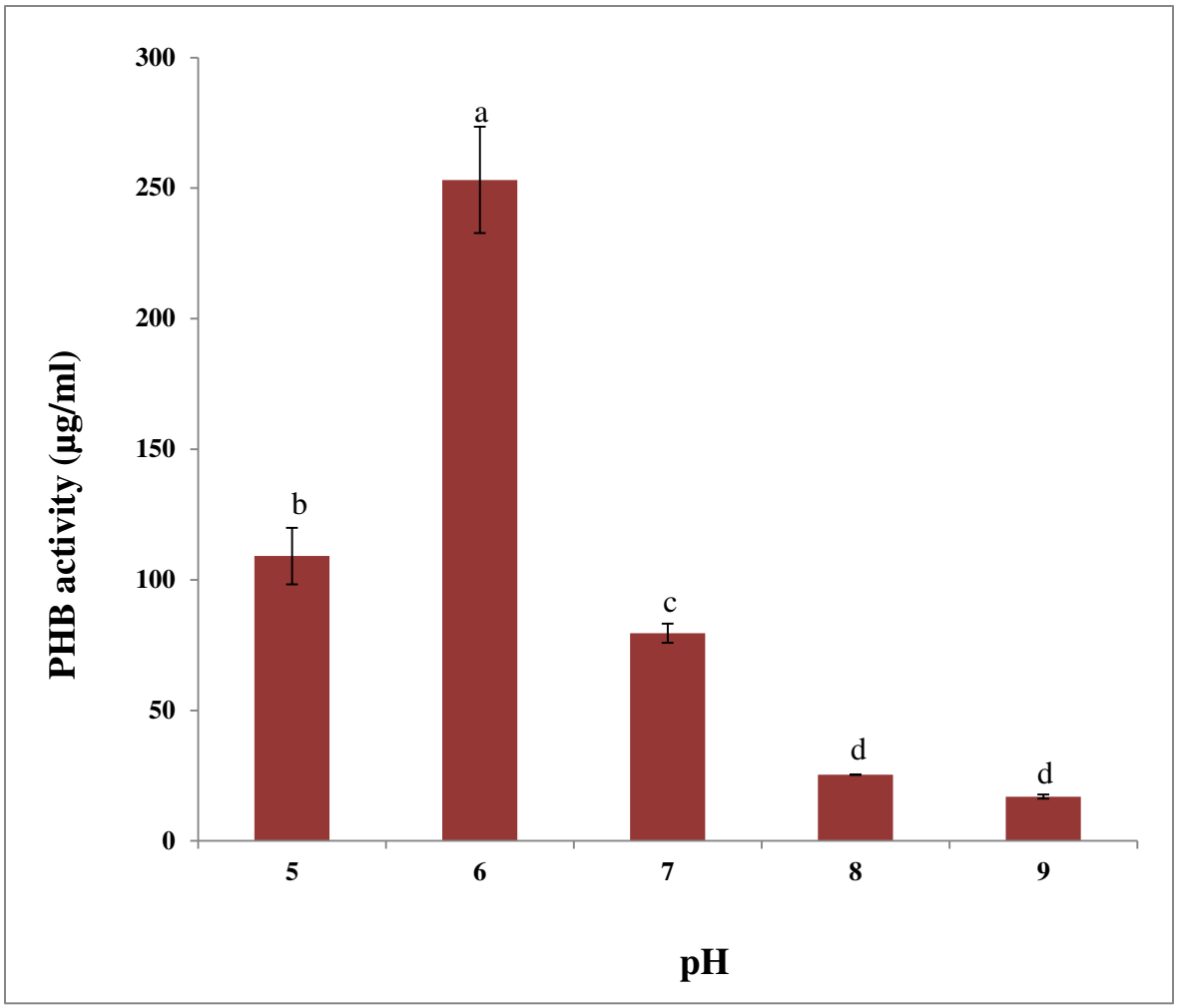


Fig. 3.29: Effects of pH on PHB production by *Bacillus thuringiensis* B43. Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

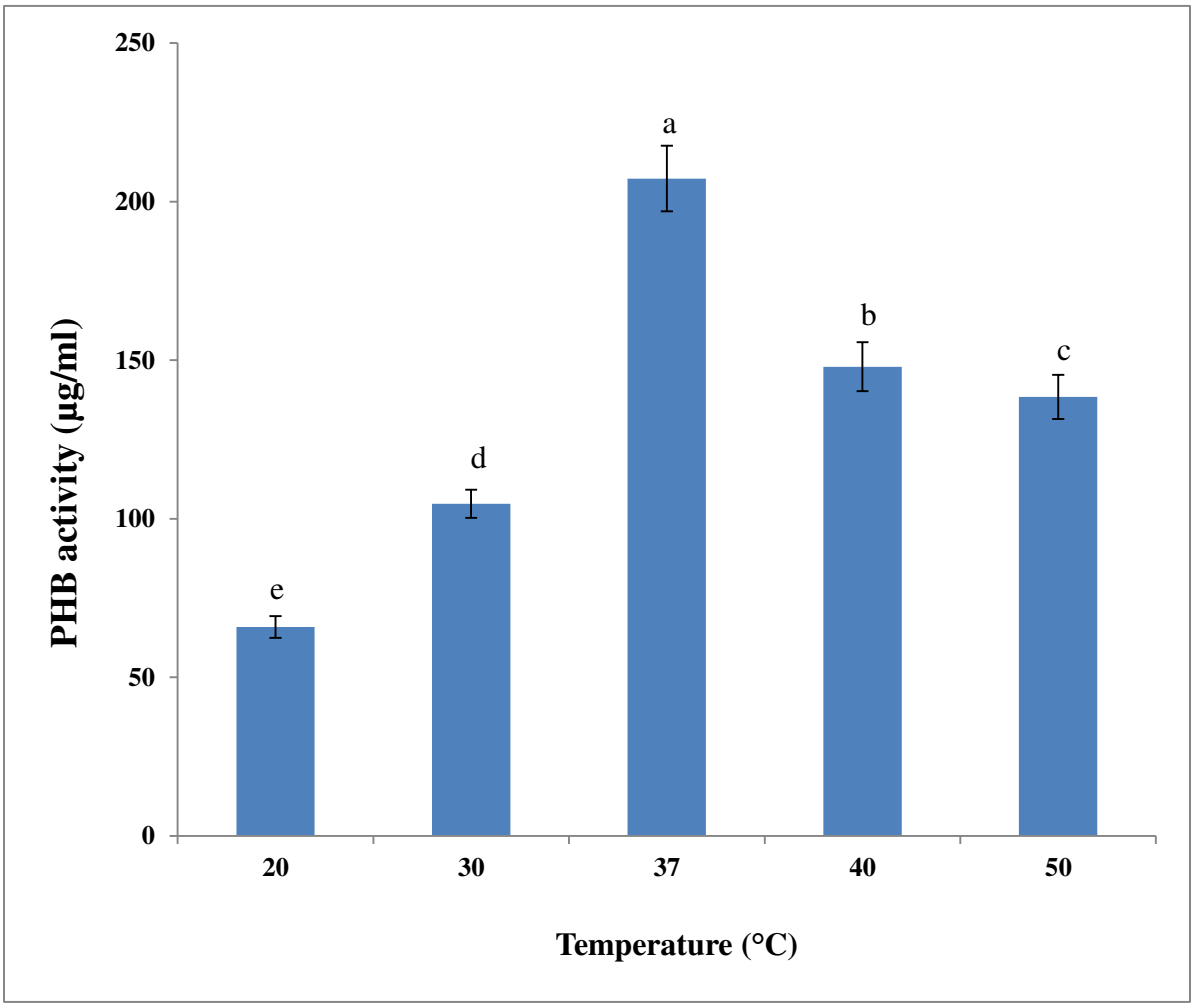


Fig. 3.30: Effects of temperature on PHB production by *Bacillus cereus* HB45. Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

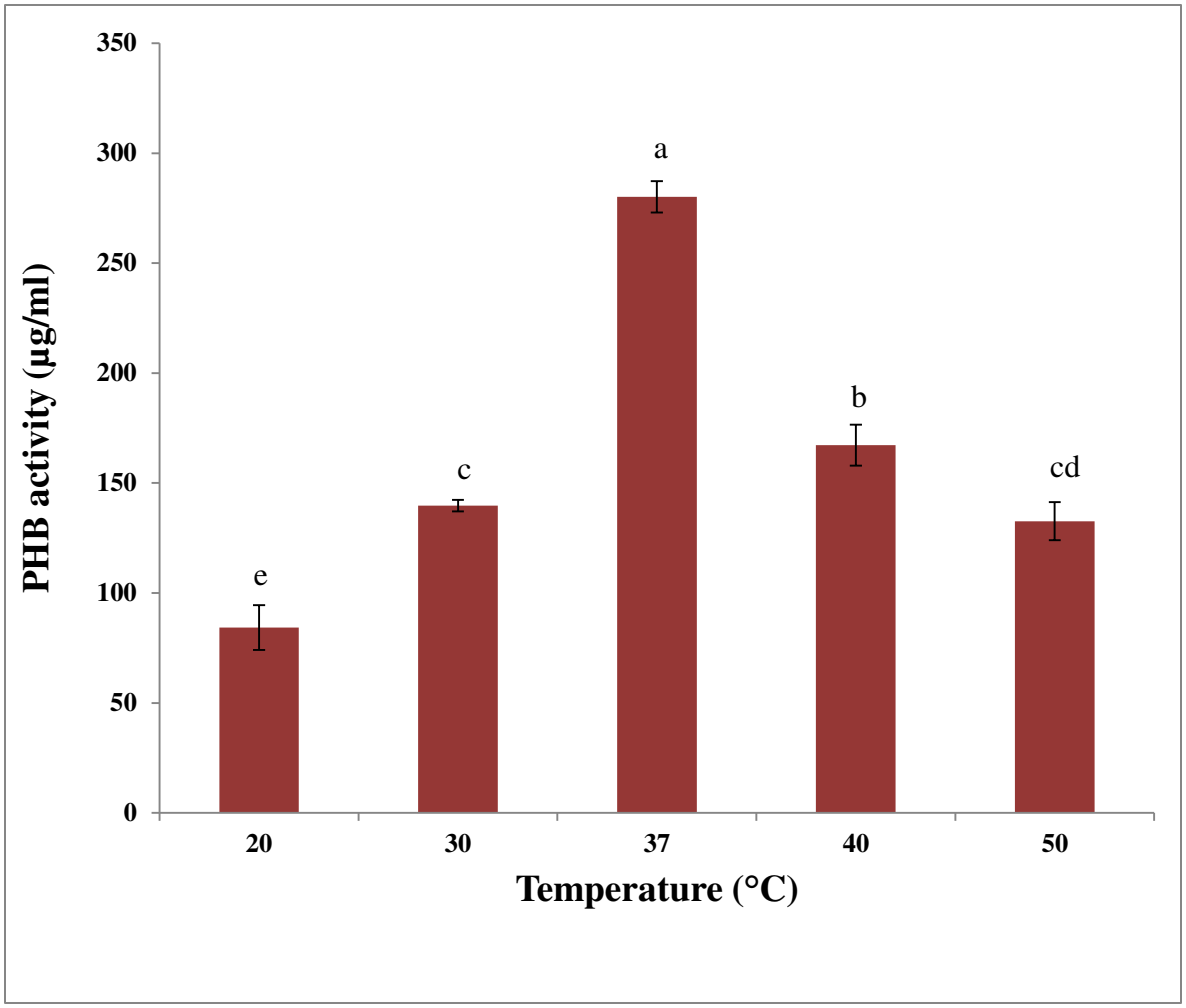


Fig. 3.31: Effects of temperature on PHB production by *Bacillus thuringiensis* B43. Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

3.14.3 Effects of carbon source on PHB production

PHB production might be affected by the carbon source used during its production process. In this study, various carbon sources at 1% concentration were added to the production medium and their effects on PHB production were evaluated. Glucose, sucrose, starch, arabinose, xylose and mannitol were used as major carbon sources for identifying the most suitable carbon source for PHB production.

Effects of carbon source were shown in Fig. 3.32 and Fig. 3.33. In case of carbon source *Bacillus cereus* HB45 showed positive response with supplementation of mannitol and *Bacillus thuringiensis* B43 showed positive response with supplementation of sucrose in the culture medium as the carbon source. Before adding any carbon source *Bacillus cereus* HB45 and *Bacillus thuringiensis* B43 could produce PHB 79.75 ± 6.10 and 72.72 ± 6.59 µg/ml, respectively. The maximum PHB activity as observed 338.99 ± 26.40 and 449.77 ± 11.03 µg/ml were achieved after 72h of incubation by *Bacillus cereus* HB45 and *Bacillus thuringiensis* B43, respectively when medium provided with mannitol and sucrose. The addition of mannitol enhanced PHB production up to 4.25 fold higher than without adding mannitol in case of *B. cereus* HB45 and in case of *B. thuringiensis* B43 production enhanced 6.18 fold higher than without adding sucrose. Arabinose showed minimum response for PHB activity (85.16 ± 1.95 and 24.75 ±0.29µg/ml) in both the isolates.

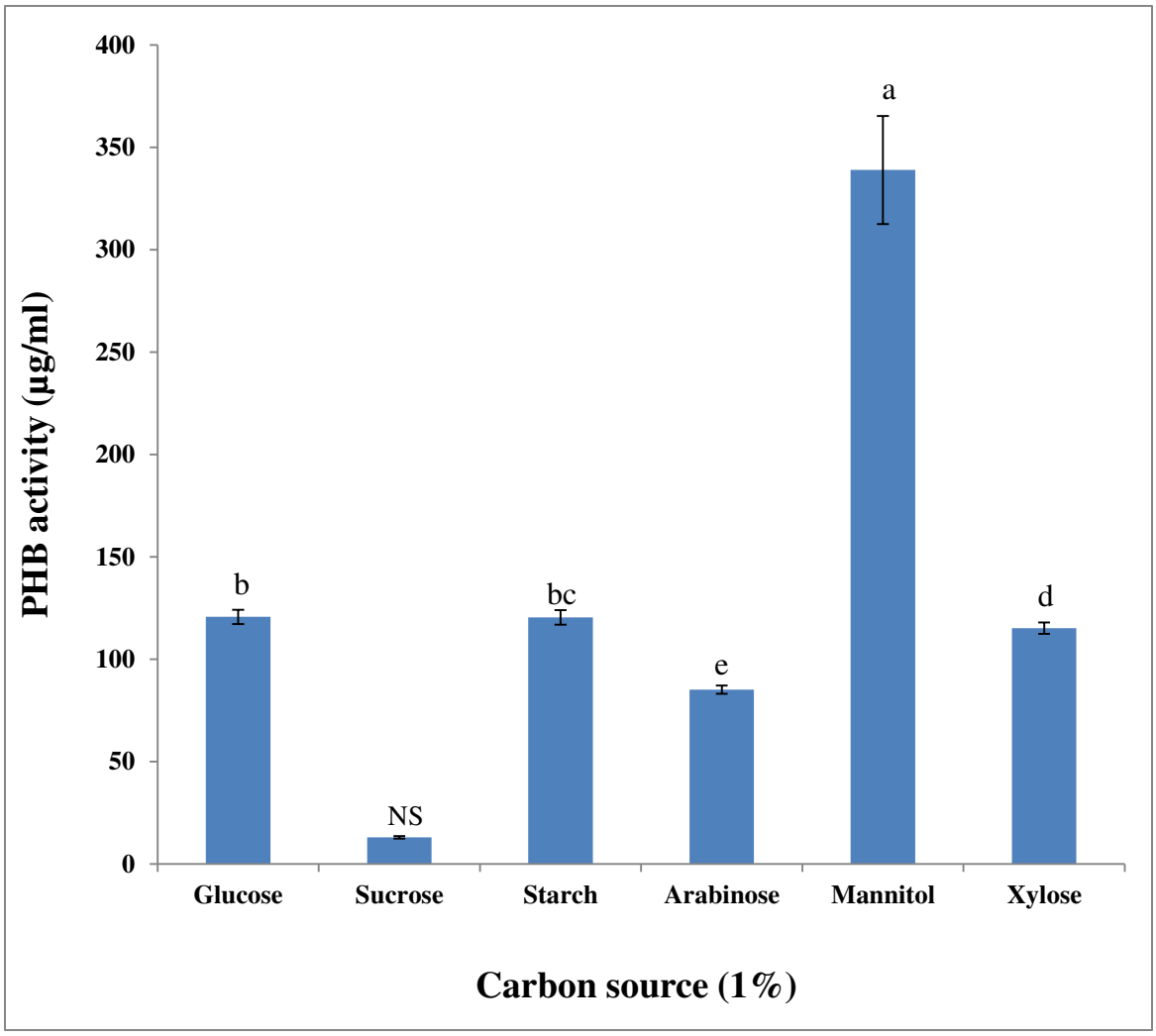


Fig. 3.32: Effects of carbon source on PHB production by *Bacillus cereus* HB45. Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3). NS= Not significant.

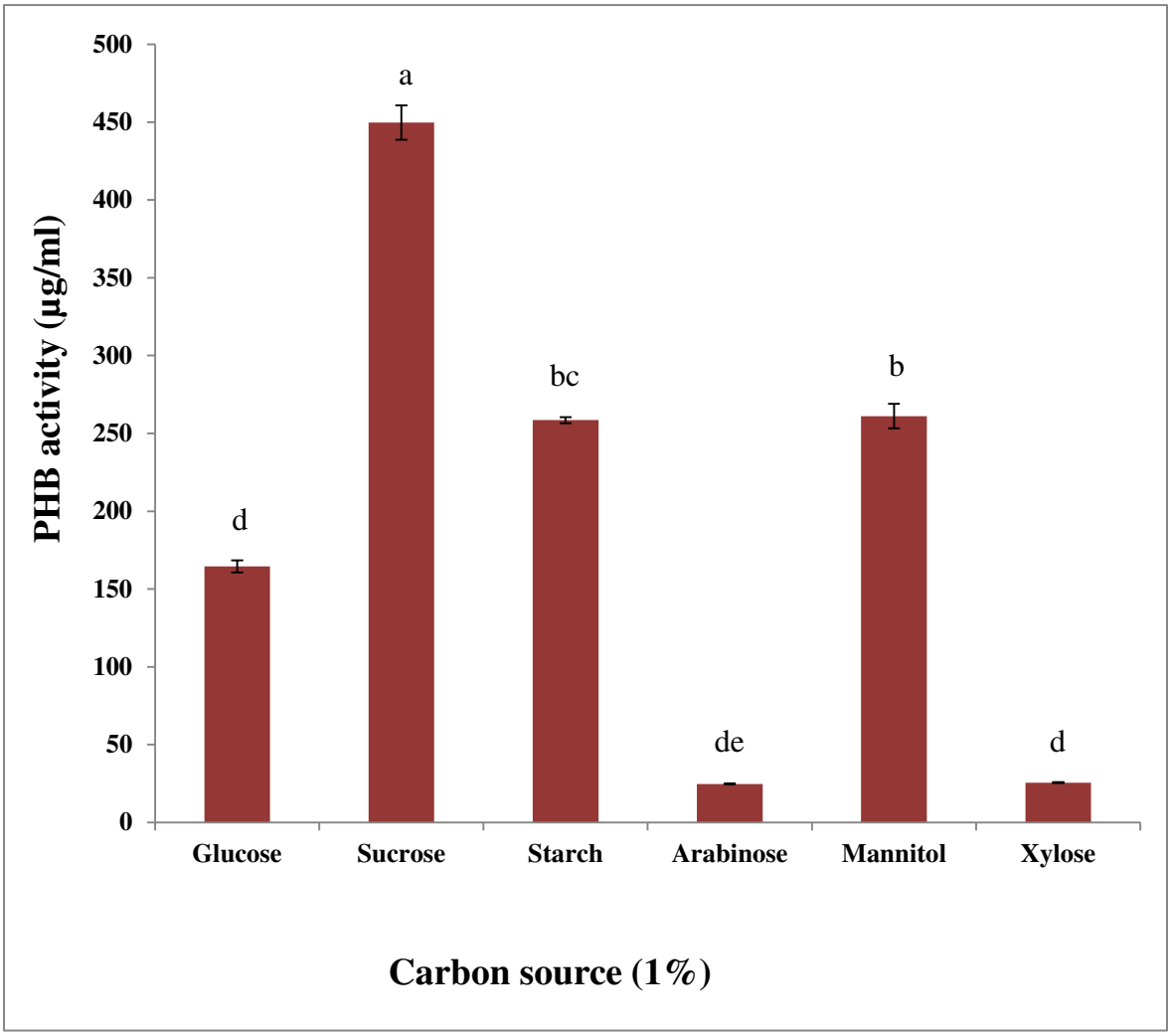


Fig. 3.33: Effects of carbon source on PHB production by *Bacillus thuringiensis* B43. Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

3.14.4 Effects of better carbon source concentration on PHB production

After screening the carbon sources, varying concentrations of better carbon source were added to the production medium and incubated at 37°C and PHB activity was estimated after 72 h of incubation.

PHB produced by the *Bacillus cereus* HB45 when grown on different concentrations of the best carbon source (Mannitol) is presented in Fig. 3.34. Various concentrations of mannitol (0.5–3.0%, w/v) were supplemented in the medium to investigate the effect on PHB production of *Bacillus cereus* HB45. Result showed that, 1.0% (w/v) mannitol responded the highest (335.80 ± 3.47 µg/ml) PHB production and thereafter, a decrease concentration was observed. PHB production was found to be the lowest (83.08 ± 4.90 µg/ml) by *Bacillus cereus* HB45 at 3% of mannitol.

Like mannitol for *Bacillus cereus* HB45, various concentrations of sucrose (0.5–3.0%, w/v) were supplemented in the medium to investigate the effect on PHB production of *Bacillus thuringiensis* B43 since sucrose was found to be suitable for PHB production. The highest PHB activity (425.50 ± 13.99 µg/ml) showed at 1% and the lowest (27.83 ± 1.44 µg/ml) at 3% of sucrose (Fig. 3.35).

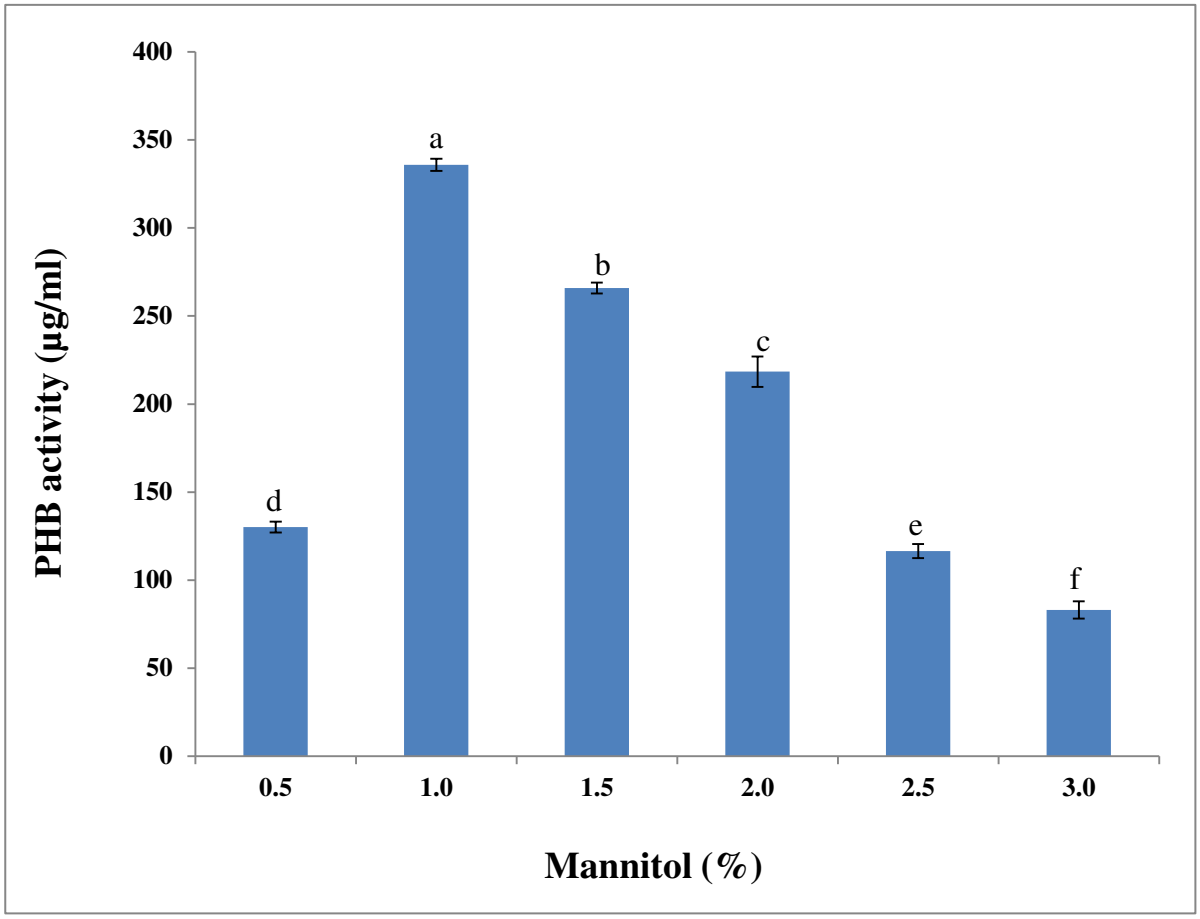


Fig. 3.34: Effects of mannitol concentration on PHB production by *Bacillus cereus* HB45.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

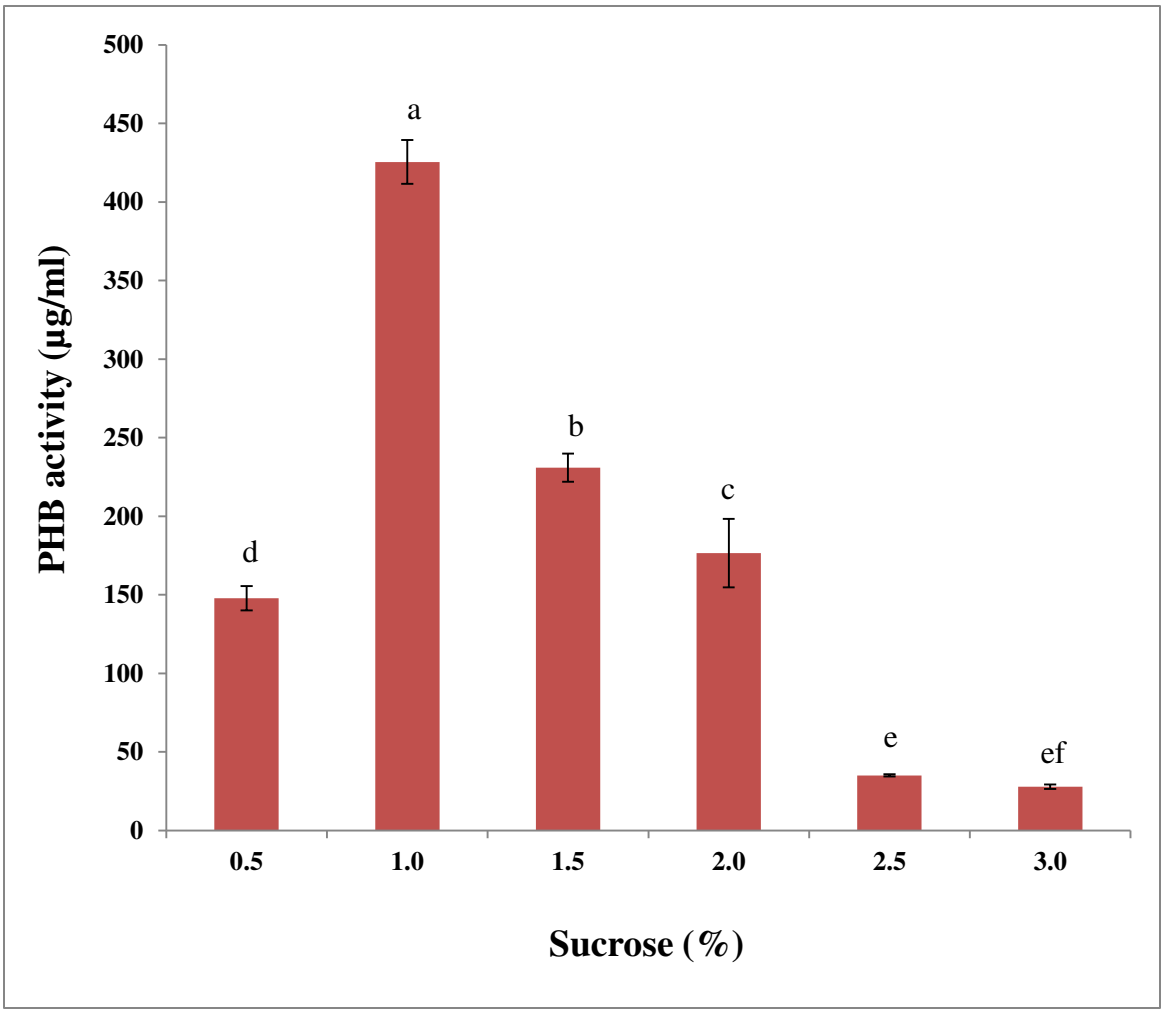


Fig. 3.35: Effects of sucrose concentration on PHB production by *Bacillus thuringiensis* B43.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

3.14.5 Effects of organic nitrogen source on PHB production

The effect of nitrogen sources on the PHB production was tested by supplementing production medium with different organic nitrogen sources (1% w/v) *viz.* peptone, tryptone, beef extract and yeast extract and PHB activity was estimated.

Effects of organic nitrogen source experiment showed that, tryptone and yeast extract were found to be the most effective nitrogen source for PHB production by *Bacillus cereus* HB45 and *Bacillus thuringiensis* B43, respectively. The maximum PHB production by *Bacillus cereus* HB45 was obtained in case of tryptone which attained the PHB activity up to 432.86 ± 8.13 µg/ml and in case of yeast extract it was found to be 280.16 ± 2.24 µg/ml by *Bacillus thuringiensis* B43 (Fig. 3.36 and Fig. 3.37).

3.14.6 Effects of better organic nitrogen source concentration on PHB production

The best organic nitrogen source was added in different concentration to the production medium for maximum PHB production. Nitrogen source tryptone was found to be better for the isolate *Bacillus cereus* HB45. Therefore, various concentrations of tryptone (0.5–3.0%, w/v) were supplemented in the medium. Results showed that there was an increase in PHB production with the increase in the concentration of tryptone up to 1.5% (Fig. 3.38). The highest PHB activity attained at 1.5% tryptone concentration and it was 444.62 ± 7.36 µg/ml and PHB activity was the lowest at 3% concentration (49.25 ± 3.08 µg/ml).

Yeast extract was found to be better for *Bacillus thuringiensis* B43 so that various concentrations of yeast extract (0.5–3.0%, w/v) were supplemented in the medium to

investigate the effect on PHB production by *Bacillus thuringiensis* B43. The highest amount of PHB production (273.05 ±13.97 µg/ml) showed at 1.0% and the lowest (69.28 ± 3.07 µg/ml) at 0.5% (Fig. 3.39).

3.14.7 Effects of inorganic nitrogen source on PHB production

Effects of inorganic nitrogen source on PHB production was carried out by using different inorganic nitrogen sources such as ammonium chloride, ammonium nitrate and ammonium orthophosphate at 1% (w/v) concentration to the medium. PHB activity was estimated after 72 h of incubation. In the present study ammonium orthophosphate was found to be the best inorganic nitrogen source for PHB production by the *Bacillus cereus* HB45 (423.54 ± 3.62 µg/ml) and ammonium chloride for *Bacillus thuringiensis* B43 (432.86 ± 4.43 µg/ml) (Fig. 3.40 and Fig. 3.41).

The lowest amount of PHB was produced with ammonium chloride which gave 341.68 ± 10.51 µg/ml by *Bacillus cereus* HB45 and *Bacillus thuringiensis* B43 gave lowest PHB (133.59 ± 3.70 µg/ml) with ammonium sulphate. The result showed that the supplementation of the inorganic nitrogen source prolong PHB production by both of the isolates.

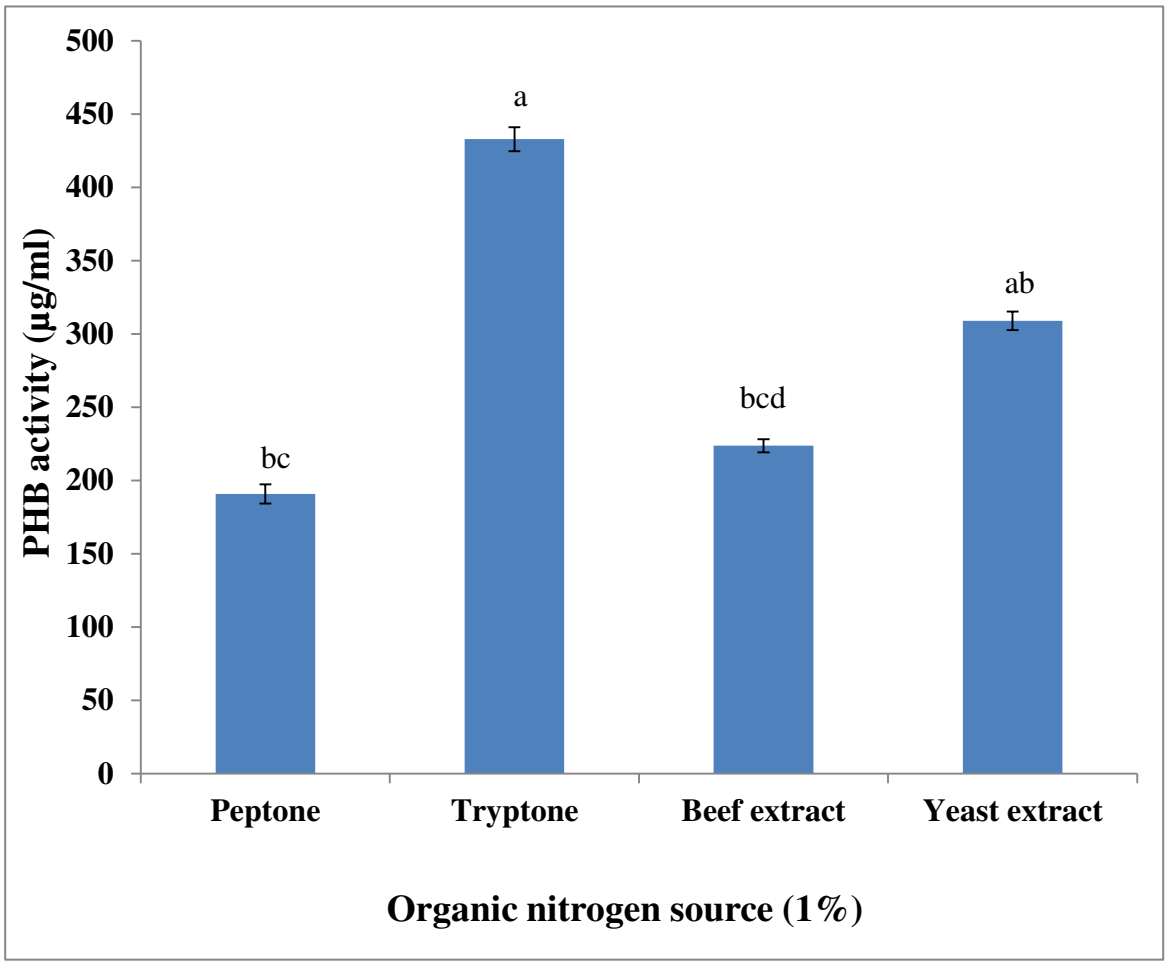


Fig. 3.36: Effects of organic nitrogen source on PHB production by *Bacillus cereus* HB45.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

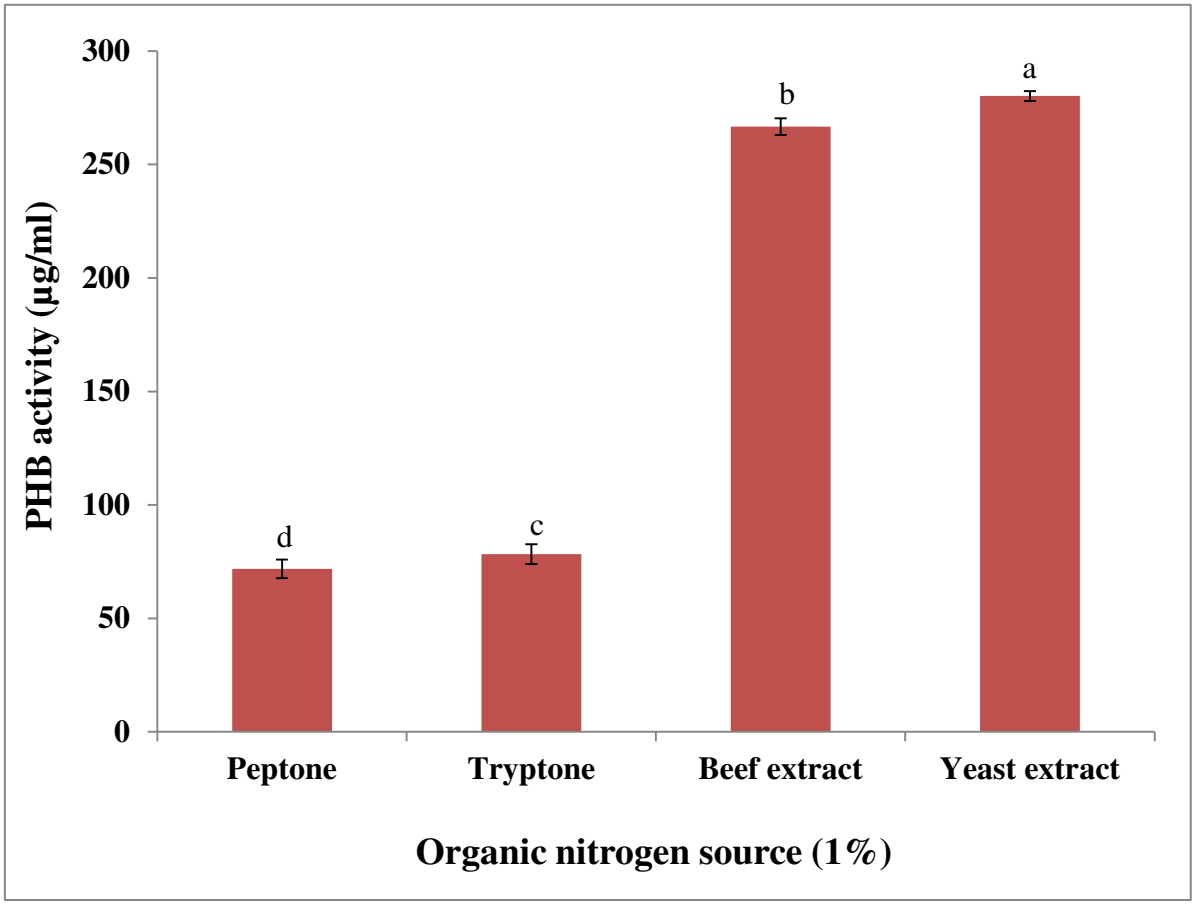


Fig. 3.37: Effects of organic nitrogen source on PHB production by *Bacillus thuringiensis* B43.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

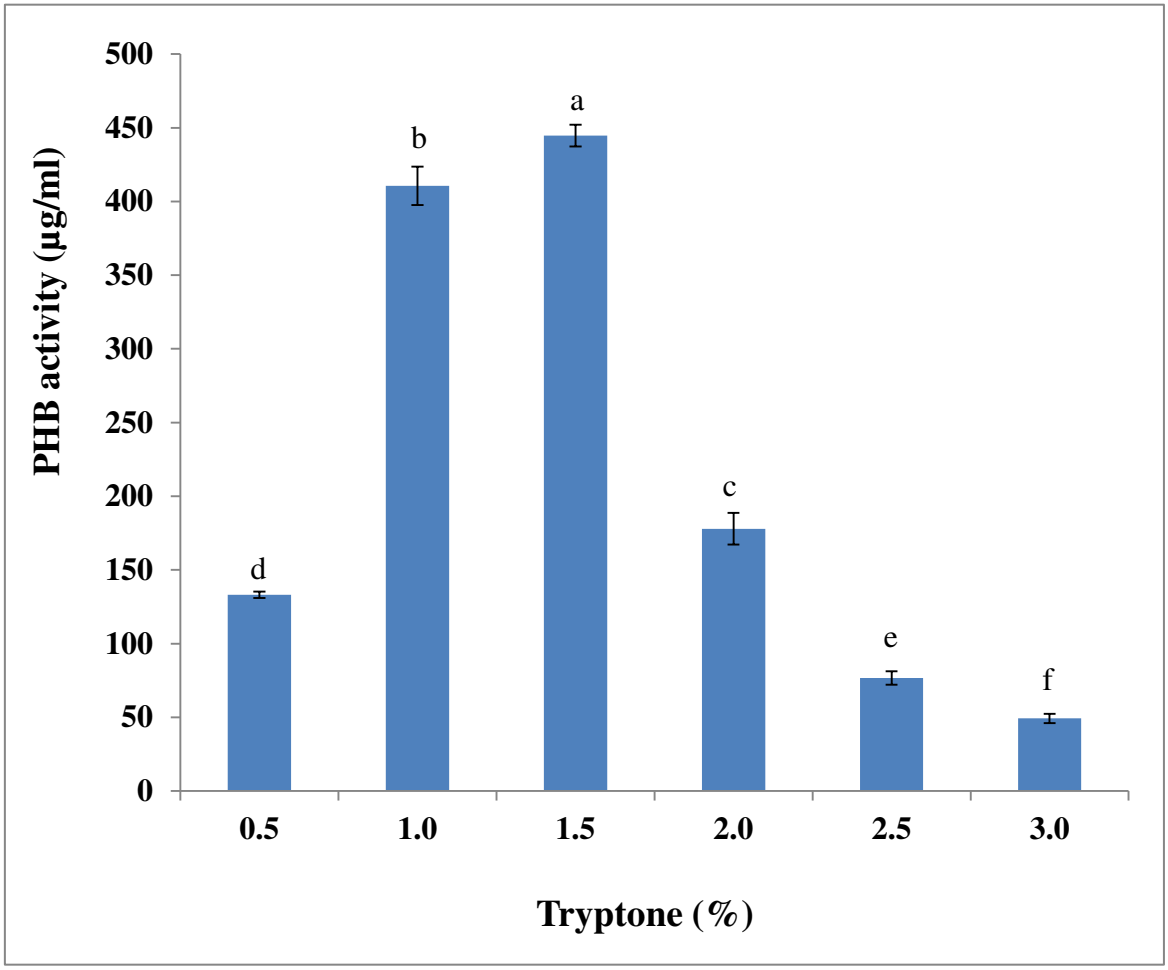


Fig. 3.38: Effects of tryptone concentration on PHB production by *Bacillus cereus* HB45. Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

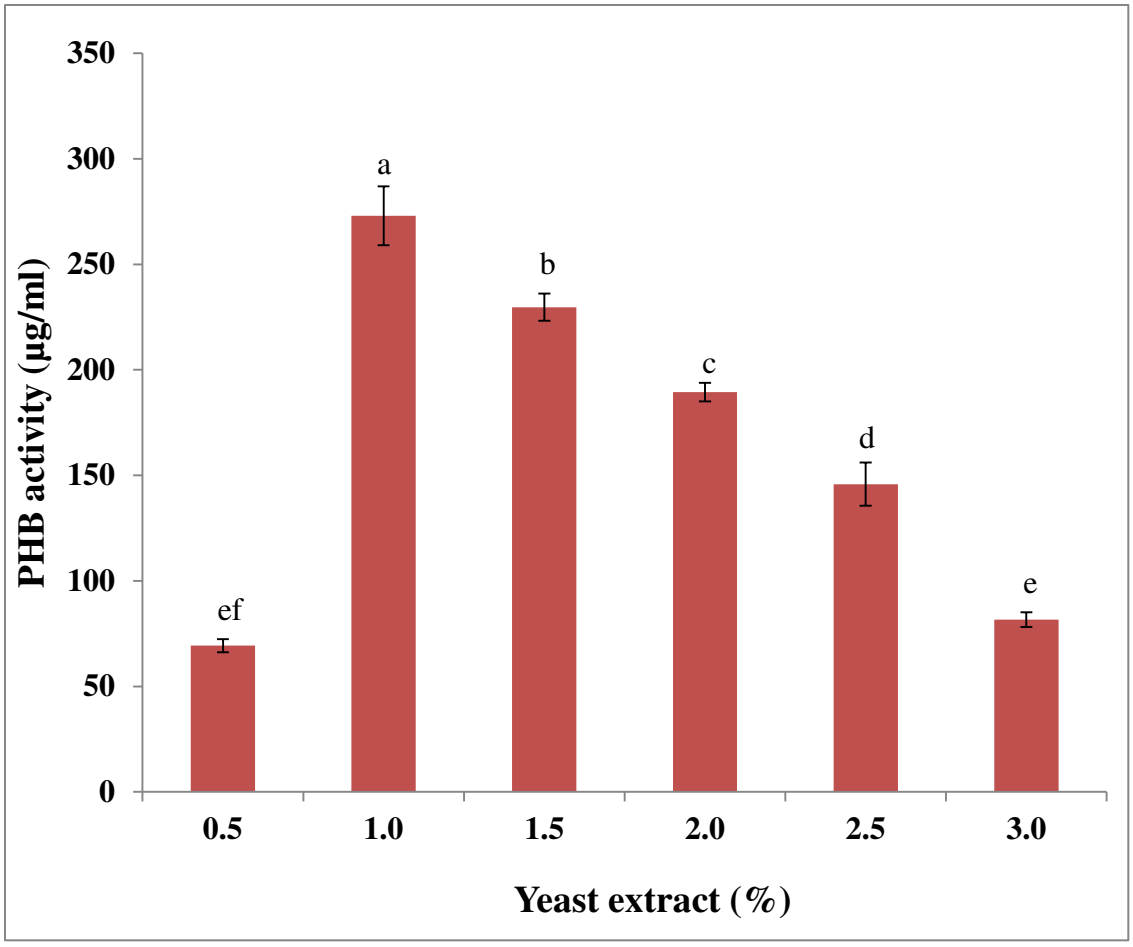


Fig. 3.39: Effects of Yeast extract concentration on PHB production by *Bacillus thuringiensis* B43.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

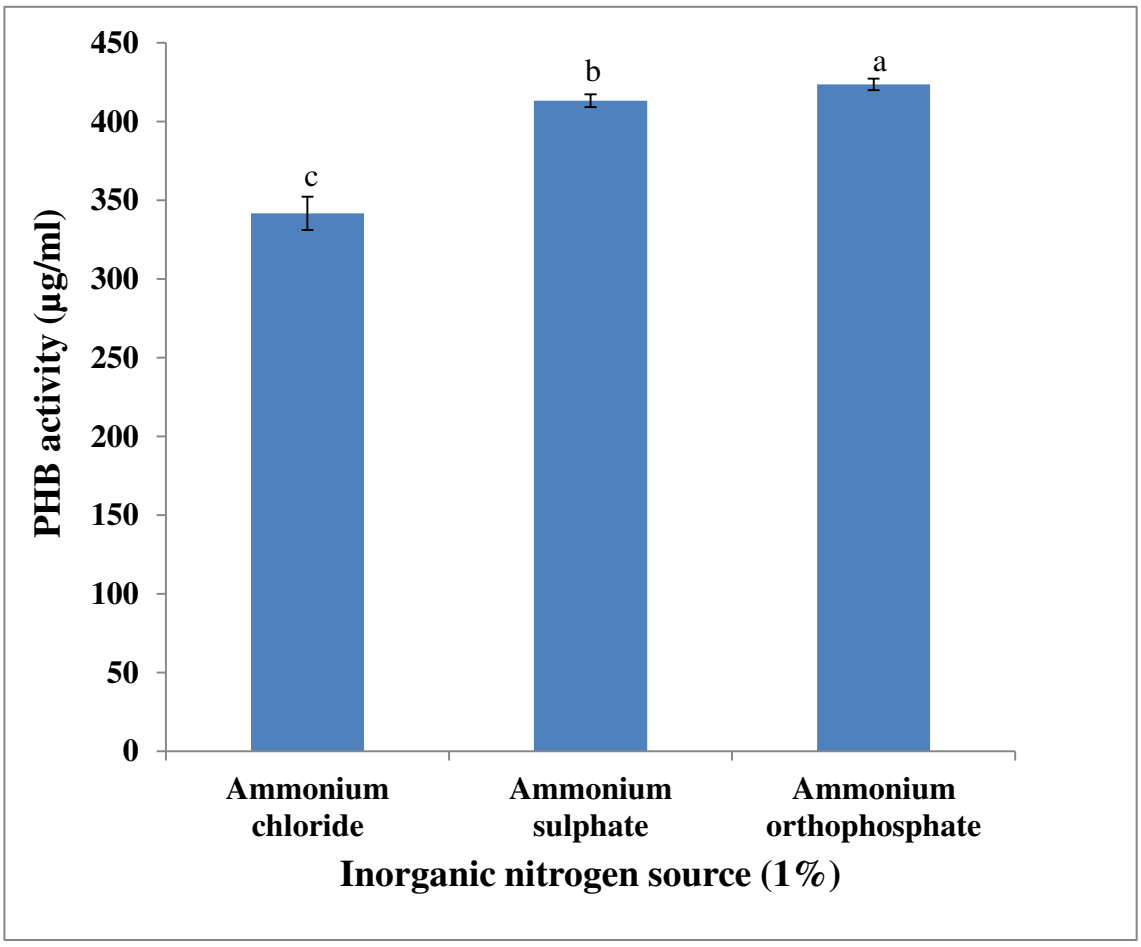


Fig. 3.40: Effects of inorganic nitrogen source on PHB production by *Bacillus cereus* HB45.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

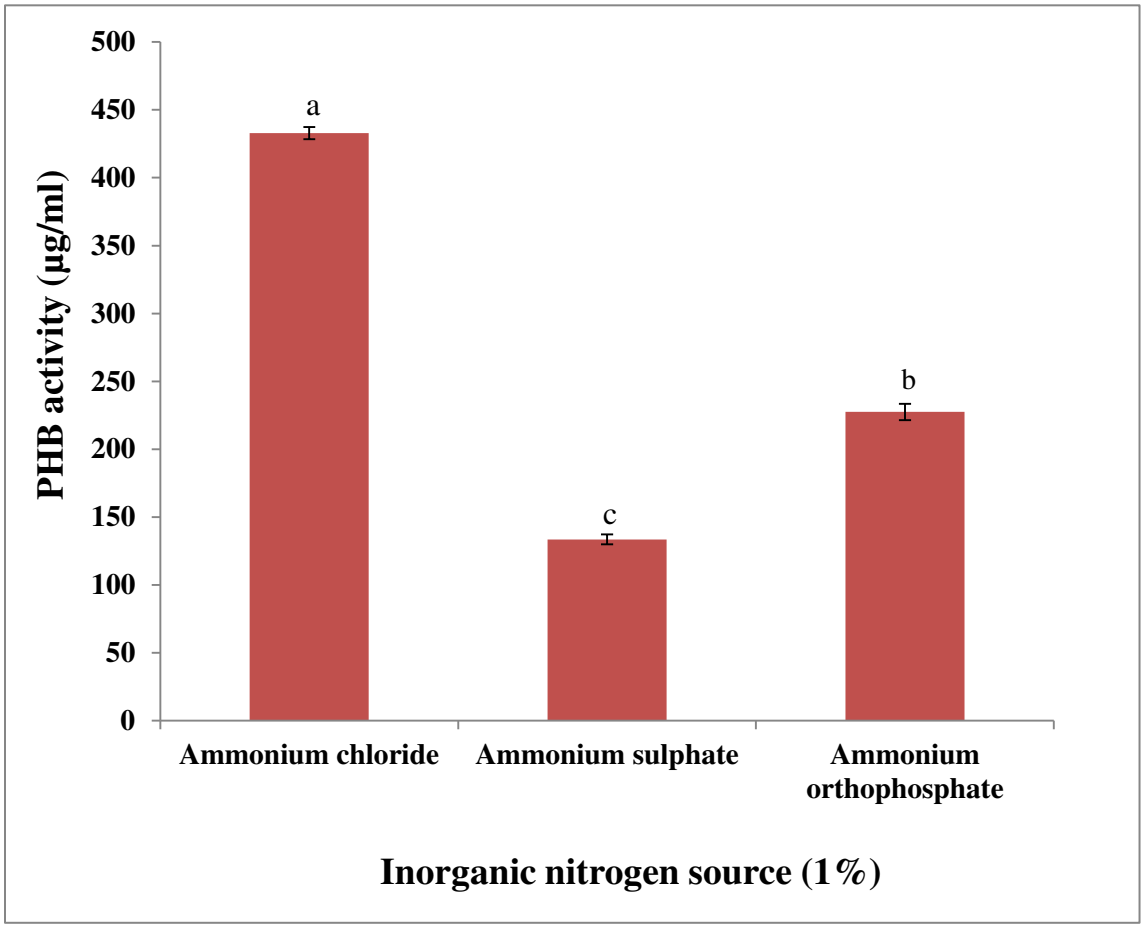


Fig. 3.41: Effects of inorganic nitrogen source on PHB production by *Bacillus thuringiensis* B43.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

3.14.8 Effects of better inorganic nitrogen source concentration on PHB production

Various concentrations (0.5-3.0%, w/v) of better inorganic nitrogen source were added in the medium to investigate the effect on PHB production. Ammonium orthophosphate was found to be better inorganic nitrogen source for *Bacillus cereus* HB45. Therefore, different concentrations of ammonium orthophosphate were added to the medium for *Bacillus cereus* HB45. It was observed that PHB production was increased with increase of ammonium orthophosphate up to 2.0% and thereafter, PHB production was declined (Fig. 3.42). At this concentration ammonium orthophosphate PHB activity attained 516.68 ± 7.35 µg/ml by *Bacillus cereus* HB45.

Ammonium chloride was found to be better inorganic nitrogen for *Bacillus thuringiensis* B43. Therefore, different concentrations of ammonium chloride (0.5 -3.0%, w/v) were supplemented in the medium for *Bacillus thuringiensis* B43. The highest PHB production (508.10 ± 13.66 µg/ml) showed at 2.0% ammonium chloride and the lowest (180.41 ± 9.21µg/ml) at 0.5% (Fig. 3.43).

3.14.9 Effects of inoculum concentration on PHB production

Effects of inoculum concentration were tested for PHB production. For this purpose, 1-6% fresh inoculum was used for the PHB production. Maximum PHB activity was obtained at 2% inoculum for *Bacillus cereus* HB45 and 1% for *Bacillus thuringiensis* B43 (Fig. 3.44 and Fig. 3.45). The highest PHB production was 179.87 ± 4.31 µg/ml by *Bacillus cereus* HB45 at 2% inoculum and 242.59 ± 9.02 µg/ml by *Bacillus thuringiensis* B43 at 1%.

This study revealed that increase of inoculum concentration up to 2% in production media there was a considerable increase in the PHB production for *Bacillus cereus* HB45 and 1% for *Bacillus thuringiensis* B43.

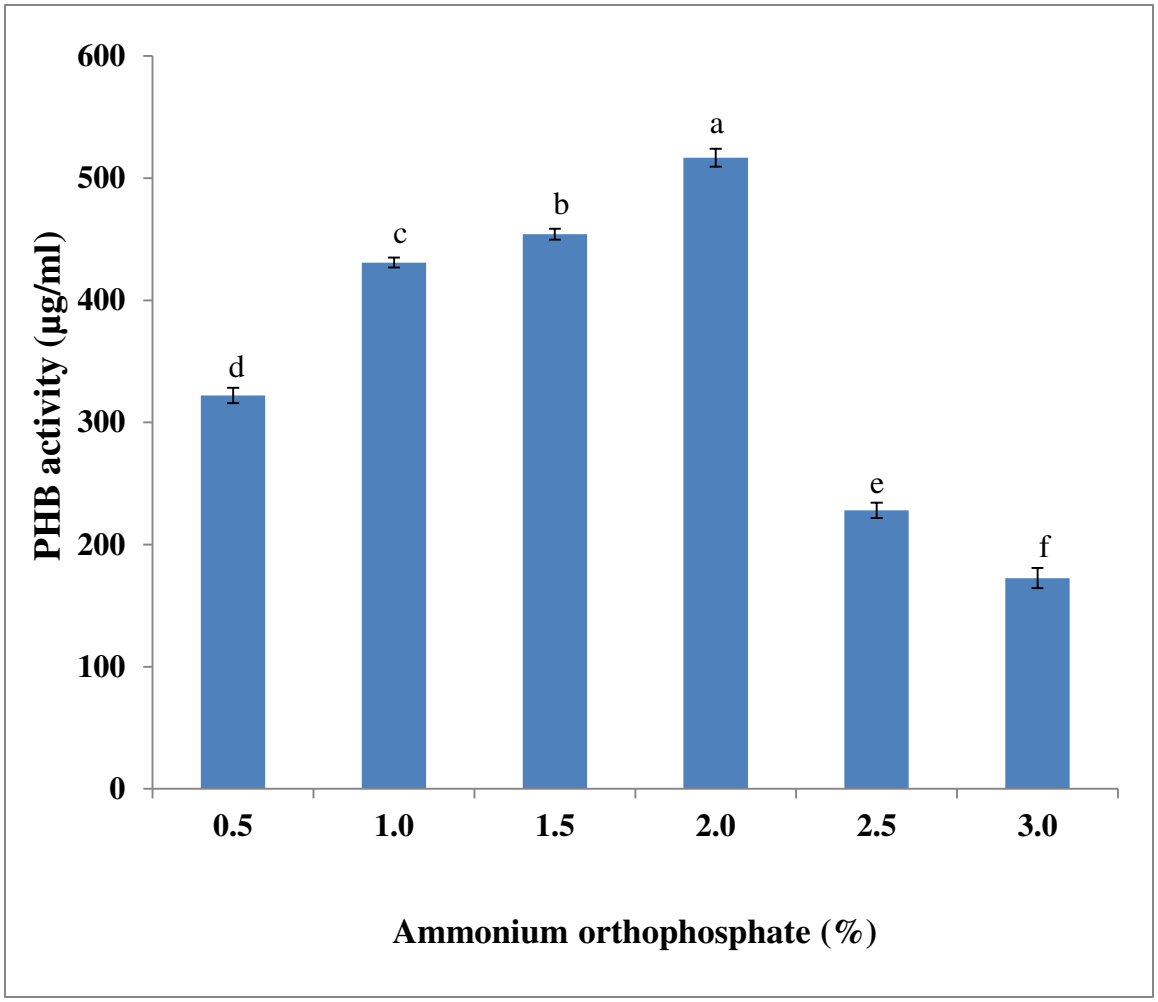


Fig. 3.42: Effects of ammonium orthophosphate concentration on PHB production by *Bacillus cereus* HB45.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

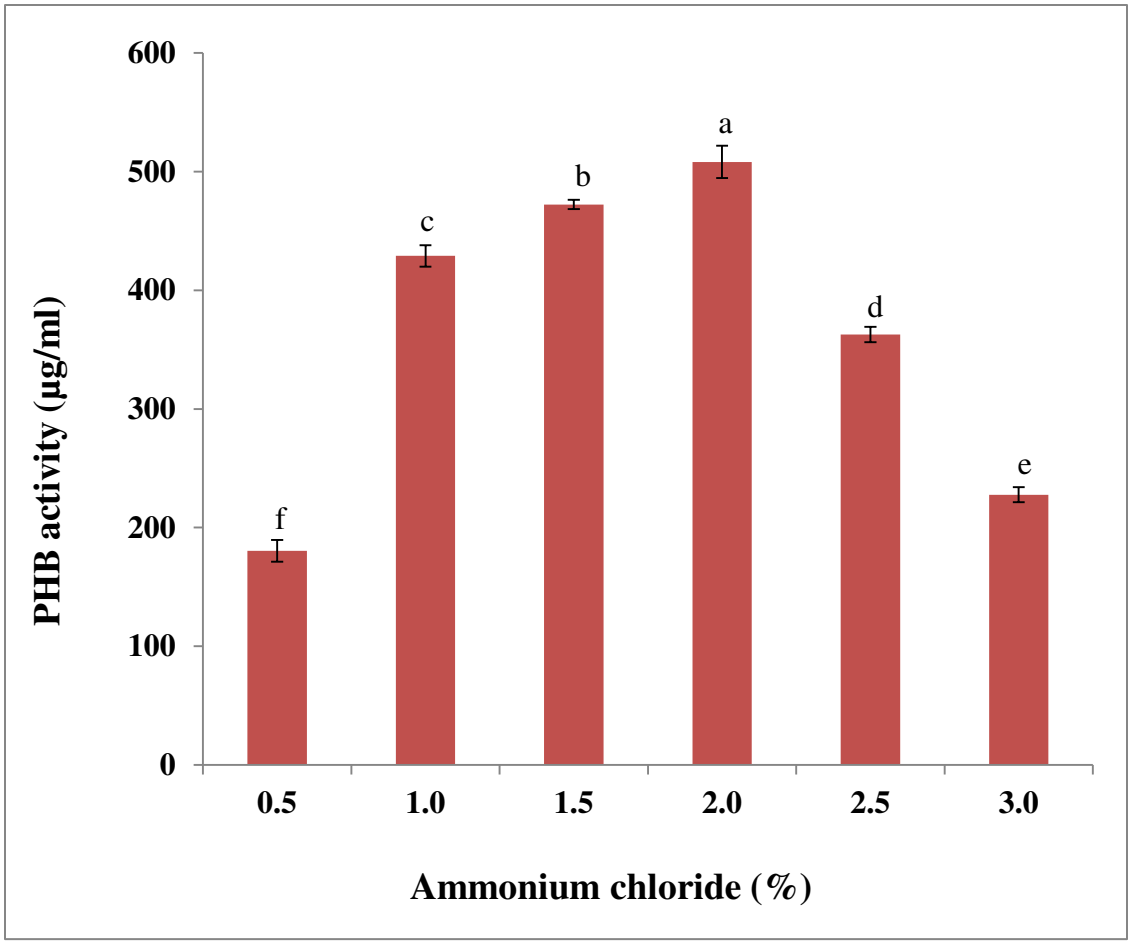


Fig. 3.43: Effects of ammonium chloride concentration on PHB production by *Bacillus thuringiensis* B43.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

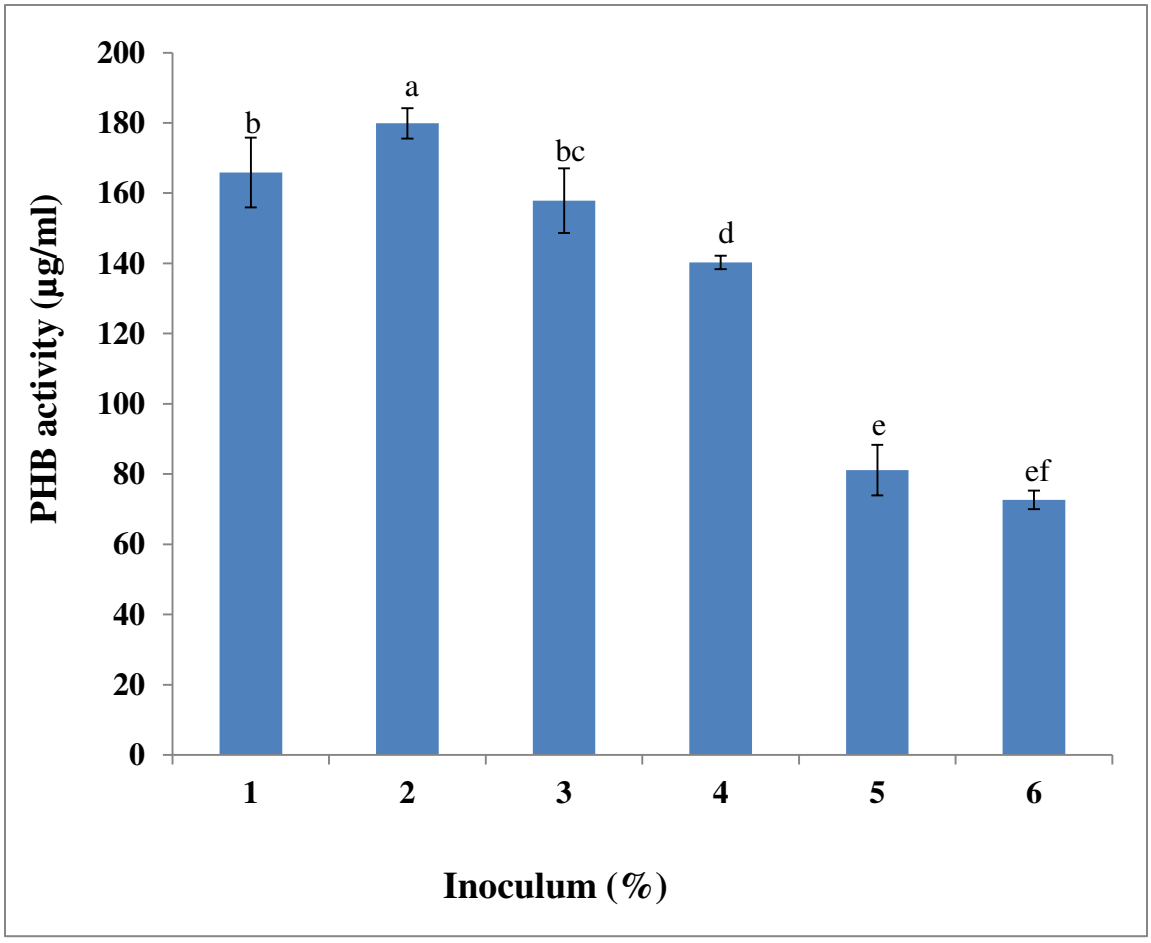


Fig. 3.44: Effects of inoculum concentration on PHB production by *Bacillus cereus* HB45.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

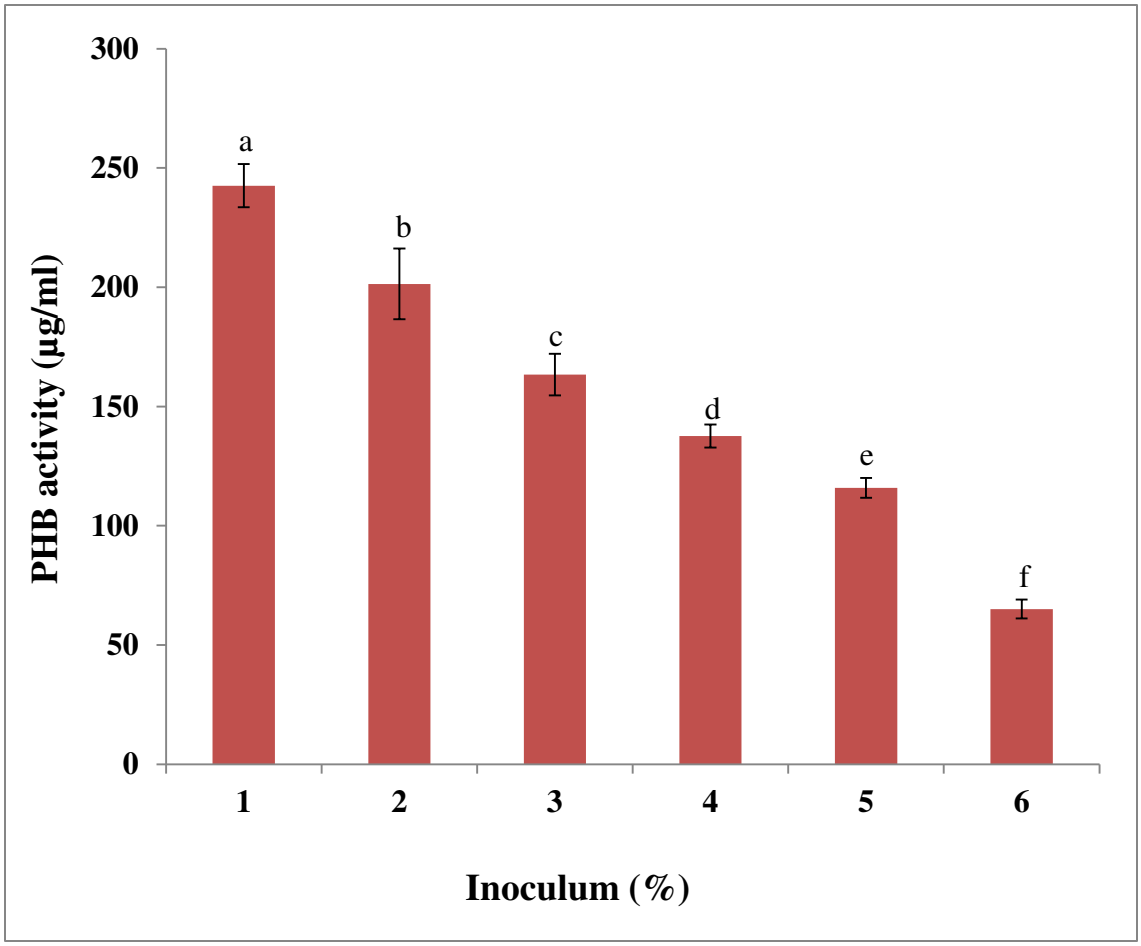


Fig. 3.45: Effects of inoculum concentration on PHB production by *Bacillus thuringiensis* B43.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

3.15 PHB production in all studied optimum condition

After optimization of all studied parameters, the best condition (Table 3.12) was set for the maximum PHB production by the selected indigenous bacterial isolates. Inoculated flasks were incubated at 37°C for 72 h and samples were analyzed in every 24 h intervals. The result clearly reflected an enhanced production of PHB in the possible optimum condition (Table 3.13 and Fig. 3.46, 3.47). The results showed that the maximum PHB production by *Bacillus cereus* HB45 and *Bacillus thuringiensis* B43 occurred at 72 h of incubation where PHB activity was achieved to 867.17 ± 7.35 and 955.41 ± 7.35 µg/ml, respectively. These were about 11 times higher in case of *Bacillus cereus* HB45 and 13 fold higher in case of *Bacillus thuringiensis* B43 before optimization. Both the isolates showed minimum activity at 24 h of incubation. Comparative analysis of PHB production is shown in Table 3.14. All the bacterial isolates showed considerably better performances after optimization.

Table 3.12: Best optimum conditions for PHB production.

Parameters	***Bacillus cereus* HB45**	***Bacillus thuringiensis* B43**
pH	6	6
Temperature	37º C	37º C
Carbon source	1% Mannitol	1% Sucrose
Organic nitrogen source	1.5% Tryptone	1.5% Yeast extract
Inorganic nitrogen source	2% $NH_4H_2PO_4$	2% NH_4Cl
Inoculum concentration	2%	1%

Table 3.13: PHB production in all optimum conditions.

	PHB Production (µg/ml)	
Incubation time (h)	***Bacillus cereus* HB45**	***Bacillus thuringiensis* B43**
24	98.30 ± 3.69	113.50 ± 3.89
48	413.99 ± 3.70	403.94 ± 4.82
72	**867.17 ± 7.35**	**955.41 ± 7.35**
96	636.78 ± 3.62	654.67 ± 3.89
120	306.39 ± 2.78	291.93 ± 3.06

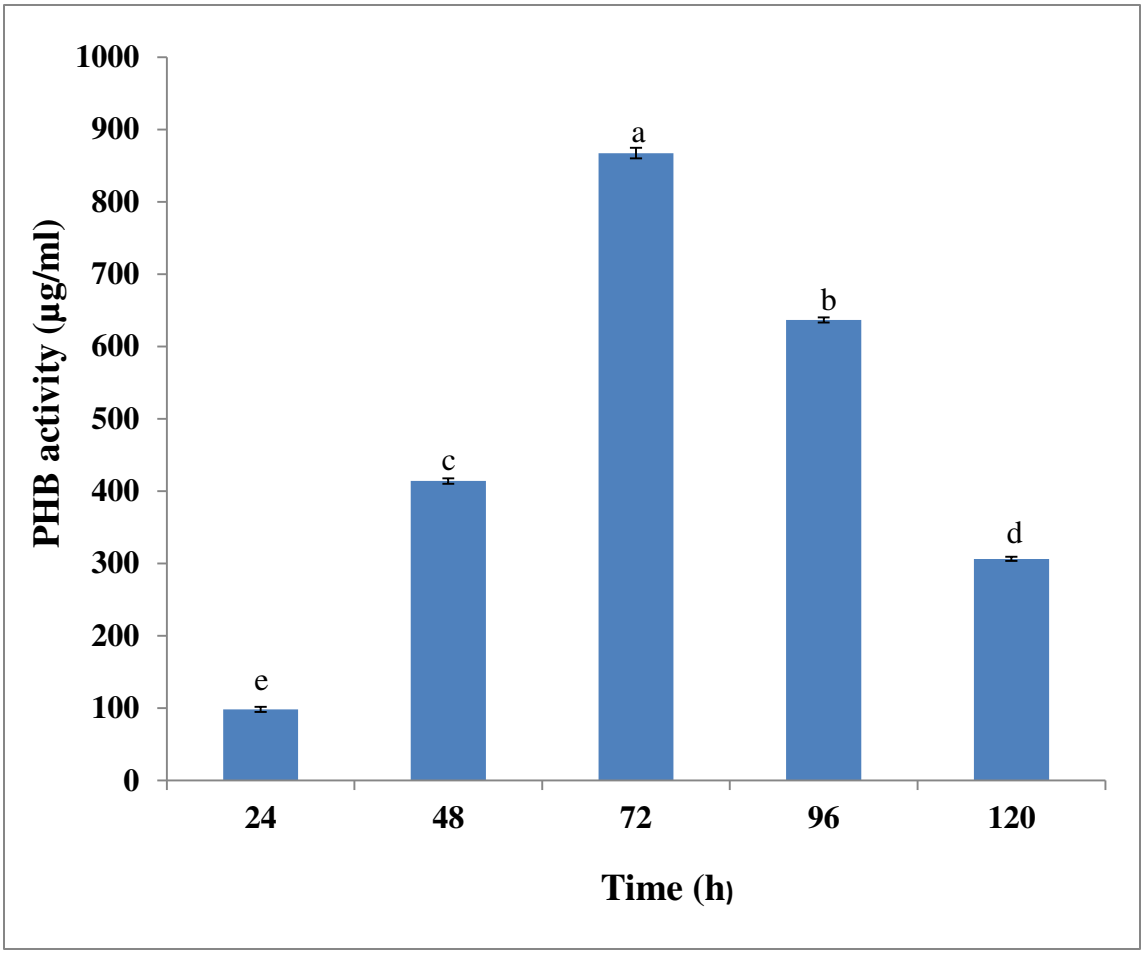


Fig. 3.46: PHB production in all studied optimum conditions by *Bacillus cereus* HB45.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

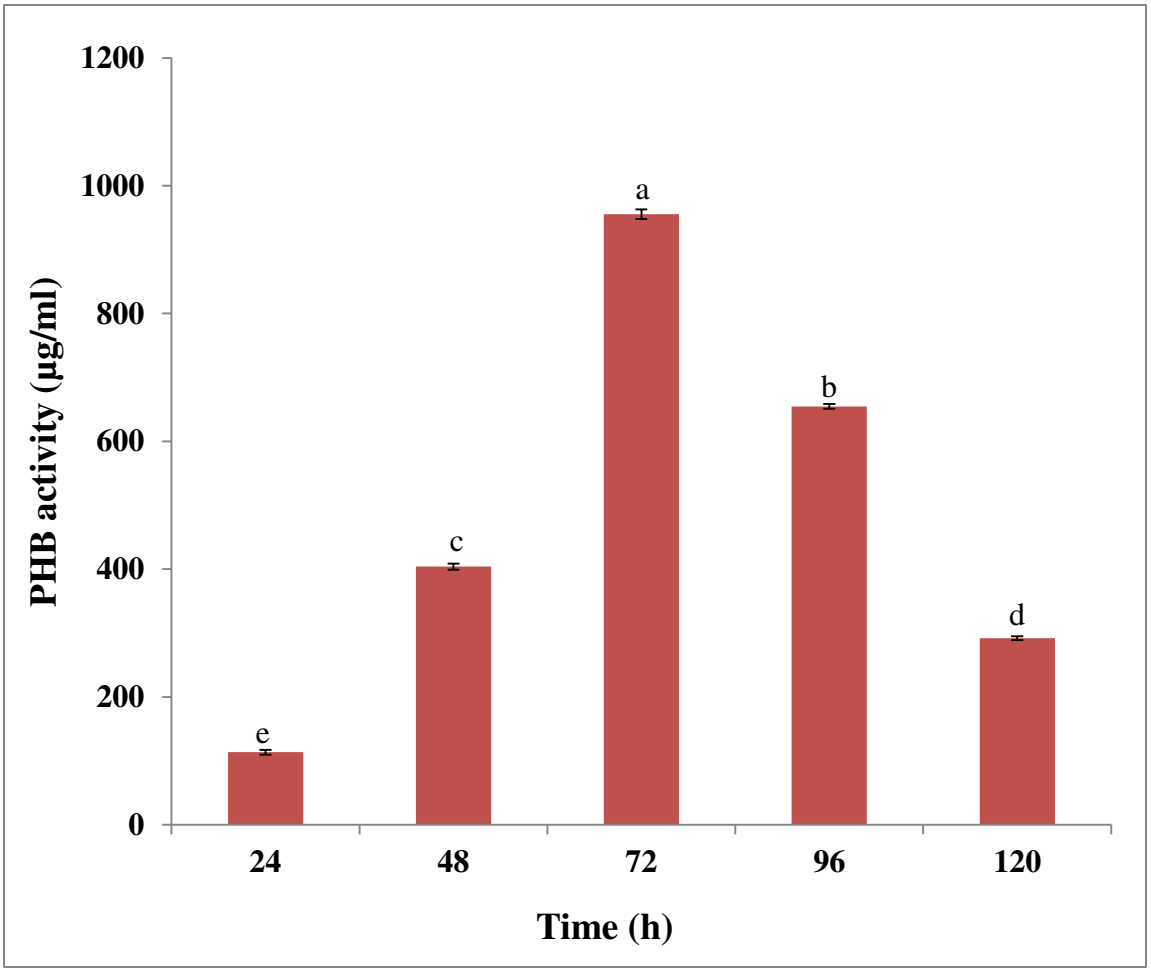


Fig. 3.47: PHB production in all studied optimum conditions by *Bacillus thuringiensis* B43.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ (n=3).

Table 3.14: Comparative analysis of PHB production.

Bacterial isolates	PHB Production (µg/ml)	
	Before optimization	After optimization
Bacillus cereus HB45	79.75 ± 6.10	867.17 ± 7.35
Bacillus thuringiensis B43	72.72 ± 6.59	955.41 ± 7.35

3.16 Fourier transforms infrared spectroscopy analysis

Fourier transforms infrared spectroscopy (FTIR) is one of the significant and fast means to acquire information on polymer structure because every chemical compound in the sample creates its own distinct contribution to the transmittance spectrum. FTIR analysis was done at resolution of 4000–400 cm^{-1}.

Polymer extracted from *B. cereus* HB45 and *B. thuringiensis* B43 was analyzed by FTIR using 8400S Shimadzu spectrophotometer (Fig. 3.48 and Fig. 3.49). From the obtained spectrum of *B. cereus* HB45, it could be inferred that the band at 3441.01 cm^{-1} corresponds to OH (Hydroxyl) group, whereas band at 1730.15 cm^{-1} represents C=O (Carbonyl) and COO (ester) groups. Peak at 1730.15 cm^{-1} corresponds to the ester carbonyl group indicating anhydride groups grafted onto PHB backbone and another illustrated band at 2931.80 cm^{-1} corresponding to C-H stretch. The band at 1456.26 cm^{-1} corresponds to CH showing asymmetrical stretching and bending vibration in CH_3 group, whereas band at 1381.03 cm^{-1} representing COH bond. Stretch of bands ranging from 1055-1286 cm^{-1} corresponds to the stretching of the C–O bond of the ester group. Based on the observed FTIR data it could be confirmed that the synthesized polymer extracted from the isolated bacteria was found to be PHB.

In case of *B. thuringiensis* B43, FTIR analysis confirms the PHB and been compared with the earlier reported articles, the peak at 3441.01 cm^{-1} revealed the intramolecular polymeric hydrogen bond, whereas band at 1728.22 cm^{-1} represents C=O (Carbonyl) and COO (ester) groups. Peak at 1728.22 cm^{-1} corresponds to the ester carbonyl group indicating anhydride groups grafted onto PHB backbone and another illustrated band at 2929.87cm^{-1} corresponding to C-H stretch. The band at 1456.26 cm^{-1} corresponds to CH showing asymmetrical stretching and bending vibration in CH_3 group, whereas band at 1382.96 cm^{-1} representing COH bond. Stretch of bands ranging from 1053-1284 cm^{-1} showed C-O bonding. The spectroscopic analysis presented a correct insight for the chemical structure of PHB by representing the functional groups present in the PHB polymers. The spectral peaks were compared with the standard polyhydroxybutyrate obtained from Sigma Aldrich. The analyzed results correlated with the report of Arun *et al.* (2009) confirming that the isolated compound was PHB.

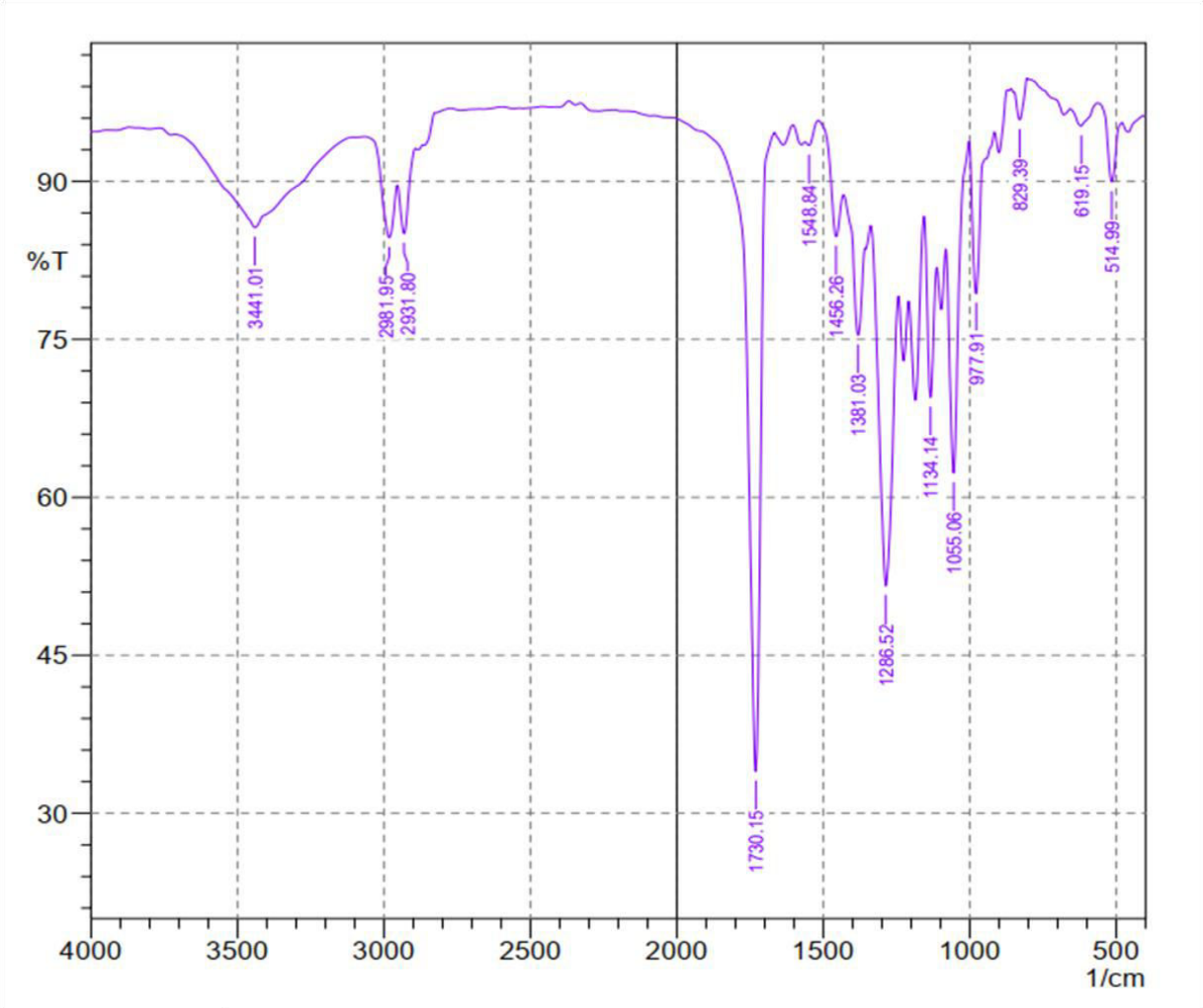


Fig. 3.48: FTIR spectra of PHB produced by *Bacillus cereus* HB45.

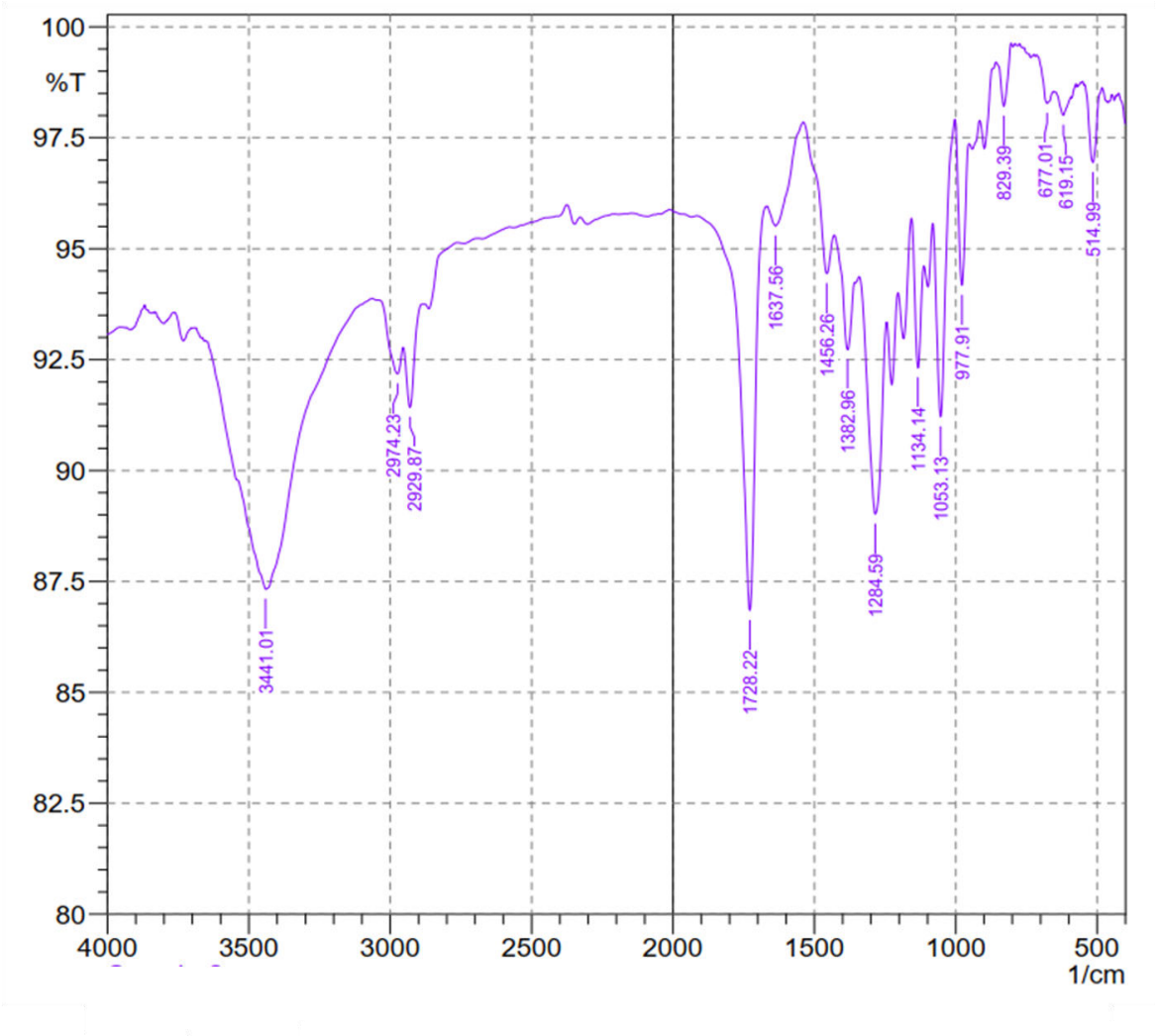


Fig. 3.49: FTIR spectra of PHB produced by *Bacillus thuringiensis* B43.

3.17 Production of PHB film

PHB films were produced by *Bacillus cereus* HB45 and *Bacillus thuringiensis* B43 using best carbon and nitrogen source (Fig. 3.50). PHB film from *B. cereus* HB45 was produced using mannitol at 1% concentration, 1.5% tryptone and 2% ammonium orthophosphate. PHB films from *B. thuringiensis* B43 was also produced using 1% sucrose, 1.5% Yeast extract and 2% ammonium chloride; evaporating the filtrate at 4°C. The PHB films produced were very stiff and brittle in appearance and were found to be fragile.

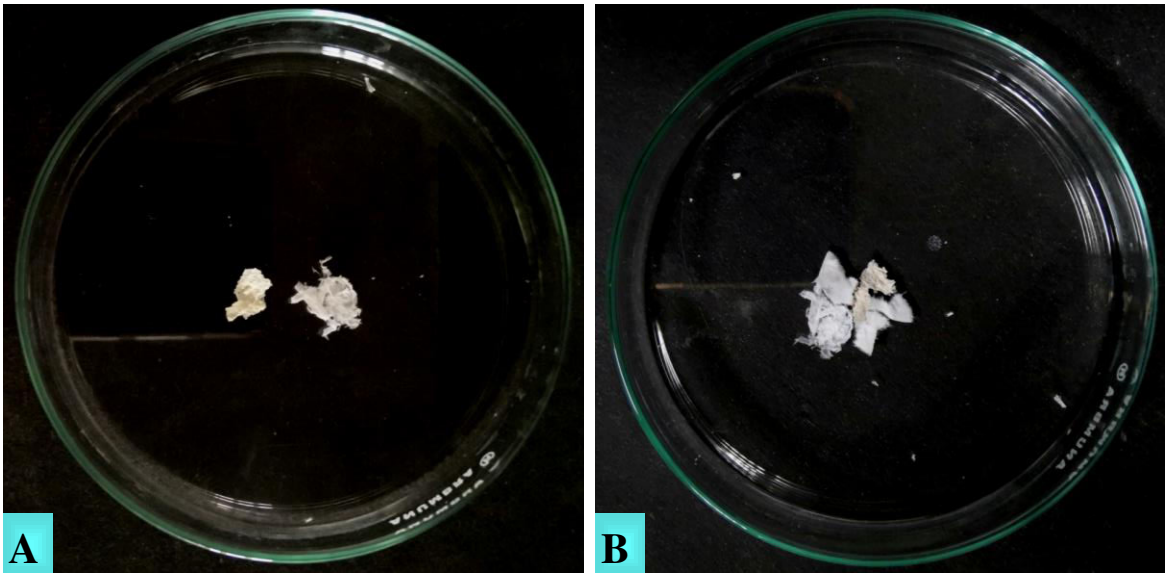
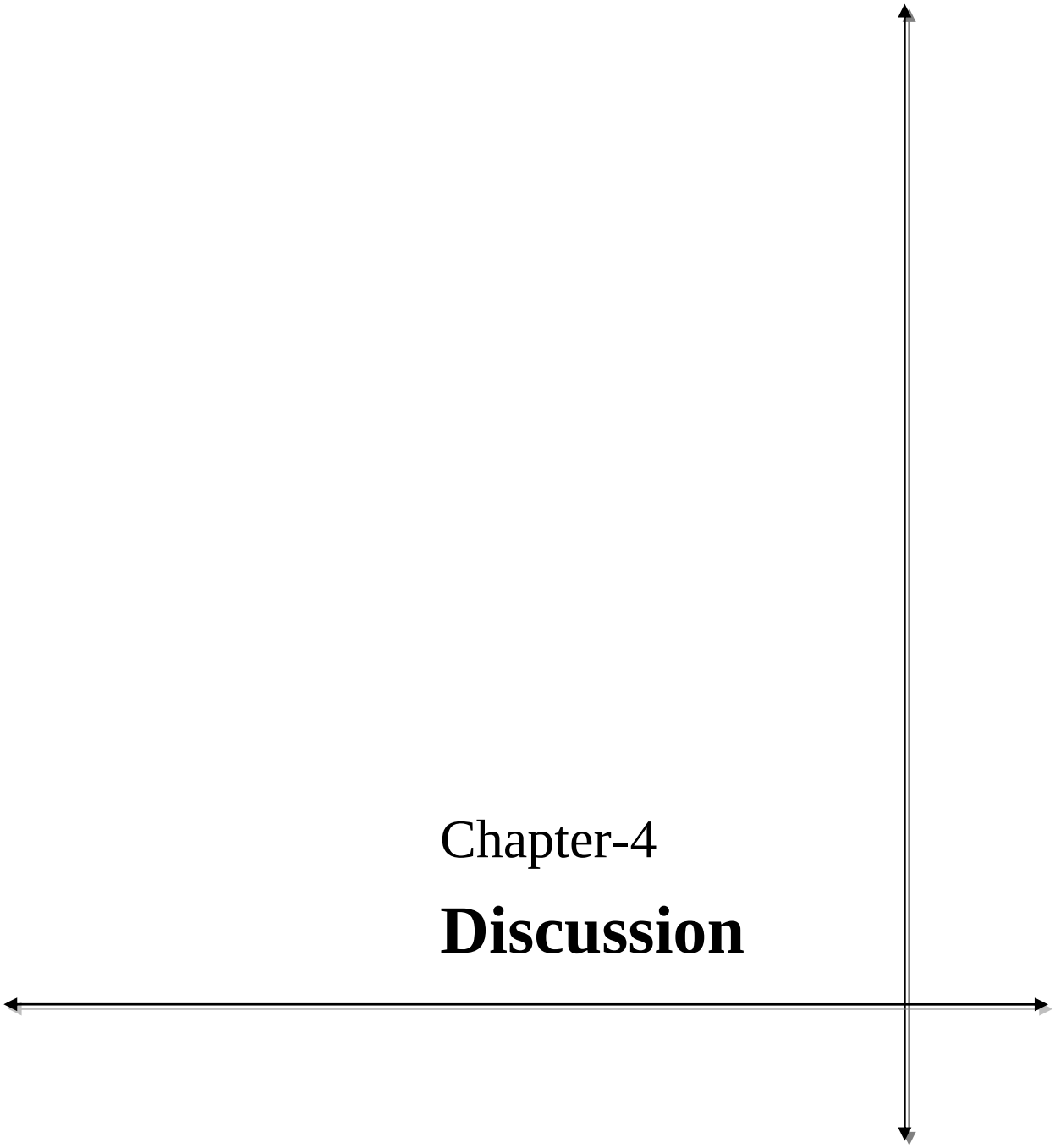


Fig. 3.50: Photographs showing the PHB films produced by A) *Bacillus cereus* HB45 and B) *Bacillus thuringiensis* B43

Chapter-4

Discussion



Discussion

Bio-plastics made up of polyhydroxyalkonate (PHA) could be a major substitute to traditional plastics. PHA is produced inside the bacteria naturally under stressful condition. They are found as intracellular granules in bacteria (Peters and Rehm 2005). PHA based plastics can be synthesized using various renewable sources such as low-molecular weight, fatty acids, alcohols, saccharides etc. Such bioplastics are completely degradable to carbon dioxide and water through natural microbial mineralization. PHAs have been a polymer producing factories to switch from ecologically harmful end-of-the-pipe production towards sustainable technologies (Verlinden *et al.* 2007).

PHB is synthesized from acetate or butyrate via betahydroxyybutyrl–CoA. It's described as "the first example of a true thermoplastic from biotechnology". PHB can be produced from bacteria under "stressful condition" and substantiated with other "carbon sources". In response, it built up the sugar into granules of PHB essentially in an effort to preserve them for late rmetabolization (Vieira *et al*. 2011). PHB is a stiff and rather brittle polymer of high crystallinity whose mechanical property is not unlike polystyrene which is less brittle and more temperate resistant. PHB based plastic substitutes are less flexible than traditional plastics; they are completely biodegradable and leave behind no residue (Mikkili *et al.* 2014).

PHB is the simplest biopolyester of the PHA family and accumulated as intracellular carbon and energy storage compound in many bacteria that are challenged by nutrient constraints.

Nature is the most blooming and divergent source for getting industrially important microbes. Natural microbial diversity in environment can be harnessed for getting the desired organisms. Natural sources may have extreme environmental factors and nutrient limiting circumstances, these factors can trigger PHB production in adept producers. Many efforts have been commenced on the isolation and characterization of PHB producers from various natural sources. PHB producers are adapted to inhabit in various biological niches such as dairy wastes, hydrocarbon contaminated sites, agricultural wastes, activated sludges, pulp and paper mill wastes and industrial effluents which are directly or indirectly exposed to high organic stuff or growth limiting conditions (Saharan *et al.* 2014). In the current study, PHB producing bacteria were isolated from varied sources viz. domestic and industrial waste of Dhaka Metropolitan City.

Considering importance and applications of the PHB, five water and five soil samples were collected from ten different habitats in and around Dhaka Metropolitan City. Nutrient Agar supplemented with 1% glucose (NAG), Peptone Yeast Extract Glucose (PYG) and Luria Bartani (LB) media were used for the enumeration and isolation of aerobic heterotrophic bacteria. The bacterial load of the collected samples ranged in between 5.55×10^3 and 2.52×10^7, 3.10×10^4 and 2.23×10^7, 7.50×10^3 and 1.01×10^7 cfu/g or cfu/ml in NAG, PYG and LB media, respectively. The maximum bacterial count (2.52×10^7cfu/g) was observed in NAG medium from soil sample of BDR market. On the other hand lowest bacterial load (5.5×10^3cfu/ml) was observed in NAG medium from the garments waste water. Among the media used in this experiment for isolation NAG could be considered as good isolating medium for PHB producing bacteria. The highest number (33) of PHB producing bacteria was found in kitchen waste soil and

the lowest number (9) was found from the Turag River water sample (Table 3.1). The findings of this study clearly reflected that kitchen waste could be considered as good source of PHB producers since it contains good nutrients in all respect.

A total of 160 PHB positive bacterial colonies were primarily screened from a range of samples from different habitats. After purification, 148 bacterial isolates were found to be PHB positive. Out of 148 isolates, 30 were found to be better PHB producers.

Staining properties and microscopic observation of the selected bacterial isolates revealed that 21 isolates were Gram positive rod and 9 were Gram negative short rods. The results showed that most of the PHB producing bacteria were Gram positive. This was in accordance with the findings of Shah (2014) in which it was reported 10 Gram positive bacilli and 5 Gram negative bacilli out of 15 isolates cultured from soil sample. Similarly, Raju *et al*. (2012) also found that among 23 isolates 12 were Gram positive bacilli, 2 were Gram positive cocci and 9 were Gram negative bacilli isolated from soil and waste water samples.

For provisional identification, the morphologically, physiologically and biochemically characterized 30 isolates were compared with the standard descriptions of the Bergey's Manual of Systematic Bacteriology Vol. 1 (Krieg and Holt 1984) and Bergey's Manual of Systematic Bacteriology Vol. 2 (Sneath *et al*. 1986). Among 30 bacterial isolates all 21 Gram positive isolates were belonged to the genus *Bacillus*. Under the genus *Bacillus*, there were 9 distinct species such as *Bacillus anthracis*, *B. cereus*, *B. lentus*, *B. megaterium*, *B. mycoides*, *B. pumilus*, *B. schlegelli*, *B. subtilis* and *B. thuringiensis*. Different species of *Bacillus* have been reported as PHB producer from various

environments such as wastewater, sewage and sludge ecosystems (Sangkharak and Prasertsan 2012). Moreover, *Bacillus* is the predominant genus in soil and water and capable of growing in cheap raw materials than other bacterial isolates (Khiyami *et al*. 2011). In a study, Yuksekdag *et al.* (2004) reported PHA production by *Bacillus subtilis* and *Bacillus megaterium*. Ghate *et al.* (2011) reported *Bacillus cereus* and *Bacillus megaterium* as good PHB producers. Production and characterization of PHB produced by *Bacillus megaterium* NCIM 2475 was reported by Otari *et al*. (2009). The production of PHB by *Bacillus* species from industrial wastes was reported by Full *et al*. (2006). *B. thuringiensis* R1 (Rohini *et al.* 2006) and *B. mycoides* DFC1 (Narayanan and Ramana 2012) reported as a PHB accumulating bacteria. Swati *et al.* (2017) reported that wide arrays of PHAs producer *Bacillus* species are recorded with diverse biosynthetic mechanism and functional properties.

Gram negative isolates were identified as *Acetobacter aceti*, *A. liquefaciens*, *Enterobacter cloaceae*, *Klebsiella oxytoca*, *Neisseria elongata*, *Pseudomonas aeruginosa*, *Rhizobium leguminosarum* and *Tatumella ptyseos*. Mercan *et al*. (2002) reported *Pseudomonas* and *Rhizobium* having the ability of PHB production. Priya and Harish (2013) isolated and characterized *Rhizobium leguminosarum* which was capable of producing PHB. Nair (2013) mentioned PHB production from different purified cultures *viz.* *Alcaligenes* sp., *Neisseria* sp., *Bacillus* sp. and *Pseudomonas* sp. etc.

Molecular identification on the basis of 16S rRNA sequence analysis could be considered as more accurate identification of microbial species than that of conventional identification due to the process being troublesome and time consuming (Poorani *et al.* 2009). Identification of bacterial isolates based upon 16S rRNA gene sequence analysis

had widely been used (Bajaj and Sharma 2011; Charen *et al*. 2014 and Liu *et al.* 2014). In the present study, 10 better PHB producer bacterial isolates were selected for molecular identification. In molecular identification, the bacterial isolate S_2N-4 was identified as *Bacillus cereus* HB45 having 93% identity match and maximum coverage score was 680. The bacterial isolate S_4P-2 was identified as *Bacillus subtilis* NXUSASNFB008 having 97% identity match and maximum coverage score was 913. The bacterial isolate S_4L-3 was identified as *Bacillus thuringiensis* B43 having 99% identity match and maximum coverage score was 1007. The bacterial isolate S_7N-5 was identified as *Bacillus cereus* AK9 having 95% identity match and maximum coverage score was 784. The bacterial isolate S_7P-29 was identified as *Bacillus anthracis* S2CB42 having 91% identity match and maximum coverage score was 691. The bacterial isolate S_8P-2 was identified as *Sinorhizobium* sp. R25067 having 98% identity match and maximum coverage score was 961. The bacterial isolate S_8N-6 was identified as *Bacillus mycoides* TCCC11292 having 98% identity match and maximum coverage score was 1007. The bacterial isolate S_8P-11 was identified as *Bacillus cereus* WCF2 having 99% identity match and maximum coverage score was 1013. The bacterial isolate S_8L-18 was identified as *Bacillus cereus* PU having 99% identity match and maximum coverage score was 1007. The bacterial isolate S_{10}L-16 was identified as *Bacillus megaterium* LY-6 having 97% identity match and maximum coverage score was 913. Mizunoa *et al.* (2010) studied PHB producing *Bacillus cereus* YB-4 group with 16S rDNA sequence analysis. Narayanan and Ramana (2012) performed 16S rRNA gene sequence analysis with NCBI-BLAST homology search program and identified the bacterium as *Bacillus mycoides* DFC1 strain.

A comparative analysis was done in between provisional and molecular identification of 10 bacterial isolates (Table 3.11). Among 10 isolates, 9 genera were matched with their conventional provisional identification. Molecularly identified *Sinorhizobium* sp. R25067 differs with its provisional identification. The differed isolate was provisionally identified as *Rhizobium leguminosarum*. On this point it could be mentioned that conventional identification based on morphological, physiological and biochemical characters were still found to be valid with minor exceptions. Therefore conventional identification could be practiced where molecular identification facilities are not available.

A phylogenetic tree among the 10 bacterial isolates was generated using neighbor joining (NJ) distance based algorithm of phylogenetic analysis based on 16S rRNA sequences acquired for the 10 isolates (Fig 3.23). In the phylogenetic tree the closely related bacterial strains were grouped together while strains having distant relationships were placed separately.

Molecularly identified and better PHB producer 10 bacterial isolates were chosen for quantitative analysis of PHB activity. The PHB producing capabilities of the isolated indigenous 10 isolates ranged in between 3.43 ± 0.24 and 79.75 ± 6.10 µg/ml. All the isolates showed maximum PHB activity after 72 h of incubation except *Bacillus megaterium* LY6. *Bacillus megaterium* LY6 showed the highest PHB activity after 96 h of incubation. All the isolates showed decreased PHB production after 72 h of incubation with the increase of the incubation period. This is in agreement with work of Singh *et al.* (2011) where they mentioned that PHB production increases as the incubation time increases but later falls with the increase in incubation time. The present study also found to be similar to the PHB production reported by Navarro *et al.* (2006). Majority of the

bacterial isolates exhibited the highest PHB yield after 48-72 h of fermentation. During stringent conditions PHB is used by the cell as an internal reserve of carbon and energy. Singh *et al.* (2013) reported that maximum PHB production time varies among different bacteria and depends mostly upon cultural or environmental conditions used during fermentation and genetic make-up of the particular organism. The PHB production in *Bacillus subtilis* NG220 increased up to 72 h and got declined afterwards. This decrease in production of PHB after 72 h specified that the bacteria used PHB as nutrient source when normal nutrients exhausted. Time course analysis indicated that PHB was a growth-associated product. The reduction in PHB production after 72 h might be due to availability of micronutrients as well as increase in toxic metabolites during the PHB production (Baikar *et al.* 2017). In this research the only Gram negative *Sinorhizobium* sp. produced a little amount of PHB after 72 h of incubation. So far there was no literature available about PHB production by *Sinorhizobium* sp. Therefore it could be the first report here in Bangladesh. During this study two isolates *viz*. *Bacillus cereus* HB45 and *Bacillus thuringiensis* B43 showed PHB activity more than 70 µg/ml and were selected for detailed study for PHB production.

Optimization of fermentation conditions has been used to enhance yields and productivities of many bioprocesses. Hence, in order to maximize PHB production by the selected indigenous isolates, various factors such as pH, temperature, carbon and nitrogen source, inoculum concentration etc. were studied for optimum PHB production. Optimization of different parameters for PHB production was studied by Singh *et al.* (2011) and Shaaban *et al.* (2012). Among different parameters, the pH of fermentation medium and the temperature of the fermentation process might play a major role on the

PHB activity. Optimization of PHB production was carried out at 72 h of incubation by two better PHB producer *viz*. *Bacillus cereus* HB45 and *Bacillus thuringiensis* B43.

pH of the culture medium is one of the most critical parameters affecting both growth and PHB production. In this study a pH range in between 5 and 9 was set to investigate for the optimum pH of PHB production. The optimum pH of the PHB production was found to be pH 6 by *Bacillus cereus* HB45 and *B. thuringiensis* B43 and showed PHB activity 161.24 ± 3.94 and 253.16 ± 20.41µg/ml, respectively at 72 h of incubation.

The most favourable pH for bacterial growth and PHB production was obtained at pH range of 6.0 -7.0 in case of *Bacillus* sp. (Singh *et al.* 2011), *Bacillus sphaericus* NCIM 5149 (Ramadas *et al.* 2009), *Bacillus thuringiensis* IAM 12077 (Shivakumar 2012) *Rhizobium elti* E1 and *Pseudomonas stutzeri* E114 (Belal 2013). Grothe *et al.* (1999) reported that pH value ranged from 6.0 to 7.5 for optimum PHB production by *Alcaligens latus*.

The pH of the culture media influences the amount of PHB production. The present findings showed that the bacteria were capable of producing PHB in the range in between pH 5 and 9. This was in agreement with Shaaban *et al.* (2012) and reported that good bacterial growth occurred at pH 5 to 9 whereas PHB production was maximum at pH 6 to 7 and decreased above or below this pH level. Aslim *et al.* (2002) also reported that maximum PHB activity from *Rhizobium meliloti* was at pH 7. Sindhu *et al*. (2011) observed that the effect of initial pH on PHB production by *Bacillus sphaericus* NII 0838 showed that pH 7 was optimum for maximum PHB synthesis. Considering all available information it could be concluded that maximum PHB activity would be ranged between pH 6 to 7.

Temperature would be another important parameter shows influence on the development of the organisms and the production of PHB. Therefore, the effect of the temperature, five temperature ranges were tested *viz.* 20, 30, 37, 40 and 50°C for the PHB production. It had been ascertained that the chosen strain encompasses an optimum temperature at 37°C. *Bacillus cereus* HB45 and *B. thuringiensis* B43 showed maximum PHB activity (207.29 ± 10.35 and 280.09 ± 10.35 µg/ml) at 37°C (Fig. 3.30, 3.31). In all isolates, there was a direct relationship between PHB production, growth and temperature. The increase of temperature beyond 37°C had negative impact on PHB production. Bhuwal *et al*. (2013) optimized different incubation temperatures for the production of PHB. The effect of different temperatures ranging from 25 to 55°C were studied for PHB production. It was observed that optimum temperature was at 35°C. Fathima and Krishnaswamy (2016) observed maximum PHB production at 35°C. Bellard *et al*. (1995) reported maximum cell density and PHB accumulation at 37°C. In a study, Gayathiri *et al*. (2017) reported similar type of observation and reported 284 µg/ml of PHB by *Bacillus subtilis* at 37°C.

The PHB activity of the present study droped rapidly above the temperature 40°C in both of the isolates. This decrease in PHB production at high temperature is also supported by the report of Grothe *et al.* (1999) and Yuksekdag *et al.* (2004). The decrease in PHB production at high temperature could be due to low PHB polymerase enzyme activity. Tamdogan and Sidal (2011) reported that higher and lower temperatures than 30°C lead to decrease in cell biomass and PHB synthesis by *Bacillus subtilis* ATCC6633. This result also supported by Aslim *et al*. (2002) and Hamieh *et al*. (2013), who reported that optimum incubation temperature for PHB production by *Bacillus subtilis*, *Bacillus pumilis* and *Bacillus thuringiensis* was 37°C.

PHB synthesis proceed best in an environment with high carbon to low nutrient ratio. Role of carbon sources are significant as they serve different functions within the organism: biomass synthesis, cell upholding and PHB polymerization (Hungund *et al.* 2013).

Sugars are the most regular substrate used for PHB synthesis by bacteria. The major content of PHB production media is the carbon source that also acts as an initiator for PHB production. Present study was aimed to find most ideal carbon source for PHB accumulation and due to this reason a wide range of cost-effective carbon sources were examined for PHB production. In this study glucose, sucrose, starch, arabinose, xylose and mannitol were used as carbon source (1%, w/v) to investigate the better carbon in PHB production by two selected isolates. *Bacillus cereus* HB45 showed the highest activity (338.99 ± 26.40 µg/ml) when production medium provided with mannitol as carbon source (Fig. 3.32). *Bacillus thuringiensis* B43 showed the highest PHB activity (449.77 ± 11.03 µg/ml) when medium containing sucrose (Fig. 3.33). Some carbon sources like arabinose and sucrose gave comparably the lowest yield (85.16 ± 1.95, 12.96 ± 0.62 µg/ml) in case of *Bacillus cereus* HB45. Arabinose and xylose showed the lowest yield (24.75 ± 0.29, 25.58 ± 0.30 µg/ml) in case of *Bacillus thuringiensis* B43. The result clearly reflected that mannitol and sucrose could be considered as good carbon sources for PHB production.

Gayathiri *et al*. (2017) showed that sucrose was found to be more suitable for PHB accumulation (294 µg/ml) by *Bacillus subtilis*. Anderson and Dawes (1990) showed accumulation of PHB by *Alcaligenes faecalis* using fructose as the carbon source. Belal and Farid (2016) showed the highest PHB productivity by *B. cereus* growing on sucrose.

Borah *et al*. (2002) reported the use of sucrose as the cheaper source for the production of PHB by *B. mycoides* RLJ B-017. Similar results were reported for *Rhizobium meliloti* by Kshama (2004). Thapa *et al*. (2018) showed that *B. pumilus*, *B. pasteurii* and *B. sphaericus* preferred sucrose while *B. megaterium* preferred glucose and *B. cereus* preferred both glucose and sucrose as the carbon source. Ponnusamy *et al*. (2019) reported production medium supplemented with starch produced maximum PHB by *B. thuringiensis* E101. From this discussion it could be inferred that carbon sources varied case by case for PHB production.

After screening the carbon sources, optimization of better carbon sources were carried out for the production of PHB. Various concentrations of better carbon source, mannitol were supplemented in the medium with 0.5-3.0%, (w/v) to investigate the effect on the PHB production by *Bacillus cereus* HB45. Out of six concentrations of mannitol, 1.0% (w/v) supported the highest PHB production when compared to other levels and thereafter, a decrease concentration was observed. The PHB activity with 1% mannitol as carbon source was 335.80 ± 3.47µg/ml by the *Bacillus cereus* HB45 (Fig. 3.34). Various concentrations of sucrose (0.5-3.0%, w/v) were supplemented in the medium to investigate the effect on PHB production by the isolate of *Bacillus thuringiensis* B43 since sucrose was found to be suitable for PHB production. The highest PHB activity (425.50 ± 13.99 µg/ml) showed at 1% concentration of sucrose (Fig. 3.35). Gayathiri *et al*. (2017) reported that 2% concentration of sucrose supported the highest PHB production by *Bacillus subtilis*. The PHB production using different fermentable sugars was tested and it was found that maximum biomass was produced with 2% (w/v) sucrose (Halami 2008). Sindhu *et al.* (2011) used medium with different concentrations of crude

glycerol as sole carbon source ranging from 1-5% (w/v), and found that maximum PHB yield was obtained at 1% glycerol concentration by *Bacillus sphaericus* NII 0838. There were very few reports available on PHB production with different concentrations of mannitol.

Nitrogen sources are of secondary energy sources for the organisms which play an important role in the growth of the organism and the production of PHB. Generally, nutrient constraints enhance PHB production whereas high nitrogen source leads to growth of biomass with no PHB production (Albuquerque *et al*. 2010). Low nitrogen concentration is good for PHB accumulation. Under normal conditions, bacteria synthesize their cell materials like proteins and grow but in nutrient limiting conditions bacteria may shift their protein synthesis to PHB synthesis for survival. Therefore, nutrient control is required to prompt PHB accumulation (Elsayed *et al.* 2013).

Different organic nitrogen sources such as peptone, tryptone, yeast extract and beef extract were tested for maximum production of PHB by the selected bacterial isolates. Results clearly showed that tryptone and yeast extract were found to be better for PHB production than other nitrogen sources used. In this study *Bacillus cereus* HB45 showed the highest PHB activity (432.86 ± 8.13 µg/ml) when tryptone was added to the medium whereas *Bacillus thuringiensis* B43 showed the highest activity (280.16 ± 2.24 µg/ml) when yeast extract was added in the medium (Fig. 3.36 and Fig. 3.37). Yeast extract gave similar results in case of *Bacillus thuringiensis* E101 reported by Ponnusamy *et al*. (2019). In another experiment, Chaijamrus and Udpuay (2008) was used peptone as the most effective nitrogen source for PHB production by *Bacillus cereus* PS 10. They also reported that higher nitrogen concentration in the medium decrease the PHB yield. Singh *et al*. (2011) showed that when tryptone as organic nitrogen source was used, the

bacterial isolate *Bacillus* sp. was able to produce higher amount of PHB. Paul *et al.* (2017) showed that peptone was found to be more suitable nitrogen source for PHB accumulation (382 µg/ml) by *Bacillus cereus*.

The best organic nitrogen source was added in different concentration for maximum PHB production. Tryptone was found to be better for the isolate *Bacillus cereus* HB45. Therefore, various concentrations (0.5-3.0%, w/v) of tryptone were supplemented in the medium for *Bacillus cereus* HB45. The PHB activity showed the highest (444.62 ± 7.36 µg/ml) at 1.5% concentration of tryptone by *Bacillus cereus* HB45 (Fig. 3.38). Yeast extract was found to be better for *Bacillus thuringiensis* B43, so that various concentrations (0.5-3.0%, w/v) of yeast extract were supplemented in the medium to investigate the effect on PHB production of *Bacillus thuringiensis* B43. The highest amount of PHB production was shown by *Bacillus thuringiensis* B43 (273.05 ± 13.97 µg/ml) at 1% of yeast extract (Fig. 3.39). The result reflected that sources and concentration of organic nitrogen varied case by case. In another experiment, peptone the most effective nitrogen source was used at different concentrations (0.5-5 %) for PHB production from *Bacillus cereus* PS 10. It was observed that with increase in peptone concentration i.e. 0.5-1 %, PHB yield increased considerably. However, above 2 % PHB synthesis decreased. High nitrogen concentration in the medium has been reported to decrease the PHB yield (Chaijamrus and Udpuay 2008).

Besides organic nitrogen sources, various inorganic nitrogen sources *viz.* ammonium chloride, ammonium nitrate and ammonium orthophosphate were also tested to the PHB production. In this study ammonium orthophosphate was found to be the best inorganic nitrogen source for PHB production by the *Bacillus cereus* HB45 (423.54 ± 3.62 µg/ml)

and ammonium chloride was found to be more suitable for *Bacillus thuringiensis* B43 (432.86 ± 4.43 µg/ml) (Fig. 3.40 and Fig. 3.41). Singh *et al*. (2011) also observed ammonium orthophosphate as the best inorganic nitrogen source for *Bacillus* sp. Ammonium chloride was also reported as nitrogen source for efficient PHB production from *Azotobacter beijerinckii* DSMZ-1041 (Montaser *et al*. 2011). Inorganic nitrogen source such as ammonium chloride has been reported to be effective in case of *Stenotrophomonas maltophilia* (Singh and Parmar 2011). However, contrary to current results, ammonium sulphate was found to be the best nitrogen source for PHB production from various bacteria such as *Alcaligenes eutrophus*, *Methylobacterium* sp. and *Sinorhizobium fredii*. Ammonium sulphate as nitrogen source gave maximum PHB yield by halotolerant photosynthetic bacteria *Rhodobacter sphaeroides* when cultured under aerobic and dark conditions (Sangkharak and Prasertsan 2008).

Six different concentrations (0.5-3.0%, w/v) of better inorganic nitrogen source were added to the medium for PHB production. Ammonium orthophosphate was found to be better inorganic nitrogen source for *Bacillus cereus* HB45. Therefore, different concentrations of ammonium orthophosphate was added in the medium to investigate the effect on PHB production of *Bacillus cereus* HB45. At 2% concentration, ammonium orthophosphate attained maximum PHB activity 516.68 ± 7.35 µg/ml was observed by *Bacillus cereus* HB45. Different concentrations of ammonium chloride (0.5-3.0%, w/v) were supplemented in the medium for PHB production by *Bacillus thuringiensis* B43. The highest PHB production (508.10 ± 13.66 µg/ml) recorded at 2% concentration of ammonium chloride (Fig. 3.43). Singh *et al*. (2011) reported the highest PHB activity by *Bacillus* sp. at 1% concentration of ammonium orthophosphate. Belal and Farid (2016)

showed the highest PHB productivity by *B. cereus* growing on ammonium sulphate. They reported that increasing of PHB production with an increase in ammonium sulphate concentration from 0.5% to 1.0%. They found that *B. cereus* was found to produce the highest PHB yield at 1 % concentration. The presence of higher concentration of nitrogen in the culture medium was found to be inhibitor for the accumulation of PHB.

Bacteria have been found to accumulate PHBs when there is a limitation of nutrients, specifically nitrogen sources (Steinbuchel and Fuchtenbusch, 1998). In this study, PHB production was increased with increase concentration of ammonium orthophosphate and ammonium chloride up to 2 % and thereafter, PHB production was declined. Present study indicated that this finding cannot be generalized for every PHB producing bacteria. This leads us to hypothesize that PHB might be synthesized by different microbes depending on their individual physiological requirements and that in certain species, PHB accumulation may not truly occur in response to nutrient stress as has been generally accepted. More detailed studies need to be performed before this authentication.

Inoculum size could be considered as one of the most important parameter for process optimization. Amount of initial biomass might influence the final yield of PHB. Current study showed that 2% inoculum was found to be the best for maximum PHB yield by *Bacillus cereus* HB45 and 1% was best for *Bacillus thuringiensis* B43 (Fig. 3.44 and Fig. 3.45). The highest PHB production was 179.87 ± 4.31 µg/ml by *Bacillus cereus* HB45 at 2% inoculum concentration and 242.59 ± 9.02 µg/ml by *Bacillus thuringiensis* B43 at 1% inoculum. *Bacillus sphaericus* NCIM 5149 produced maximum PHB when inoculum was used at 2% (Ramadas *et al.* 2009) while *Bacillus subtilis* NG220 responded at 1% inoculum (Singh *et al.* 2013). Studies on effect of inoculum level showed that 5×10^4 cells

in 100 ml medium gave maximum PHA accumulation. However, higher or lower inoculum range resulted in a major reduction in PHA production (Kumbhakar *et al.* 2012). Inoculum size might affect on PHB production by *Bacillus sphaericus* NII 0838 showed that inoculum concentration of 3% was the best for the highest PHB accumulation. However, decline in PHB production was noted with increased inoculum size beyond 3% and this indicated the crucial role of inoculum in PHB production. Most established approach for PHB production is that firstly very high cell densities are attained and then the limiting conditions are developed to trigger PHB accumulation (Sindhu *et al*. 2011).

A small amount of inoculum can lead to insufficient number of microbial cells and a reduced amount of PHB while a much higher inoculum could cause oxygen limitation, poor mixing and fast depletion of nutrients in the culture media, results in low PHB yield (Abusham *et al.* 2009). Decreased PHB yield at inoculum size (3-6%) is due to less amount of biomass while high inoculum size above 2-3% might had caused inadequate mixing and aeration due to increased viscosity of the medium. Increase in medium viscosity may lead to unfavorable conditions for production of extracellular metabolites; PHB yield may decrease due to presence of an intracellular PHB depolymerase, depletion of essential nutrients in the medium or self utilization of PHB by bacteria due to nutrient depletion (Madison and Huisman 1999 and Prasanna *et al.* 2011).

PHB production with different parameters was studied in current study to maximize the production of PHB. Considering all available findings all optimized conditions were provided for maximum PHB production. *Bacillus cereus* HB45 and *Bacillus thuringiensis* B43 showed PHB activity 867.17 ± 7.35 and 955.41 ± 7.35 µg/ml, respectively after optimization of different parameters (Fig. 3.46 and Fig. 3.47). Both the

isolate showed minimum activity at 24 h of incubation. The time of incubation showed sharp decrease in PHB activity after 72 h of incubation. The production of PHB, however, was noted at 72 h after which the production ceased as the bacteria utilized the PHB on reaching the starvation period. This could be due to the reason that there must have been a shift in metabolic pathway to other metabolism, so that the PHB yield reduced with the time progress (Thirumala *et al*. 2009).

Comparative analysis of PHB production by *Bacillus cereus* HB45 and *Bacillus thuringiensis* B43 is shown in Table 3.14. *Bacillus cereus* HB45 and *Bacillus thuringiensis* B43 showed the highest PHB activity (867.17 ± 7.35 and 955.41 ± 7.35 µg/ml) after 72 h of incubation. Comparative analysis showed that there were about 11 and 13 fold higher than that of before optimization. Interestingly, before optimization *Bacillus cereus* HB45 was better than *Bacillus thuringiensis* B43 but after optimization *B. thuringiensis* performed better than *B. cereus.* Therefore, optimization is very important for better production.

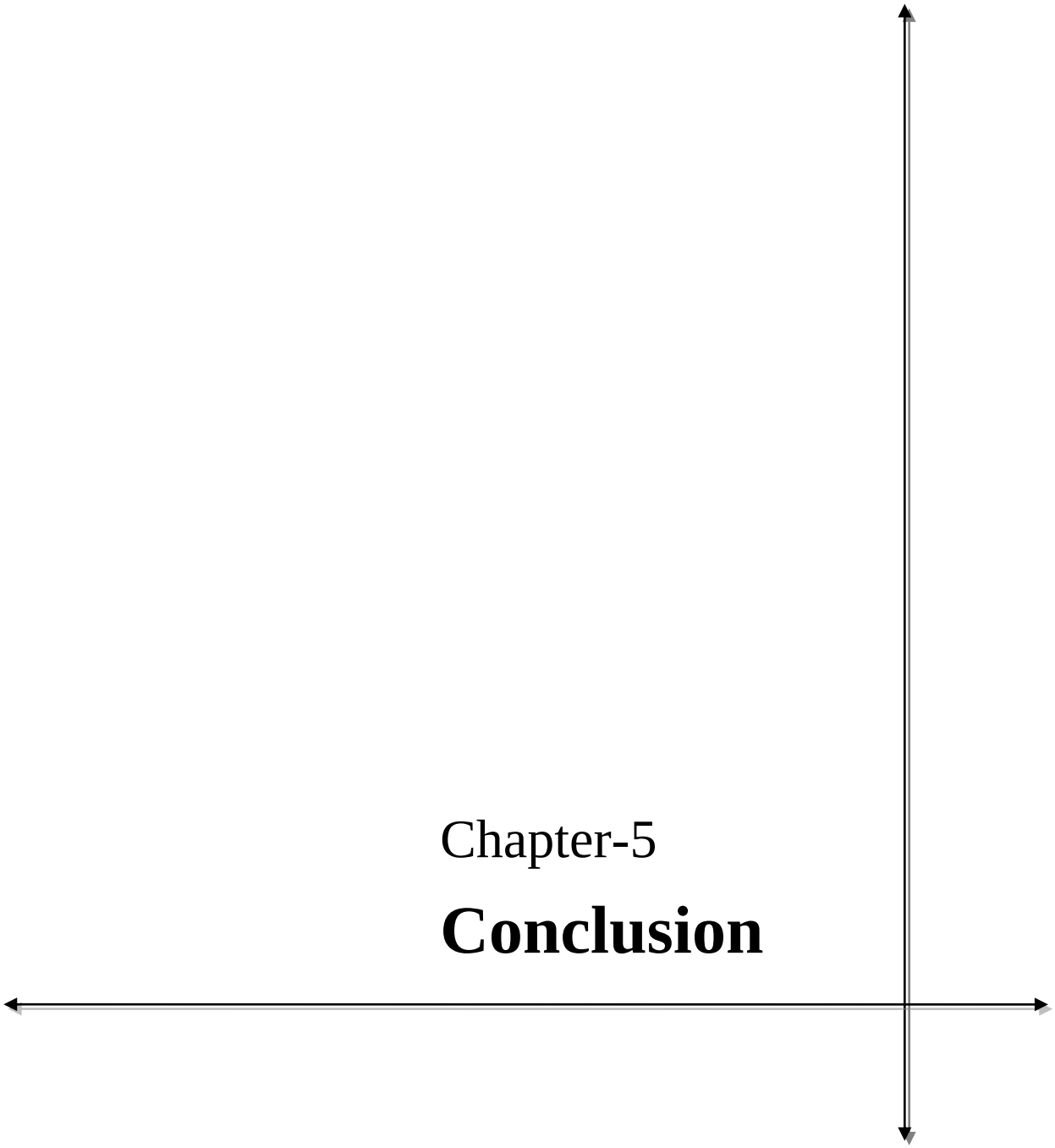
FT-IR is one of the rapid and powerful tools to obtain information about polymer structure, because every chemical compound makes its own distinct contribution to the absorbance spectrum. In the present study, it was observed that a spectrum of PHAs containing short chain length monomers such as HB and HV coincides with the previous reports. As described earlier in the results section, the FT-IR spectra (Fig. 3.48 and Fig. 3.49) of the extracted sample conferred the characteristic peaks of PHB-HV polymer.

The results described earlier in the results section are congruent with the previous findings. Band absorption between 1720 to1740 cm^{-1} is typical feature of PHB (Tripathi

et al. 2013). Two strong absorption peaks of PHB extracted from *Bacillus megaterium* MTCC 8075 were obtained at 1724.2 cm^{-1} and 1280 cm^{-1}, matching to C=O and C-O stretching groups (Muralidharan and Radha 2014). The FTIR spectra of PHA produced by *Bacillus circulans* MTCC 8167 showed high absorbance at 3360, 2922, 1735, and 1206 cm^{-1} and were supposed to be due to O-H, CH, C=O and C-O-C, respectively (Zribi-Maaloul *et al.* 2013). It had been identified that the most apparent spectral changes during PHB crystallization are in the carbonyl band at 1740-1720 cm^{-1}, whereas the amorphous phase presents a broad band near 1738 cm^{-1}, while the crystalline phase band transfer to lower wave numbers near 1722 cm^{-1}(Pachekoski *et al*. 2009). The most prominent marker (ester carbonyl) band for PHB was at 1740 cm^{-1} and 1724.03 cm^{-1} for pure PHB (Lo´pez-Corte´s *et al.* 2010). In pure PHB granule, asymmetrical deformation of C-H bond in CH_2 groups and CH_3 groups, C=O bond stretching and C-O ester bond are represented by wave numbers 1460, 1379, 1726 and 1150 cm^{-1}, respectively. FTIR spectrum of the polymer produced by *Bacillus firmus* NII 0830 showed peaks at 1724 cm^{-1} and 1279 cm^{-1}equivalent to specific rotations around carbon atoms. The PHB polymer extracted from *Bacillus thuringiensis* GVP was characterized by FTIR spectroscopy. The bands were recorded at 1724.04 cm^{-1} and 1280.69 cm^{-1} matching to C=O and C-O stretching group respectively. The peaks for CH_2, CH_3, C-O-C and terminal OH group occurred at 1458.15 cm^{-1},1381.71 cm^{-1}, 1184.33 cm^{-1} and 3444.40 cm^{-1}, respectively (Charen *et al.* 2014). PHA extracted from the fermentation with *Bacillus megaterium* S29 was characterized by IR transmission spectrum and exhibited main bands at 1726, 2960–2850, 1390–1370 and 1230–1050 cm^{-1} corresponding to the carbonyl group, methyl and methylene groups, the methyl group, and the ester group,

respectively (Rodriguez-Contreras *et al*. 2013). IR spectra for the polymer extracted from *Bacillus subtilis* NG220 was recorded in the range of 4000–600 cm^{-1}. FTIR spectra illustrated two strong absorption bands at 1705 and 1034 cm^{-1}, definite for C=O and C–O stretching vibrations, respectively. The absorption peaks at 2916 and 2955 cm^{-1} were due to C–H stretching vibrations of methyl, methylene groups. These prominent bands validated the structure of poly-*β*-hydroxybutyrate (Singh *et al*. 2013).

During this study, PHB films were prepared with the PHB produced by *Bacillus cereus* HB45 and *Bacillus thuringiensis* B43 using best carbon and nitrogen source (Fig. 3.50). The PHB films produced were very stiff, brittle in appearance and were fragile. PHB is a semi-crystalline material with an elevated melting temperature and a high degree of crystallinity. Its mechanical properties are equivalent to those of isotactic polypropylene. Furthermore, PHB has a comparatively high glass transition temperature which is near to room temperature and thus it is rigid and fragile at ambient temperature. Polymer blending offers appealing possibilities of preparing cheap biodegradable materials with useful mechanical properties. Copolymers generally have enhanced thermo-mechanical properties as compared to homopolymers. Copolymers (blended film) are glossy, easy to hold and less brittle in character as compared to only PHB film. Blends of PHB and polyhydroxyvalerate (PHV) can be reduced the brittleness of PHB. However, further investigation is required to understand the suitability of using these organisms in the homopolymer (PHB film) and copolymer production.



Chapter-5

Conclusion

Conclusion

Bacterial poly-3-hydroxybutyrate (PHB) is perceived to be the most suitable alternative to conventional plastics due to its absolute degradation and biocompatible properties. In the current study, primarily a total of 160 bacterial isolates from various waste materials were screened for PHB producing ability. After purification, 148 bacterial isolates were secondary screened. Based on microscopic observation, 30 isolates were found to be better PHB producers and were selected for detailed study. Staining properties and microscopic observation of the selected bacterial isolates revealed that 21 isolates were Gram positive rod and 9 were Gram negative short rods. These 30 PHB producers were provisionally identified on the basis of cell morphology, Gram reaction, physiological and biochemical characteristics. Out of these 10 potential PHB producers were molecularly identified using 16S rRNA gene technology. Molecularly identified 10 better PHB producers were studied in detail with special reference to biotechnological point of view.

The results clearly demonstrated that PHB producing bacteria are widely distributed in nature and different species of *Bacillus* were found to be the predominating genus. A comparative analysis was done in between provisional and molecular identification of 10 PHB producing potential bacterial isolates. Among 10 isolates, nine genera were matched with their conventional identification except *Rhizobium leguminosarum*, where conventionally identified *Rhizobium leguminosarum* was found to be *Sinorhizobium* sp. in case of molecular identification. The result clearly showed that conventional

identification technique could be still valid to some extent and can be used where molecular identification facility is not available.

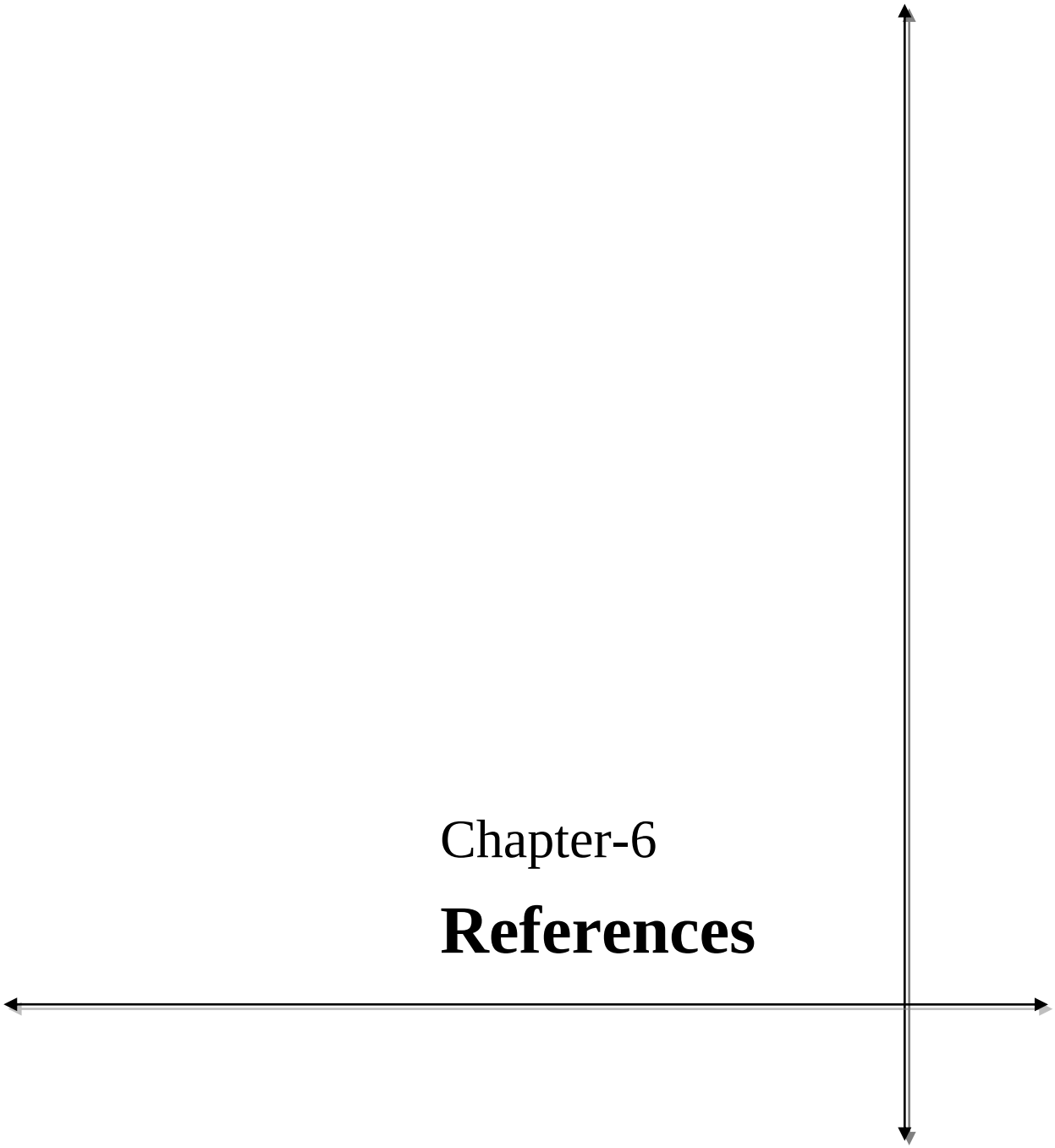
Extraction and quantification of PHB by sodium hypochlorite method and crotonic acid method respectively are well established analytical methods in PHB production and were used in this work. Two locally isolated bacteria showed PHB activity above 70 µg/ml. *Bacillus cereus* HB45 and *Bacillus thuringiensis* B43 were better PHB producers among all the indigenous bacterial isolates.

The cultural conditions had a considerable effects on PHB production especially carbon, nitrogen and inoculum concentration played important role in PHB production. The current study demonstrated that *Bacillus thuringiensis* B43, a bacterial isolate from industrial waste water has got enormous metabolic potential to use carbon sources for growth and PHB production. Sucrose supported maximum PHB production by *Bacillus thuringiensis* B43. In case of organic nitrogen sources, tryptone supported maximum PHB production by *Bacillus cereus* HB45. In addition to this, ammonium chloride was found to be better inorganic nitrogen source for maximum production of PHB by *Bacillus thuringiensis* B43.

Optimum conditions of the parameters investigated gave a baseline for further study involving large scale and cost-effective production of PHB by two isolates. The finally optimized medium increased PHB production than that of before optimization. The maximum PHB production was recorded as 955.41 ± 7.35 µg/ml by the *Bacillus thuringiensis* B43 which could be considered as biotechnologically important indigenous bacteria isolated from Bangladesh.

PHB extracted from the indigenous isolates was characterized by FTIR which gave thioester containing group which might be contain 2-methyl-3-hydroxybutyric acid. PHB films were prepared with the PHB produced by the two bacterial isolates using best carbon and nitrogen source. For large scale production of PHB, further scale-up studies using the optimized media must be needed.

Finally, it could be concluded that the two potential indigenous bacteria viz. *Bacillus cereus* HB45 and *Bacillus thuringiensis* B43 were found to be important for commercially PHB production. The environment of Bangladesh is very much potential for bacterial resources. More studies are to be needed for new potential isolates with special reference to PHB production and to address the new issue of environmental challenge.



Chapter-6

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Chapter-7

Appendix

Appendix

Composition of the media and reagents used in this study are as follows. In each case, pH was adjusted before sterilization.

1. Ammonium oxalate crystal violet solution (Claus 1995)

Solution A

Crystal violet (85% dye content)	2.0 g
Ethyl alcohol (95%)	20.0 ml

Solution B

Ammonium oxalate	0.8 g
Distilled water	80.0 ml

Solution **A** and **B** were mixed, Stored for 24 hrs before use.

2. Basal medium for fermentation (SAB 1957)

Diamonium hydrogen phosphate	1.0 g
Magnesium sulphate	0.2 g
Potassium chloride	0.2 g
Yeast extract	0.2 g
Agar	15.0 g
Distilled water	100.0 ml
pH	6.8

pH of the medium was adjusted before adding 15 ml of a 0.04% (w/v) solution of Bromocresol Purple.

0.5% Sugar (Carbohydrate) was added sterilized separately.

3. Buffer solution

pH of the buffer	Name and quantity of the chemical(s) used (g)	Volume of deionized water (ml)
4.5	KH_2P0_4- 5.45	200
6.5	KH_2P0_4- 2.72	200
	K_2HP0_4- 1.74	
	Na_2HP0_4-1.39	
8.5	K_2HP0_4- 3.48	200
	Na_2HP0_4- 2.78	

4. Deep glucose agar medium (SAB 1957)

Beef extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Agar	15.0 g
Distilled Water	100.0 ml
pH	7.2

5. Egg-yolk lecithinase medium (SAB 1957)

Peptone	40.0 g
Na_2HPO_4	5.0 g
KH_2PO_4	1.0 g
NaCl	2.0 g
$MgSO_4$	0.1 g
Glucose	2.0 g
Agar	15.0 g
Distilled Water	1000 ml
pH	7.2

Egg-yolk (1.5 ml) was added to 100 ml of basal medium.

6. Indole nitrate broth (Biolife Manual 1991)

Peptone	20.0 g
Disodium phosphate	2.0 g
Glucose	1.0 g
Potassium nitrate	1.0 g
Distilled water	1000 ml
pH	6.8

7. Iodine solution (SAB 1957)

Iodine	0.33 g
Potassium Iodide	0.66 g
Distilled water	100 ml

8. Kligler Iron Agar (Atlas 1997)

Peptone	40.0 g
Agar	12.0 g
Lactose	10.0 g
NaCl	5.0 g
Beef extract	3.0 g
Yeast extract	3.0 g
Glucose	1.0 g
Ferric citrate	0.3 g
$Na_2S_2O_3$	0.3 g
Phenol Red	0.3 g
Distilled Water	1000 ml
pH	7.2

9. KOH-creatine solution (SAB 1957)

KOH	40.0 g
Creatine	0.3 g
Distilled water	100 ml

10. Kovac's reagent (SAB 1957)

Para-dimethyl-amino-benzaldehyde	5.0 g
Butyl alcohol	75 ml
HCl (Conc.)	25 ml

11. Malachite green solution (Claus 1995)

Malachite green	5.0 g
Distilled water	100 ml

12. Methyl Red / Voges-Proskauer broth medium (Sneath *et al.* 1986)

Peptone	7.0 g
Glucose	5.0 g
NaCl	5.0 g
Distilled water	1000 ml
pH	6.5

13. Methyl red solution (Bryan 1950)

Methyl red	0.1 g
Ethyl alcohol (95%)	300 ml
Distilled water	200 ml

14. Motility medium (Eklund and Lankford 1967)

Nutrient broth	100 ml
Agar	0.3 g
TTC	0.001 g
pH	7.0 ± 0.2

15. α-Napthol solution (Bryan 1950)

α –Napthol	15.0 g
Ethyl alcohol (95%)	100 ml

16. Nutrient agar medium (Eklund and Lankford 1967)

Beef extract	3.0 g
Peptone	5.0 g
NaCl	5.0 g
Agar	15.0 g
Distilled water	1000 ml
pH	7.0 ± 0.2

17. Nitrate broth medium (SAB 1957)

Beef extract	3.0 g
Peptone	5.0 g
Potassium nitrate	1.0 g
Distilled water	1000 ml
pH	7.2

18. Oxidase test reagent (Collins and Lyne 1984)

Tetramethyl-p-phenylene-diamine dihydro-chloride	1.0 g
Distilled water	100 ml

19. Physiological saline

Sodium chloride	0.85 g
Distilled water	100 ml

20. Peptone Broth

Peptone	1.0 g
Distilled water	100 ml

21. Peptone Yeast Extract Glucose Agar (PYG) medium (Atlas 1997)

Glucose	10.0 g
Peptone	5.0 g
Yeast extract	5.0 g
Agar	15.0 g
Distilled water	1000 ml
pH	7.2

22. Propionate agar medium (Sneath *et al.* 1986)

Sodium propionate	2.0 g
$MgS0_4.7H_20$	1.2 g
NH_4HP0_4	0.5 g
KCI	1.0 g
Trace element solution	40 ml
Agar	15.0 g
Distilled water	920 ml
Phenol red (0.04% w/v)	20 ml
pH	6.8

23. Safranin solution (SAB 1957)

Safranin	0.5 g
Distilled water	100 ml

24. Simmon's citrate agar (Atlas 1997)

Sodium citrate	2.0 g
$MgS0_4.7H_20$	1.2 g
NH_4HP0_4	0.5 g
KCl	1.0 g
Trace element solution	40 ml

Agar	15.0 g
Distilled water	920 ml
Bromothymol blue (0.04% w/v)	20 ml
pH	6.8

25. Skim Milk Agar (SMA) medium (Collins and Lyne 1984)

Skim Milk	6.6 ml
Nutrient agar	100 ml
pH	7.2

Skim milk was sterilized separately for 5 minutes and was added to the medium before plating.

26. Starch Nutrient Agar medium (Claus 1995)

Beef extract	3.0 g
Peptone	5.0 g
Soluble starch	10.0 g
Agar	15.0 g
Distilled water	1000 ml
pH	7.2

27. Trace element solution (Sneath *et al.* 1986)

EDTA	500.0 mg
$FeSO_4.7H_2O$	200.0 mg
$ZnSO_4.7H_2O$	10.0 mg
$MnCl_2.4H_2O$	3.0 mg
H_3BO_3	30.0 mg
$CoCl_2.6H_2O$	20.0 mg
$NiCl_2.6H_2O$	2.0 mg
$Na_2MoO_4.2H_2O$	3.0 mg
Distilled water	1000 ml

28. Tyrosine Agar medium (Sneath *et al.* 1986)

L-tyrosine	0.5 g
Distilled water	10 ml
Nutrient agar	100 ml

Tyrosine was sterilized separately and was added to the medium before plating.

Publication

Khan FI, Aktar L, Islam T and Saha ML 2019. Isolation and Identification of Indigenous Poly-β-Hydroxybutyrate (PHB) Producing Bacteria from Different Waste Materials. Plant Tissue Cult. Biotech. **29**(1): 15-24.