Fermentative Production of Poly-hydroxybutyrate (PHB) from Sucrose Based Renewable Resources

A dissertation report submitted in partial fulfilment of the requirement for the award of degree of

Master of Technology in Biotechnology

Under the guidance of

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PATIALA

July 2014

CERTIFICATE

This is to certify that the report entitled "Fermentative Production of Polyhydroxybutyrate (PHB) from sucrose based renewable resources" submitted by Shweta Aggarwal in partial fulfilment of the requirement for the award of Degree of Masters in Technology in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by her under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university.

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I hereby declare that the work being presented in the report entitled "Fermentative Production of Polyhydroxybutyrate (PHB) from sucrose based renewable resources" in partial fulfilment of the requirement for the award of Degree of Masters in Technology in Biotechnology to Thapar University, Patiala is my own work during the period of July 2013 to June 2014, under the supervision of Dr. Pinaki Dey, Lecturer, Department of Biotechnology, Thapar University, Patiala. I have not submitted the matter embodied in this report for the award of any other degree.

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It is certified that the above statement made by the student is correct to the best of my knowledge and belief.

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Date: **Shweta Aggarwal**
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CONTENTS

LIST OF ABBREVIATIONS

PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PHBV	Polyhydroxybutyrate-co-hydroxyvalerate
PHBHHx	Polyhydroxybutyrate-co-hydroxyhexanoate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
ICI	Imperial Chemical Industries
ATCC	American Type Culture Collection
MTCC	Microbial Type Culture Collection
SDS	Sodium Dodecyl Sulphate
DCW	Dry Cell Weight
DNSA	3,5-dinitrosalicylic acid
PVDF	Polyvinylidene Difluoride
HPLC	High Performance Liquid Chromatography
RSM	Response Surface Methodology
CCD	Central Composite Design
SEM	Scanning Electron Microscope
EDS	Energy Dispersive X-ray Spectroscopy
XRD	X-ray Diffraction

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Polyhydroxybutyrate (PHB), a type of biodegradable plastic, which has attracted significant interest as a replacement for petroleum-derived plastics because of its degradability, sustainability and some properties similar to conventional plastics, is still not being produced commercially for consumer products because of its high cost. Its cost of production is 5-10 times higher as compared to the conventional plastics. One the major factor leading to this high cost of production is cost of the substrate used for production. Hence, this study focused on the production of PHB from a cheap substrate - table sugar which is a sucrose based renewable source. The microbial strain used was *Cupriavidus necator* (MTCC 1472). Optimisation of media components was done for PHB production in shake flask by applying Plackett-Burman design and Response Surface Methodology. Using the optimised media scale-up of PHB production from shake flask to fermenter was carried out. In batch fermentation maximum biomass and PHB obtained were 4.724g/L and 2.824 g/L respectively with PHB productivity of 0.072g/Lh. Fed-batch fermentation studies were then done at different flow rates to enhance PHB production. At flow rate of 70 mL/h, 2.88-fold increase in productivity and at 30 mL/h, 2.57-fold increase in productivity was achieved as compared to batch cultivation. Extraction efficiencies of different methods were also investigated and surfactant hypochlorite digestion method was found to give maximum PHB extract per gram biomass.

INTRODUCTION

Today plastics are an indispensable element of nearly all industries and households and have become an essential commodity to improve the comfort and quality of life. But these conventional plastics are creating a global problem as they get accumulated in the environment due to their non-biodegradability. Replacing these plastics with completely biodegradable plastics having similar physical and mechanical properties offers the best solution to this problem.

There are three types of biodegradable plastics – (i) *Chemically synthesised polymers*: Given that all their properties are not similar to those of conventional plastics, they are not feasible as alternative for plastics commercially. They are also prone to microbial or enzymic attack. Polylactic acid, polyglycollic acid, polyvinyl alcohol, poly(ε-caprolactone), poly(ethylene oxide) are examples of polymers that fall under this category. (ii) *Starch-based biodegradable plastics:* These plastics are produced as blend of plastic and starch by adding starch as a cross-linking agent and filler. As soil microbes can degrade starch easily, they break the polymer matrix and thus the degradation time is greatly reduced. But the fragments that are left after the degradation of starch are recalcitrant thus making these plastics partially degradable. (iii) *Polyhydroxyalkanoates (PHAs):* Polyhydroxyalkanoates (PHAs) are the only polymers that are 100% biodegradable. They are polyesters of different hydroxyacids and can be synthesised by several microorganisms. They are accumulated as energy storage materials when concentration of a vital nutrient like phosphorus or nitrogen is limiting in medium and the carbon source is present in excess (D. Byrom, 1994). These compounds are metabolised and used when the vital nutrient is supplied to the cell. Also their properties are found to be similar to those of conventional plastics.

Only a few types of PHAs like, poly-3-hydroxybutyrate (P3HB), poly-4-hydroxybutyrate (P4HB), co-polymers of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV), copolymers of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx) are accessible in adequate quantities for research applications. Amongst all the PHAs, PHB has gained significant interest as a contender for biocompatible and biodegradable plastics

.

1.1 Polyhydroxybutyrate (PHB) and its biosynthesis

Polyhydroxybutyrate (PHB) is the first discovered PHA and is also the most extensively studied and characterised PHA. It is accumulated as membrane bound inclusion bodies in various bacteria at up to 80% of the dry cell weight. Its mechanical properties are comparable to those of conventional plastics like polyethylene or polypropylene. The general structure of PHB is as shown in figure:

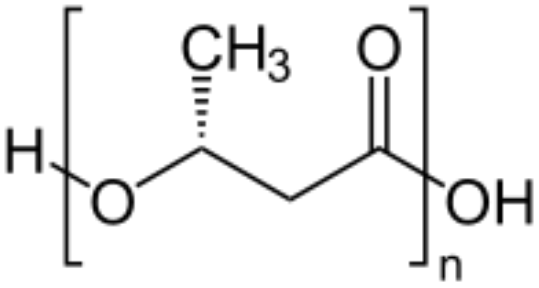

Figure 1: General structure of PHB

PHB can be chemically synthesised using β-butyrate or β- hydroxybutyric acid as monomers, but the process conditions are difficult to control and the production cost is also high. Thus, this approach is economically impractical (Wang *et al*., 2001). Comparing chemical synthesis to biosynthesis, production of PHB via biosynthesis requires simple and mild conditions during production (Li, 2007). Therefore, most desirable way of PHB production is fermentative production using specific microorganisms.

In the biosynthetic pathway of PHB in *R.eutropha*, acetyl- CoA is converted to PHB by the help of three enzymes. The first step includes the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA by the enzyme 3-ketothiolase. An NADPH-dependent acetoacetyl-CoA reductase then carries out its reduction to 3-hydroxybutyryl-CoA. In the third step, polymerization of 3-hydroxybutyryl-CoA to PHB occurs which is catalysed by PHB synthase (Anderson *et al*., 1990).

In other organisms the pathway is different than in *R. eutropha*. For example, in *Rhodopsuedomonas rubum*, the pathway differs after the second step. The acetoacetyl-CoA formed is reduced to L-(+)-3-hydroxybutyryl-CoA which is then converted to D-(−)-3-

hydroxybutyryl-CoA by two enoyl-CoA hydratases. In some *Pseudomonas* species like *P. oleovorans*, PHA consisting of 3-hydroxyalkanoic acid of medium chain length (which consists of 6-14 carbon atoms) is accumulated if cells are grown on alkanes, alkanoic acids or alkanols (Lagaveen *et al*., 1988).

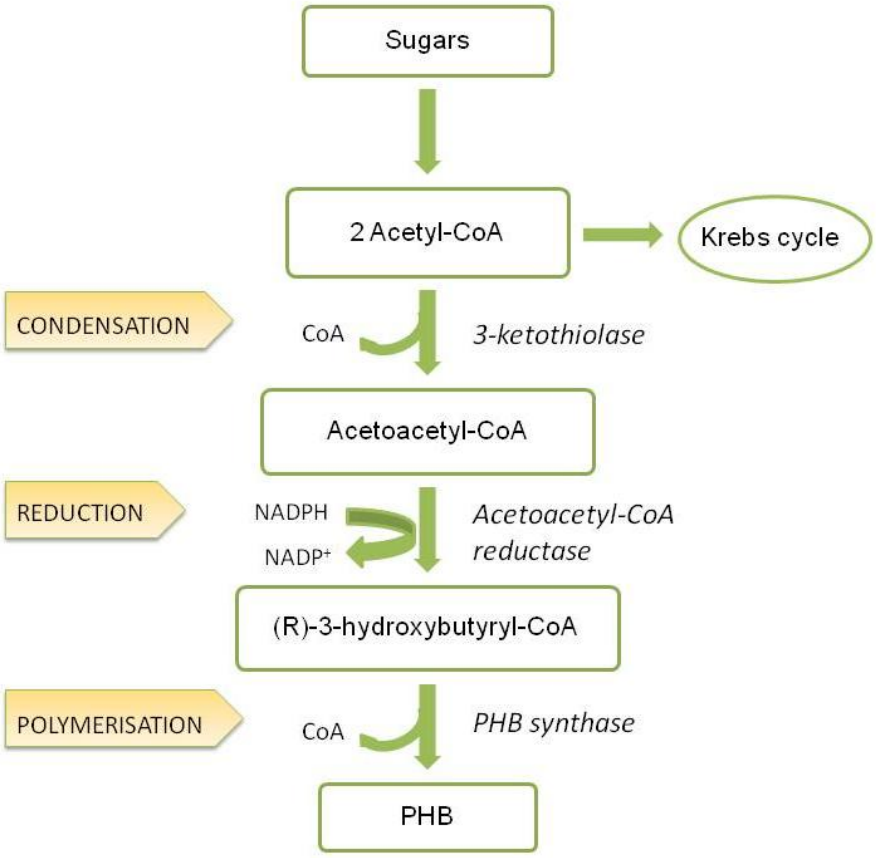

Figure 2: Metabolic pathway involved in synthesis of PHB in *R. eutropha*

1.2 Properties of PHB

- It can be completely degraded to carbon dioxide and water in aerobic conditions and in anaerobic conditions, it is degraded to methane.
- Its mechanical properties are comparable to those of conventional plastics such as polyethylene or polypropylene.
- It has good oxygen permeability.
- It shows 100% resistance to moisture in air and to water.

- The molecular weight of PHB ranges from 2×10^5 to 3×10^6 Da.
- Its melting point is 175℃.
- It can be moulded, spun into fibres, made into films and used to make heteropolymers with other synthetic polymers.

1.3 Microorganisms that can produce PHB

There are various microorganisms isolated from different sources like soil, lake, pulp industry sludge etc. and are well known to produce PHB. Table 1 summarise a list some microorganisms that produce PHB.

Table 1: List of PHB producing microorganisms

Microorganism	Gram staining	References
Ralstonia eutropha	-ve	Kim *et al*., 1994; Ryu *et al*., 1997
Alcaligenes latus	-ve	Yamane *et al.,* 1996; Wang *et al.,* 1997
Azotobacter vinelandii	-ve	Page and Comish, 1993
Pseudomonas oleovorans	-ve	Fuechtenbusch *et al*., 2000
Rhizobium	-ve	Aslim *et al*., 2002
Bacillus subtilis	+ve	Singh *et al*., 2013
Brevundimonas sp.	-ve	Bhuwal *et al*., 2013
Bacillus megaterium	+ve	Lopez *et al*., 2012
Enterococcus sp.	+ve	Bhuwal *et al*., 2013
rE.coli	-ve	Fidler and Dennis, 1992

For production of PHB, usually gram-negative bacterial strains are used because they can consume cheap carbon sources and can accumulate PHB up to 80% of the cell's dry weight while in gram positive bacteria production of PHB is low as they have a tendency to undergo sporulation. Among the numerous microbes studied, most widely used bacteria for production of PHB are *Ralstonia eutropha* and *Alcaligenes latus.*

1.4 Substrates used for PHB production

A wide variety of substrates have been used for PHB production mainly by *Ralstonia eutropha*. Initially, PHB was produced mainly using simple carbon sources like fructose, glucose and acetic acid (Ramsay *et al*., 1991; Braunegg *et al*., 1995; Wang *et al*., 1997). But as it is known that one of the factor that influences the production cost of PHB most is the cost of the substrate used for production (Kim, 2000). Thus, the investigations are being done on low cost carbon substrates to reduce the cost of production. Various carbon sources such as glycerol (Bormann *et al*., 1999), soyabean oil (Akiyama *et al*., 2003), residual oil from rhamnose production (Fuechtenbusch et al., 2000), oleic acid (Eggink *et al*., 1993), whey (Lee et al., 1997), sugarcane juice (Waranya et al., 2011), sweet sorghum juice (Tanamool et al., 2009) have been used to produce PHB.

Sucrose has been extensively used for production of PHB using *Alcaligenes latus* (Hrabak, 1992; Wang et al., 1997; Yamane et al., 1996; Grothe et al., 1999). But very less investigation has been done on production of PHB from sucrose using *Ralstonia eutropha*. Table sugar, which is a common form of sucrose, is a cheap substrate priced at 0.4 dollars/kg. Also table sugar is much cheaper in India as it is the second largest producer of sugarcane in the world after Brazil. Thus, the present study focuses on production of PHB from table sugar using *Ralstonia eutropha*.

1.5 Production process of PHB

PHB production is carried out by submerged fermentation process which can be operated in three different modes- batch, fed-batch, and continuous. Batch process was basically used for PHB production but high cell density and PHB production was not achieved due to exhaustion of nutrients. To increase PHB production, increasing the cell density and also PHB content of the cells have to be considered. Cultures with high cell density increase productivity. Although fed-batch fermentation has more complex operation than batch, yet it is used to achieve high cell densities and high PHB content ((Suzuki *et al*., 1986; Kim *et al*., 1994; Patwardhan *et al*., 2004). Kim *et al*. have reported that fed-batch fermentation with nitrogen limitation yielded maximum biomass concentration of 161g/L and PHB concentration of 124g/L with productivity of 2.42 g/Lh. Ryu *et al* report maximum biomass

concentration of 281g/L and PHB concentration of 232 g/L with an overall productivity of 3.14 g/Lh when fed-batch fermentation was carried out with phosphate limitation.

Continuous fermentation has also been investigated for production of PHB by *Alcaligenes eutrophus* (Khanna *et al*., 2010; Hafuka *et al*., 2011). The yield and productivity in continuous fermentation is generally lesser as compared to fed-batch fermentation process because the accumulation of PHB occurs in growth limiting conditions. Its accumulation during the exponential phase is very less. Thus, to achieve high production of PHB in continuous cultivation, very low dilution rates are required which lead to very low productivity.

1.6 Separation of PHB from cells

As PHB is an intracellular product which is accumulated in the form of inclusion bodies, thus, cell lysis is required for the release of PHB from cells. It is a crucial stage of the process and also adds to the production cost. There are many different processes that can be employed to lyse cells – (i) digestion of cells using surfactants or sodium hypochlorite; (ii) enzymatic digestion method; (iii) mechanical methods which include bead mill disruption, high pressure homogenisation, ultrasonication etc.

To find a comparatively cheaper method with high recovery rate for PHB extraction, a wide variety of solvents have been investigated. Sodium hypochlorite solution and chloroform are most commonly used solvents for PHB extraction because their solubility is high and recovery of PHB is easy (Hahn *et al*., 1994). Hahn *et al*. (1994) used sodium hypochlorite and chloroform to extract PHB from lyophilised and powdered *R. eutropha* and *E.coli* cells. By using dispersions of sodium hypochlorite solution and chloroform, they obtained PHB with high purity levels of 98%. Salmiati *et al*. (2009) also used the same technique. They studied the effect of varying ratio of concentrations of chloroform to sodium hypochlorite on the yield and purity of PHB from mixed cultures.

A combination of surfactant and hypochlorite was used by Dong *et al* on *Azotobacter chroococcum* G-3 for extraction of PHB. By the use of this method, they were able to obtained PHB recovery of 86.6% with purity of 98%. This method is advantageous because

of its low cost of operation and its less degradation of PHB. Comparing chloroform-hypochlorite method and surfactant-hypochlorite digestion for PHB extraction from *R. eutropha*, the surfactant hypochlorite digestion method resulted in lower cost of recovery.

1.7 Applications of PHB

PHB has a broad range of applications owing to its unique features.

- It can be used for applications similar as conventional commodity plastics which consist of the disposable items such as cups, bottles, cosmetic containers etc.

- It can be used in packaging films mainly in bags, containers and paper coatings.

- Another potential application of PHB which is in the development of implanted medical devices for orthopaedic, hernioplastic, dental, and skin surgery. Many medical devices as shown in figure 1 have been developed but they are still under investigation. (Bonartsev *et al*. 2007)

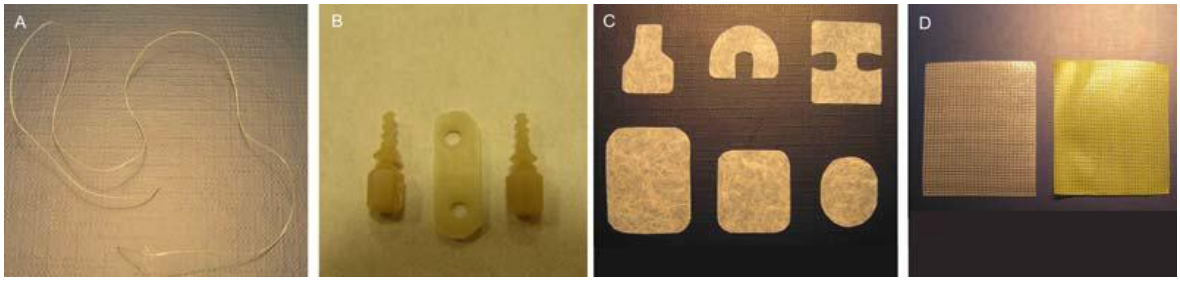

Figure 3: Different medical devices that have been developed using PHB. Bioresorbable surgical sutures (fig. A), biodegradable screws and plates for cartilage and bone fixation (fig. B), biodegradable membranes for periodontal treatment (fig. C), surgical meshes with PHB coating for hernioplastic surgery (fig. D) (Bonartsev *et al*. 2007)

- PHB as a promising material for slow release of pharmaceuticals has also been investigated because inside the body, the rate of degradation of PHB is very slow which usually varies from months to years. (Korsatko *et al*., 1983; Lafferty *et al.*, 1988).

- The monomer unit of PHB i.e. 3-hydroxybutyric acid is a chiral molecule. Thus, it can be used as basic molecules for the chemical production of complex chiral pharmaceutical or agrochemical agents (Seebach et al., 1987).

2. LITERATURE REVIEW

For production of PHB, *Alcaligenes eutrophus* is the most extensively studied bacteria because it can accumulate large quantity of PHB by using simple carbon sources like fructose, glucose, and acetic acid. Large scale production of PHB has been done by Imperial Chemical Industries (ICI) of UK using glucose as carbon source by fed-batch cultivation of *Alcaligenes eutrophus*. Kim *et al.* (1994) obtained final biomass and PHB concentration of 164, 121g/L respectively with an overall productivity of 2.42 g/(Lh) by maintaining glucose concentration at 10-20g/L during fed-batch culture.

Various other carbon sources have also been used for production of PHB by *A. eutropha*. Linko *et al*., (1993) obtained 7.1 g/L of PHB when lactic acid used as a carbon source was fed periodically. Also, *A. eutropha* has been reported to consume carbon dioxide in autotrophic conditions and thus leading to the production of PHB. Plant oils were also found to be good substrate (Fukui *et al*., 1988). Oleic acid has also been reported to produce 32.5 g/L of PHB by fed-batch cultivation of *A. eutropha* in 60 h. Glycerol, soybean oil, residual oil from rhamnose have also been used for PHB production.

After *A.eutrophus*, *A. latus* is the most studied microbe for PHB production. The accumulation of PHB in *A. latus* is growth associated. *A. latus* DSM 1123 can accumulate PHB concentration of about 80% of dry mass in only 5h. Grothe et al. (1999) reported that A. latus ATCC 29713 produced PHB up to 63% of biomass after 93 h of batch culture.

Although PHB has attracted significant interest, still there is a hurdle in its commercial application which is its high production cost which is around 5-10 times of petroleum-based plastics like polyethylene and polypropylene. The factor that is most significant for high production cost is cost of the substrate being used. Thus, scientists have been investigating various strains that are able to consume cheap carbon source and also studied different fermentation strategies. Ramsay *et al*. (1995) reported that *Pseudomonas cepacia* ATCC 17759 when grown on xylose leads to production of 2.6 g/l biomass with PHB concentration of 60%. He found that the cost of substrate (xylose) was half as compared to bulk glucose. PHB has also been produced from activated sludge, molasses and industrial waste. But there are certain disadvantages associated with them. Industrial waste requires pre-treatment before

it can be used for PHB production. Molasses contain heavy metals such as As, Al, Cu, Fe which inhibit growth.

Also different fermentation strategies have been studied in respect to enhance PHB production. Grothe *et al*. (1999) reported that *A. latus* ATCC 29713 when cultivated by fed-batch process using molasses shows maximum specific growth rate of 0.265 h^{-1} as compared to 0.075 h^{-1} during batch cultivation. Shilpi *et al*. (2008) reported enhancement in productivity of PHB by fed- batch cultivation of *A. eutropha.* They used a decreasing nutrient feed rate strategy by which they obtained PHB productivity of 0.48g/L-h as compared to 0.18 g/L-h by batch cultivation. Du *et al*. (2001) investigated continuous production of PHB by *R. eutropha* using glucose as carbon source in a two-stage culture system. They could achieve maximum PHB productivity of 1.43 g/L-h at a dilution rate of 0.12 h^{-1}. Shilpi *et al*. (2005) also investigated repeated batch culture of *R. eutropha* using fructose as carbon source for PHB production. Removal of culture broth and supplementation with an equal volume of fresh media was done at 27 and 41 h. After two cycles of repeated batch fermentation 3-fold increase in productivity was seen as compared to batch.

3. OBJECTIVES

- Optimization of PHB production in batch process from sucrose based substrates
- Scale up of PHB production from shake flask level to fermenter level (Batch process)
- Fed batch fermentation process to enhance PHB production
- Development of cost effective separation process of PHB from PHB containing cells

4. MATERIALS AND METHOD

4.1 Microorganism and Maintenance

Cupriavidus necator (MTCC 1472), an intracellular PHB producing microorganism purchased from Microbial Type Culture Collection (MTCC), Chandigarh, was used in this study. It was earlier known as *Ralstonia eutropha, Wautersia eutropha.* It is equivalent to ATCC 17699. It is well known for its ability to produce PHB from sucrose based substrates. It was maintained on nutrient agar slants at 4^{O} C and subcultured monthly.

4.2 Media and Culture Conditions

The medium which was used as the basal medium for the growth of the microorganism consisted of the following components:
Sucrose (table sugar) - 20g/l,
$(NH_4)_2SO_4$ - 1.4 g/L,
KH_2PO_4 - 1.5g/L,
Na_2HPO_4 - 1.8 g/L,
$MgSO_4$ - 0.2 g/L,
Trace metal solution - 1 mL/L

Components of trace metal solution:
Ammonium Fe (III) citrate - 6 g/L,
$CaCl_2.2H_2O$ - 10 g/L,
H_3BO_3 - 0.3 g/L,
$CoCl_2.6H_2O$ - 0.2 g/L,
$ZnSO_4.7H_2O$ - 0.1 g/L,
$MnCl_2.4H_2O$ - 0.03 g/L,
$Na_2MoO_4.2H_2O$ - 0.03 g/L,
$NiSO_4.7H_2O$ - 0.02 g/L, and
$CuSO_4.5H_2O$ - 0.01 g/L

The phosphate components of the medium were autoclaved individually to avoid precipitation. Trace metal solution was sterilized by filter sterilization method. After that, all the components of the medium were mixed in aseptic conditions i.e. in the Laminar flow hood. A loopful of culture was inoculated into the medium and kept in incubator shaker for 48 hours at 30 °C, 150 rpm.

4.3 Studies in shake flask and at fermenter level

The medium optimization studies were done at shake flask level. Plackett-Burman design and Response Surface Methodology was applied for the optimization process. Design Expert software (version 7.0.0) was used for designing the experiments. The experiments were performed in 250 mL flasks containing 100 mL media kept on an incubator shaker for 48 hrs.

After optimization of media components at shake flask level, scale up of study to 3L fermenter (Bio-Age, India) with working volume 2L was done (batch process). The optimized media composition used for fermentation was as follows:
Peptone - 2.0g/L
Trace metal solution - 9.15 mL/L
Yeast extract - 3.10g/L
$(NH_4)_2SO_4$ - 2.18g/L
Sucrose - 20g/L
$MgSO_4.7H_2O$ - 0.2g/L
Na_2HPO_4 - 0.6g/L
KH_2PO_4 - 2.0g/L
$CaCl_2$ - 20mg/L

The physical parameters were maintained as follows:
Air flow rate - 2Lpm
Temperature - 30ºC
Stirrer speed – 300 rpm
pH - 7

All the chemical constituents of the media were mixed together and poured in the fermenter and the fermenter was dressed for autoclaving. Then the fermenter containing media was

sterilized at 121ºC for 15 min before fermentation. Inoculum volume was maintained at 5% of the total volume of the fermentative media. Samples were collected aseptically from the fermenter at intervals of 3 h and analysed.

Fed-batch fermentation studies were also done to enhance the production of PHB. A 5L fermenter (Sartorious) was used for these studies. Feed was provided at 2 different flow rates- 258mL/h and 30mL/h with the help of a peristaltic pump. Samples were collected aseptically at regular intervals of time and analysed.

4.4 Extraction of PHB from the cells

As PHB is an intracellular product, the cells need to be lysed to extract the PHB from the cells. The culture was centrifuged at 10,000 rpm for 15 min to separate the cells. Supernatant was discarded and the cell pellet obtained was dried in hot air oven (90º C, 24 hr) till a constant weight is obtained. Then the dry weight of the cells containing PHB was noted. Three different methods were then used for isolation of PHB from PHB containing *C. necator* cells and their extraction efficiency and purity was then compared to get the best method. The three methods are as follows:

(i) *Use of surfactants:* Surfactants like anionic sodium dodecyl sulphate (SDS), can be used for isolation of PHB from the cells as they incorporate themselves into the phospholipid bilayer membrane and disrupt the cells. To the cell pellet, 1% SDS solution was added and kept in an orbital shaker at 200rpm, 37 ºC for 60 min. After incubation, the solution was centrifuged and the pellet obtained was washed with distilled water. It was centrifuged again and the pellet was dried in hot air oven to obtain a constant weight.

(ii) *Use of sodium hypochlorite*: Use of sodium hypochlorite is another method for recovery of PHB from cells. A heat pretreatment was given and then digestion with sodium hypochlorite at 50º C was done. The solution was centrifuged and the pellet obtained was washed with distilled water and again centrifuged. But, there is a disadvantage associated with this method. It causes degradation of PHB leading to 50% reduction in the molecular weight.

(iii) *Surfactant-hypochlorite digestion:* To this pre-weighed dry biomass, 1% SDS solution with a pH adjusted to 10.0 was added (40 ml solution was added to 3.0 gm DCW). The mixture was then incubated for 60 min in an orbital shaker at 200 rpm, 37 ºC. After incubation, the solution was centrifuged at 10,000 rpm for 15 min. Supernatant was discarded and to the pellet obtained sodium hypochlorite solution was added. The solution was again centrifuged & to the pellet obtained distilled water was added and vortexed. The final pellet was obtained after centrifugation and was kept for drying in a hot air oven at 90 ºC, till a constant weight is obtained.

4.5 Analytical Methods

4.5.1 Estimation of biomass

For the estimation of concentration of cells in the sample, optical density of the samples was taken at 600 nm. Optical density versus time graph was prepared.

For estimation of biomass, the samples were centrifuged at 10,000 rpm for 15 minutes to spin down the cells. The supernatant obtained was separated and kept for analysis and the cell pellet obtained was dried in hot air oven (90º C, 24 hr) till a constant weight is obtained. Then the dry weight of the cells containing PHB was noted.

4.5.2 Estimation of residual sugar by DNSA method

The underlying principle of this method is that 3,5-dinitrosalicylic acid (DNSA) when reacted with reducing agents such as reducing sugars, gets reduced to 3-amino-5-nitrosalicylic acid, which is a coloured compound and shows absorbance at 540 nm. Sucrose, or common table sugar, does not show this reaction with DNSA. Therefore, if estimation of sucrose is to be done, it must first be broken down into simple sugar i.e. glucose. This can be achieved by boiling the sample containing sucrose with hydrochloric acid which will hydrolyse sucrose. The pH of the sample is then adjusted using a base, as simple sugars are good reducing agents under basic conditions.

The samples collected at 3 h intervals were centrifuged at 10,000 rpm for 15 min at 4°C. 2 mL of supernatant of each sample was taken in different test tubes and 0.5 mL of 2N HCl

was added into each. The test tubes were then placed in a boiling water bath for 10 minutes. They were carefully removed and 0.5 mL of 2 N NaOH and 3 mL of 0.050 M 3,5-dinitrosalicylic acid (DNSA) was added into each and were placed again in boiling water bath for 5 minutes. The test tube were removed from the boiling-water bath and quickly placed in ice for 10 minutes. Optical Density was measured at 540 nm. Standard curve of sucrose was prepared and then used to estimate the unknown concentrations of the sugar in samples.

Figure 4: Test tubes showing increasing intensity of colour formed during sucrose estimation by DNSA method

4.5.3 Estimation of proteins by Lowry's method

The Lowry method is used to determine protein concentrations in unknown samples which is exhibited by colour change in the samples. The basic principle behind this method is that the peptide nitrogens of proteins react with Cu^{2+} ions under alkaline conditions reducing the cupric (Cu^{2+}) ions to cuprous (Cu^{+}) ions which lead to reduction of the Folin–Ciocaltaeu reagent producing a blue colour. The blue colour is formed due to reduction of phosphomolybdotungstate to hetero-polymolybdenum blue by the copper-catalysed oxidation of the aromatic amino acids tyrosine and tryptophan. Thus the intensity of the colour produced depends on the amount of these amino acids present in the sample and can be analysed by measuring absorbance of the sample at 650-700 nm. The Lowry method is advantageous as it is sensitive to low concentrations of protein.

Reagents used in this method are as follows:

A. 2% Na_2CO_3 in 0.1 N NaOH

B. 0.5% $CuSO_4.5H_2O$ in H_2O

C. 1% NaK Tartrate in H_2O

D. Reagent I: 48 ml of A, 1 ml of B, 1 ml C

E. Reagent II- 1 part Folin-Phenol [2 N]: 1 part water

For estimation of concentration of proteins present in the PHB extracted from the bacterial cells, the PHB samples were dissolved in water in different eppendorf tubes. 200µL of each sample was taken in different test tubes and 4.5 ml of reagent I was added and kept for incubation for 10 minutes. After incubation 0.5 ml of reagent II was added and kept for 30 minutes incubation. The absorbance of the samples was measured at 660 nm. Standard graph of BSA was prepared and then used to estimate the unknown concentrations of proteins in samples.

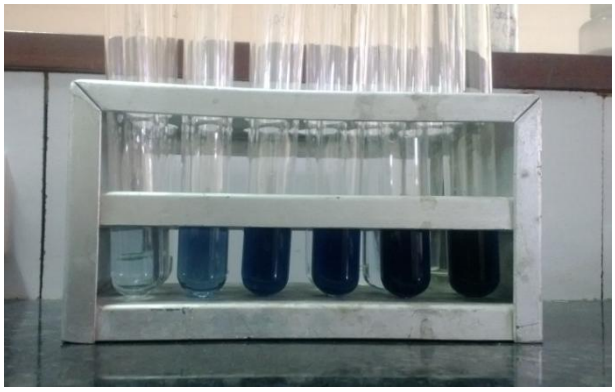

Figure 5: test tubes showing increasing intensity of blue colour formed during protein estimation by Lowry's method

4.5.4 Estimation of PHB

PHB content of the biomass was determined by acid hydrolysis of PHB to crotonic acid. The samples were centrifuged at 10,000 rpm for 15 min. To the pellets obtained, 1mL of distilled water was added, mixed by gentle shaking and then transferred to preweighed centrifuge tubes. They were centrifuged for 10 min at 10,000 rpm, dried overnight in a hot air oven at 90 °C, and weighed again to establish the total biomass.

To the dried pellets 1 mL of 96% H_2SO_4 was added for digestion and kept at 100°C for 1h to form crotonic acid. The samples were then cooled down to room temperature, and 1ml 0.014

M H_2SO_4 was added. They were then filtered through PVDF membrane. 1:10 dilution was then done with 0.014M H_2SO_4 and crotonic acid was determined by HPLC with C18 column. The solvent that was used for elution was 0.014 N H_2SO_4 and flow rate was maintained at 0.7 mL/min. Monitoring of the elution peaks was done at 210 nm with a Photodiode Array Detector. To calculate the PHB content, standard curve of commercial PHB (Sigma Aldrich,) was prepared by treating in the same manner as the samples.

5. RESULTS AND DISCUSSION

5.1 Optimization of PHB production in batch process from

5.1.1 Substrate Inhibition Studies

For optimization of the effect of substrate concentrations on specific growth rate of the bacteria, the concentration of the limiting nutrients, which are sucrose and ammonium sulphate in the present study, were varied over a range while the concentrations of the remaining components of the medium were kept constant. The experiments were performed in two steps: Firstly, the effect of changing sucrose concentration on the growth rate of *C. necator* was studied. The sucrose concentration was varied from 10 to 30 g/L and the concentrations of the other components (including ammonium sulphate) were kept constant. Secondly, the effect of changing concentration of ammonium sulphate was determined by varying its concentration from 0.2 to 6 g/L and the concentrations of the other components including sucrose were kept constant.

The experiments were performed in 250 mL conical flasks containing 100 mL medium and kept on a shaker at 30ºC. 5 mL of sample volume was collected aseptically from the flasks at intervals of 3 h. Optical density of the collected samples was measured at 600 nm using spectrophotometer (Hitachi, U-2900). Ln X_f/X_0 vs. time graphs were prepared from the obtained data. The slopes of these graphs were then used to calculate the maximum specific growth rate for each nutrient concentration during the exponential phase of culture.

Figure 6: Flasks containing different concentrations of (A) sucrose (B) ammonium sulphate

It was observed that the maximum specific growth rate of *C. necator* varies steadily as the concentration of sucrose is increased from 10 to 30 g/L. The value of maximum specific growth rate increased as the sucrose concentration was increased from 10 to 20 g/l but when the sucrose concentration was increased further, its value was found to decrease gradually due to substrate inhibition effect. The maximum value of specific growth rate achieved was 0.096 hr^{-1} at sucrose concentration of 20 g/L solution.

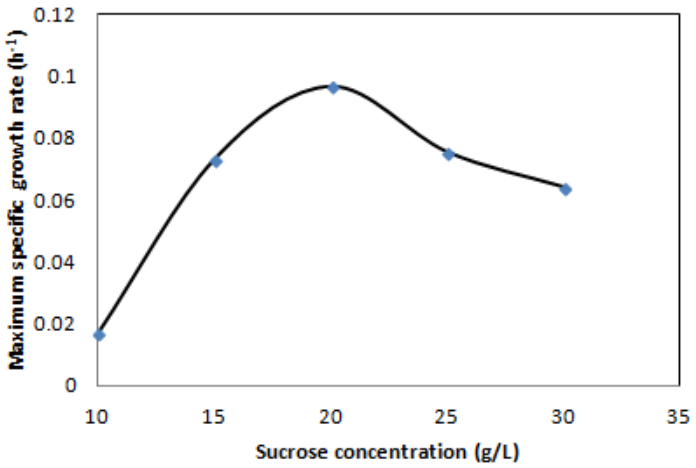

Figure 7: effect of sucrose concentration on maximum specific growth rate

Similarly like sucrose concentration, as nitrogen concentration is increased from 0.2 g/L to 2 g/L, maximum specific growth rate gradually increases, but when nitrogen concentration is increased above 2g/L, maximum specific growth rate decreases due to substrate inhibition effect (fig. 8). The highest value of maximum specific growth rate achieved was 0.1103 hr^{-1} at nitrogen concentration of 2 g/L.

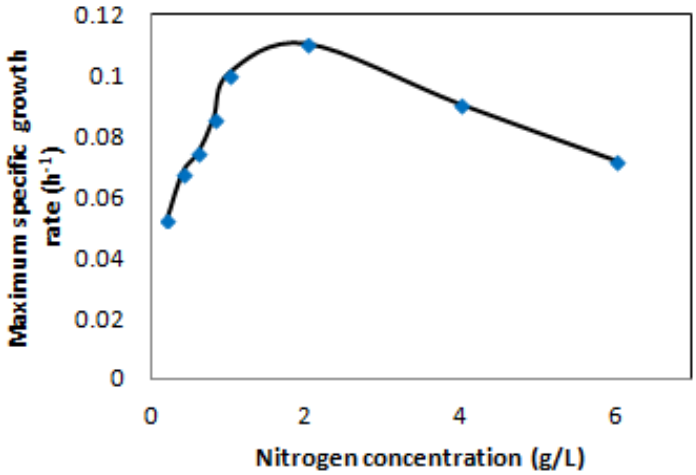

Figure 8: Effect of nitrogen concentration on maximum specific growth rate

5.1.2 Screening of most significant parameters using Plackett-Burman design

For a large number of variables, medium optimization by changing the value of one variable while keeping others constant is a time consuming process. In such a case Plackett-Burman design is a highly beneficial screening methodology. It is used to select the most significant variables or factors in fermentation process which are then optimized in further studies.

In the present study, eleven factors, which included nutrients, physical parameters, were taken up for optimization for growth and production of PHB by *C. necator*. Among the nutrients were – sucrose, $MgSO_4.7H_2O$, Na_2HPO_4, KH_2PO_4, trace element solution, $(NH_4)_2SO_4$, yeast extract, peptone, $CaCl_2$, and the physical parameters included temperature and rpm. Biomass and PHB formation were taken as the responses in designing the experiments. Each of the factor was tested at two levels, high (+1) and low (−1) (Table 2). The levels for $MgSO_4.7H_2O$, Na_2HPO_4, KH_2PO_4, trace element solution, $(NH_4)_2SO_4$, yeast extract, peptone, $CaCl_2$ were determined from extensive literature survey on fermentative production of PHB and for sucrose and $(NH_4)_2SO_4$ from initial experiments conducted with varying concentration of substrates.

Table 2: Concentrations of variables used in Plackett–Burman Design

Coded Factors	Name	Level	
		-1 (low)	+1 (high)
A (g/l)	Sucrose	15	25
B (ºC)	Temperature	25	35
C (g/l)	$MgSO_4.7H_2O$	0.15	0.25
D (g/l)	Na_2HPO_4	0.5	3
E (g/l)	KH_2PO_4	0.5	3
F (mL/l)	Trace metal solution	5	15
G (g/l)	$(NH_4)_2SO_4$	1.7	2.8
H (g/l)	Yeast extract	0.05	0.15
J (g/l)	Peptone	2	5
K (g/l)	$CaCl_2$	0.01	0.03
L	Rpm	120	180

A set of twelve experiments was designed by Plackett–Burman design using Design Expert Software (version 7.0.0). The experiments and their corresponding response values in terms of PHB are shown in table 3. The experiments were performed in 250 mL flasks containing 100 mL media. Samples were withdrawn at the end of 48 h fermentation and analyzed for the two responses using the software which yielded t coefficient values for all the factors. High positive t values of some factors with respect to the biomass and PHB responses demonstrate that they are statistically important and thus were selected for further analysis.

Table 3: Plackett–Burman experimental design for 11 variables and corresponding responses

Coded Factors											**Response**
A	B	C	D	E	F	G	H	J	K	L	PHB (g/L)
25.0	25.0	0.25	3.0	0.5	15.0	2.8	0.15	2.0	0.01	120	0.636
25.0	25.0	0.25	3.0	3.0	5.0	1.7	0.05	5.0	0.01	180	1.655
15.0	35.0	0.25	3.0	0.5	5.0	1.7	0.15	2.0	0.03	180	1.605
15.0	25.0	0.15	0.5	0.5	5.0	1.7	0.05	2.0	0.01	120	0.8
15.0	25.0	0.25	0.5	3.0	15.0	1.7	0.15	5.0	0.03	120	1.638
15.0	25.0	0.15	3.0	0.5	15.0	2.8	0.5	5.0	0.03	180	0.981
25.0	35.0	0.15	3.0	3.0	15.0	1.7	0.5	2.0	0.03	120	0.277
25.0	35.0	0.15	0.5	0.5	15.0	1.7	0.15	5.0	0.01	180	1.605
25.0	25.0	0.15	0.5	3.0	5.0	2.8	0.15	2.0	0.03	180	1.299
15.0	35.0	0.15	3.0	3.0	5.0	2.8	0.15	5.0	0.01	120	1.757
15.0	35.0	0.25	0.5	3.0	15.0	2.8	0.5	2.0	0.01	180	0.29
25.0	35.0	0.25	0.5	0.5	5.0	2.8	0.5	5.0	0.03	120	1.575

For over all analysis, order: linear, model: polynomial, transformation: none were selected. From the fit summary section in design menu, Model F-value of 853.80 and P (Probability) value less than 0.05 clearly indicate that polynomial model terms are significant in terms of analysis.

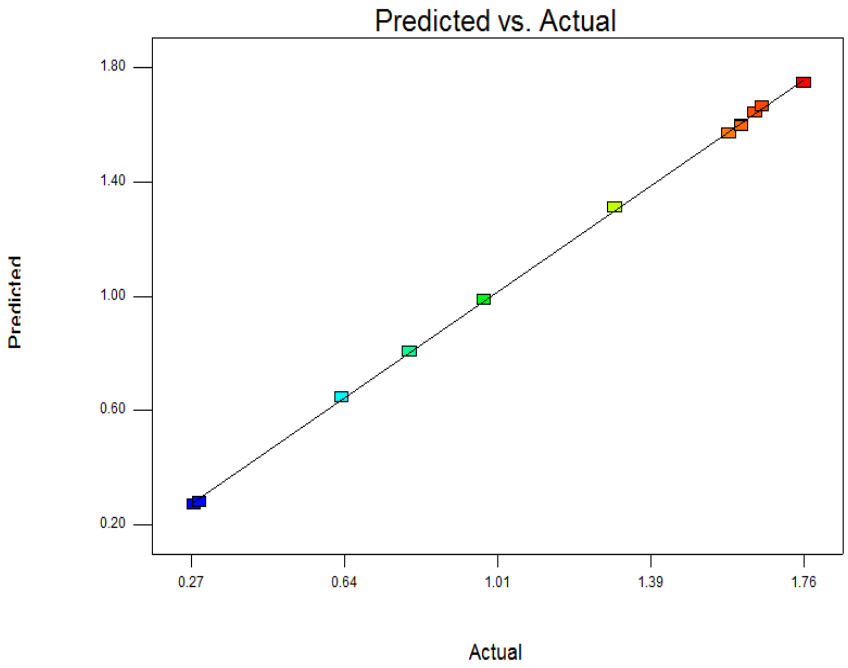

Figure 9: Distribution of predicted response vs. actual response values

As all the points are clustering over the diagonal line, thus, predicated response vs. actual response plot clearly indicates the capability of the model to predict the experiments.

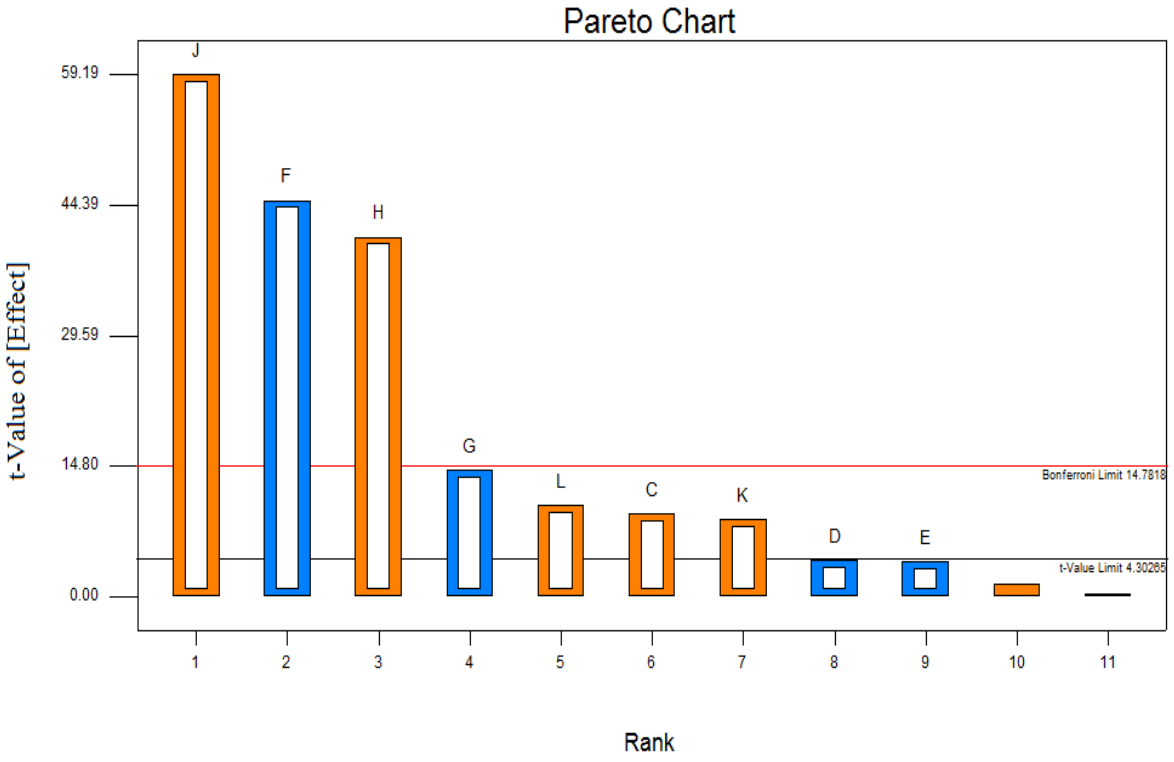

Figure 10: Pareto chart showing t-value of effects

The Pareto charts show t-values of effects or factors that are under study by two limit lines, namely, the Bonferroni limit line (t-value of effect = 14.71) and the t limit line (t-value of effect = 4.33). Factors which have t-value of effect above the Bonferroni limit line are termed as certainly significant effective factors where as the ones with t value of effect between Bonferroni limit line and t limit line are designated as factors likely to be significant.

As J, H, F have t-values far above the Bonferroni limit line, thus, they are certainly significant parameters. The t-value of G is equivalent to Bonferroni limit line, thus it is also considered as a significant parameter. Therefore, among all 11 parameters, most significant parameters are: J (Peptone), F (Trace Metal Solution), H (Yeast Extract), G ($(NH_4)_2SO_4$).

5.1.3 Optimization of the major influencing parameters by RSM

Response Surface Methodology (RSM) was then used to optimise the concentrations of the four factors namely, peptone, trace metal solution, yeast extract, $(NH_4)_2SO_4$ that were screened by the Plackett–Burman design. RSM is an efficient statistical tool which is used to determine the interactive effect of the process variables on the responses under study. PHB production was taken as the response in designing the experiments. The upper (+1) and the lower (-1) limits of the factors are shown in table 4.

Table 4: Concentration ranges of factors screened for optimization by RSM

Coded Factors	**Name**	**Level**	
		-1 (low)	+1 (high)
A (g/l)	Peptone	2	5
B (mL/L)	Trace metal solution	7.5	12.5
C (g/l)	Yeast extract	3	6
D (g/l)	$(NH_4)_2SO_4$	1.7	2.8

Using Design Expert software (version 7.0.0) a 2^n-factorial central composite design (CCD) was developed which lead to a total number of 30 experiments with different combinations of concentrations of the selected variables. The list of experiments according to the Central Composite design and the corresponding values of response are shown in table 5.

Table 5: Experimental design and corresponding response values

S. No.	Coded Factors				Response
	A	B	C	D	PHB (g/L)
1.	3.50	10.0	1.50	2.25	1.596
2.	2.00	12.5	3.00	2.80	0.993
3.	0.50	10.0	4.50	2.25	1.742
4.	2.00	7.5	6.00	1.70	1.637
5.	3.50	10.0	4.50	2.25	2.440
6.	5.00	7.5	6.00	1.70	1.326
7.	2.00	12.5	6.00	1.70	1.345
8.	5.00	7.5	3.00	1.70	1.672
9.	5.00	7.5	6.00	2.80	1.176
10.	2.00	12.5	6.00	2.80	1.258
11.	3.50	10.0	4.50	2.25	2.360
12.	3.50	10.0	4.50	3.35	1.232
13.	5.00	7.5	3.00	2.80	1.502
14.	3.50	10.0	4.50	1.15	1.627
15.	2.00	7.5	6.00	2.80	1.516
16.	5.00	12.5	3.00	1.70	1.481
17.	2.00	7.5	3.00	1.70	1.491
18.	5.00	12.5	6.00	2.80	0.7
19.	3.50	10.0	4.50	2.25	2.428
20.	5.00	12.5	6.00	1.70	0.896
21.	3.50	10.0	4.50	2.25	2.457
22.	3.50	10.0	7.50	2.25	1.281
23.	3.50	15.0	4.50	2.25	0.687
24.	3.50	10.0	4.50	2.25	2.416
25.	5.00	12.5	3.00	2.80	1.302
26.	2.00	7.5	3.00	2.80	1.267
27.	6.50	10.0	4.50	2.25	1.337
28.	3.50	5.0	4.50	2.25	1.887
29.	2.00	12.5	3.00	1.70	1.125
30.	3.5	10.0	4.50	2.25	2.431

The fermentation experiments were carried out in 250 mL flasks with working volume of 100 mL and kept in a shaker at 150 rpm at 30ºC. Samples were withdrawn at the end of 48 h and analyzed for the response. The optimum concentrations of the factors were obtained by the regression and graphical analysis of the two responses using the software.

To determine the interrelationship between these variables, Transformation: None and Quadratic Model was selected. In the fit summary section, model F-value: 32.22 and P value (0.001) being less than 0.050 indicate that the model terms are significant. The empirical relationship between PHB production and the four variables is given by the final regression equation as shown below:

$$PHB = 2.44 - 0.058A - 0.20B - 0.067C - 0.085D - 6.68 \times 10^{-3}AB - 0.17AC - 8.18 \times 10^{-3}AD - 0.027BC + 4.43 \times 10^{-3}BD + 9.43 \times 10^{-3}CD - 0.25A^2 - 0.31B^2 - 0.27C^2 - 0.27D^2$$

The adjusted determination coefficient value (adj. R^2 = 0.9378) was within reasonable agreement with the predicted R^2 of 0.8154. In this case A, B, C, D, AC, A^2, B^2, C^2, D^2 are significant model terms.

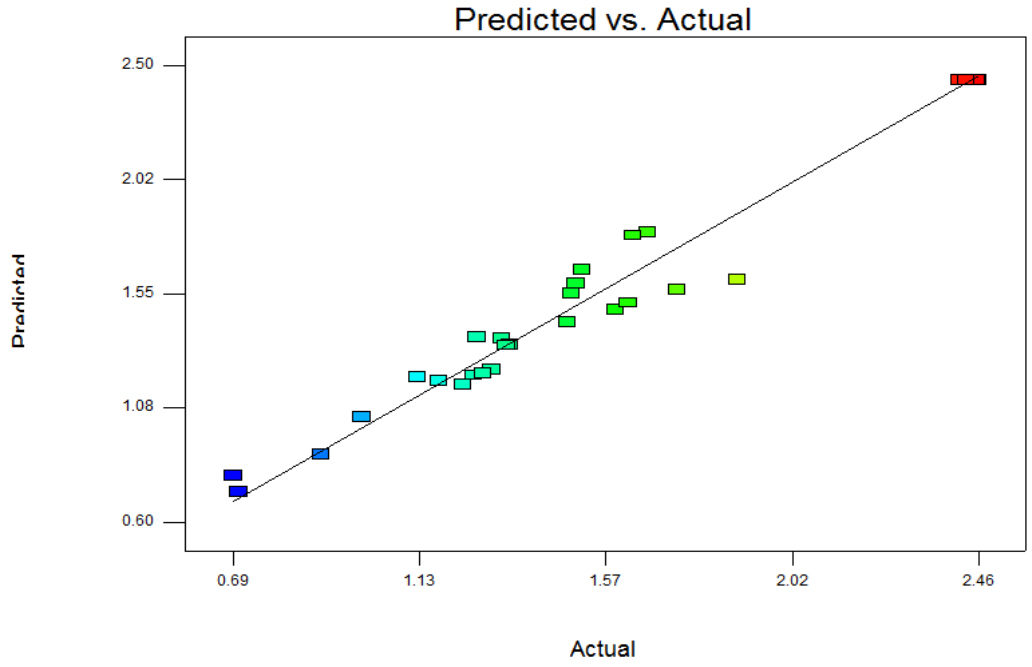

Figure 11: Distribution of predicted values versus experimentally determined values of the response

The high significance of the model is also implied by the predicted versus actual response curve. Clustering of the point around the diagonal indicates the capability of the model to predict the experiments.

- **Combined effect of Trace metal solution with peptone and yeast extract in PHB production:**

It can be seen from figure 12 and 13 that the concentration of PHB initially increased with increase in concentration of trace metal solution up to 10mL/L, but as it was increased beyond 10mL/L PHB production began to decrease. The effect of peptone was also similar. PHB production increased with increase in concentration of peptone up to 3.50g/L and started decreasing as peptone concentration was increased beyond that. Also, at high concentration of trace metal solution, when the concentration of peptone was increased beyond 3.50g/L, a large decrease in PHB production was seen due to combined substrate inhibition effect.

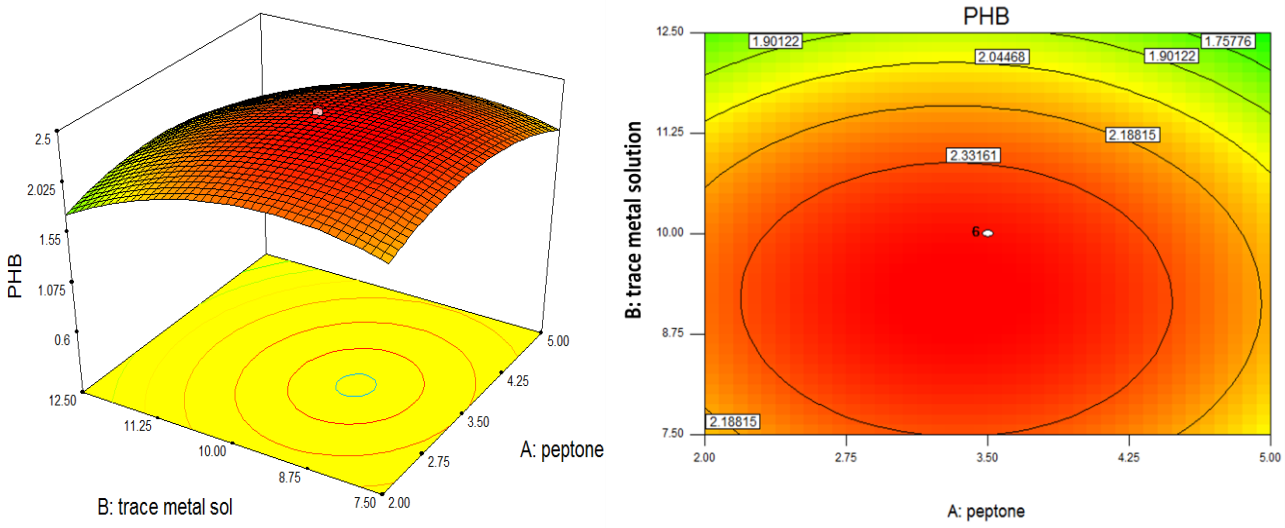

Figure 12: Response surface and contour plot showing the effect of trace metal solution and peptone in PHB production.

Similarly, figure 13 shows the conjugate effect of yeast extract and trace metal solution in PHB production. PHB production increase as the concentration of yeast extract is increased upto 4.50g/L but above this concentration, PHB production starts decreasing.

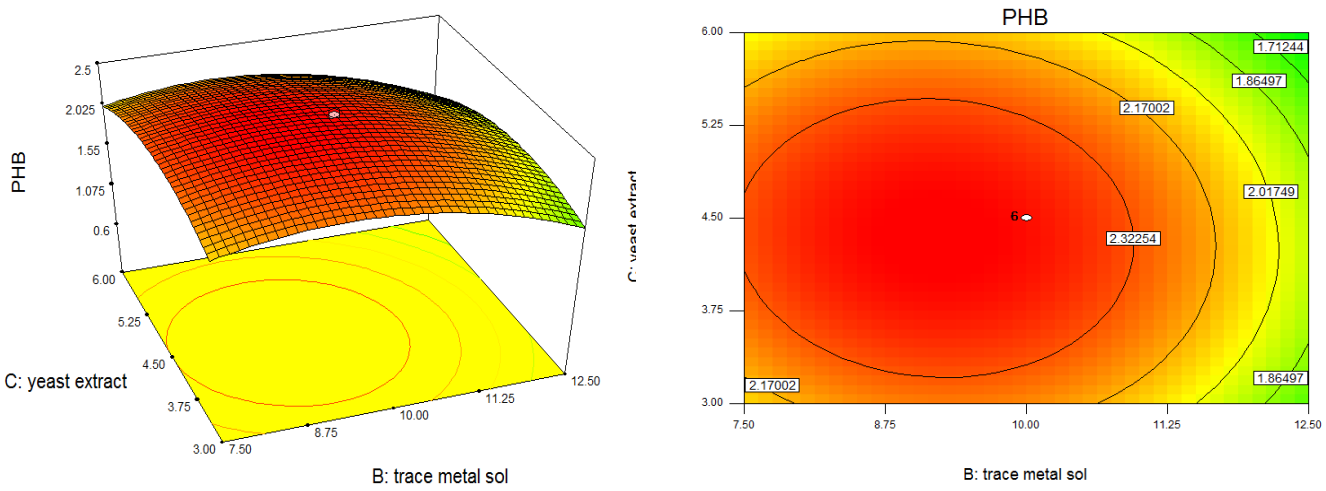

Figure 13: Response surface and contour plot showing the effect of trace metal solution and yeast extract in PHB production.

- **Combined effect of peptone with yeast extract and $(NH_4)_2SO_4$ in PHB production:**

Figure 14 shows the conjugate effect of yeast extract and peptone in PHB production. When the concentration of both these variables is low, PHB production is very low. It increases with increase in concentration of the variables and is observed to be maximum at 4.50g/L yeast extract and 3.50g/L peptone. When the concentration of both these variables is high, PHB production is greatly reduced due to the combined inhibition effect.

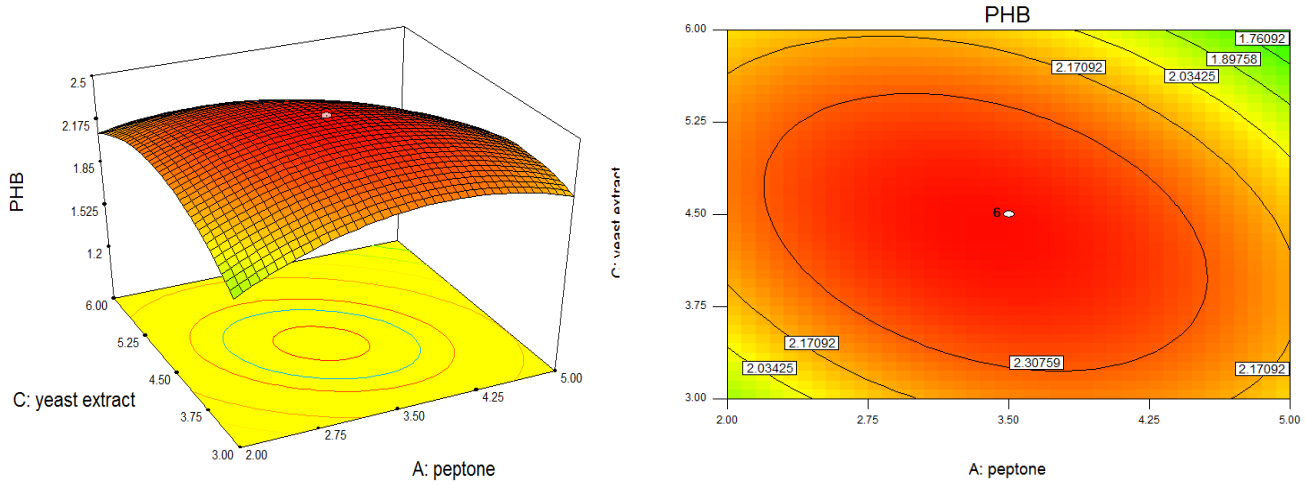

Figure 14: Response surface and contour plot showing the effect of peptone and yeast extract in PHB production.

Similarly, PHB production is low at low concentration of $(NH_4)_2SO_4$ and increases with increase in its concentration upto 2.25 g/L. Further increment in concentration leads to gradual reduction in PHB production.

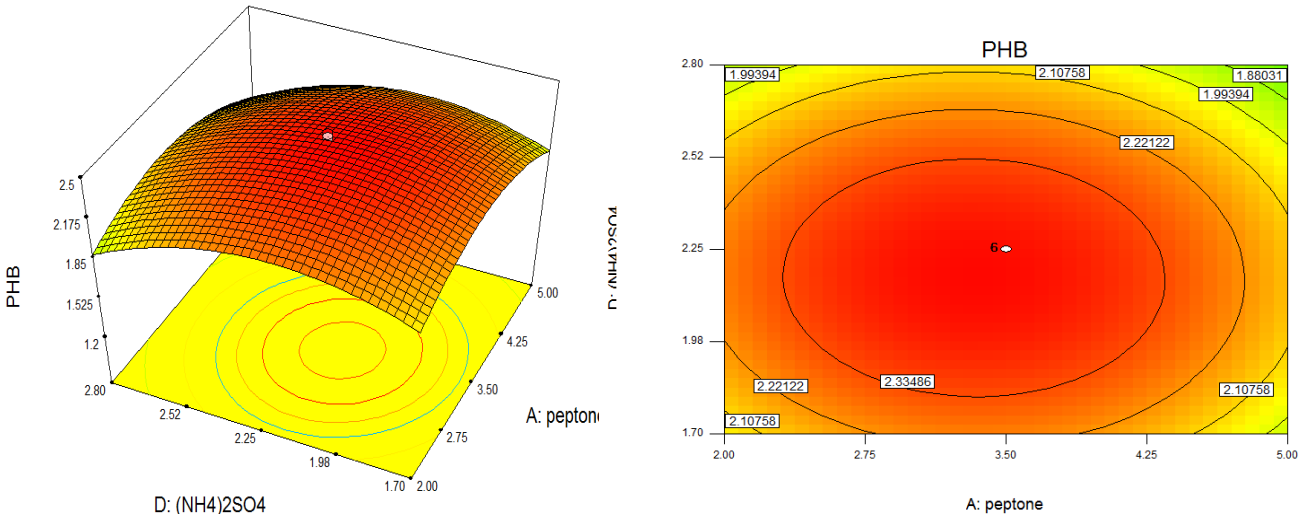

Figure 15: Response surface plot showing the effect of peptone and $(NH_4)_2SO_4$ in PHB production

- **Combined effect of $(NH_4)_2SO_4$ with yeast extract and trace metal solution in PHB production:**

The conjugate effect of $(NH_4)_2SO_4$ and yeast extract in PHB production is shown in figure 16 below. When the concentration of both these variables is low, PHB production is very low. It increases with increase in concentration of the variables and is observed to be maximum at 4.50g/L yeast extract and 2.25 g/L $(NH_4)_2SO_4$. When the concentration of both these variables is high, PHB production is also reduced due to the combined inhibition effect.

Similarly, figure 17 shows the combined effect of concentrations of $(NH_4)_2SO_4$ and trace metal solution in PHB production. It can be seen that the inhibition effect of high concentration of trace metal solution is more than the inhibition effect of high concentration of $(NH_4)_2SO_4$.

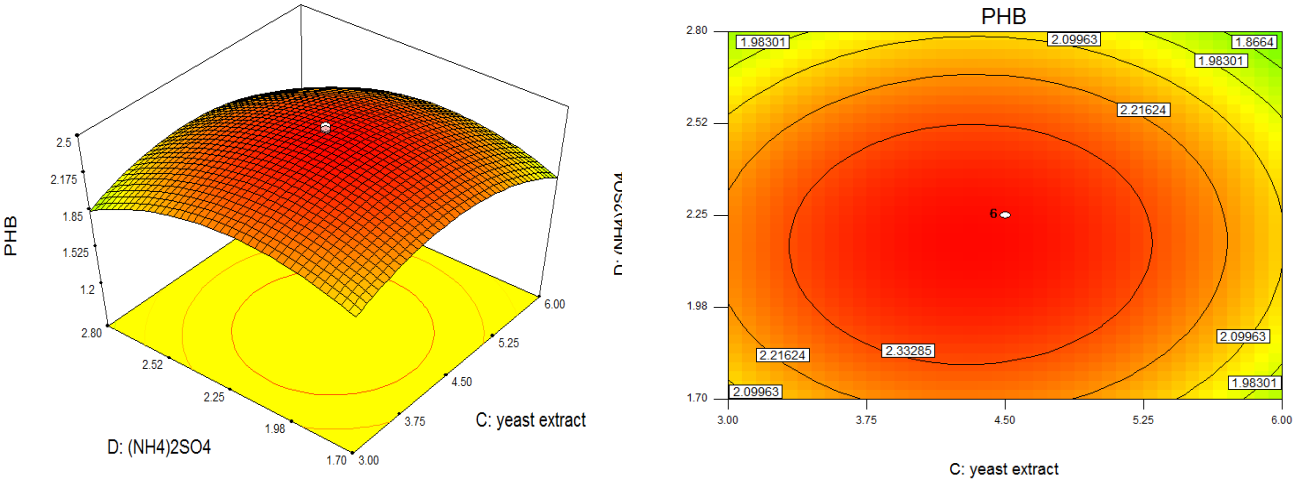

Figure 16: Response surface plot showing the effect of $(NH_4)_2SO_4$ and yeast extract in PHB production

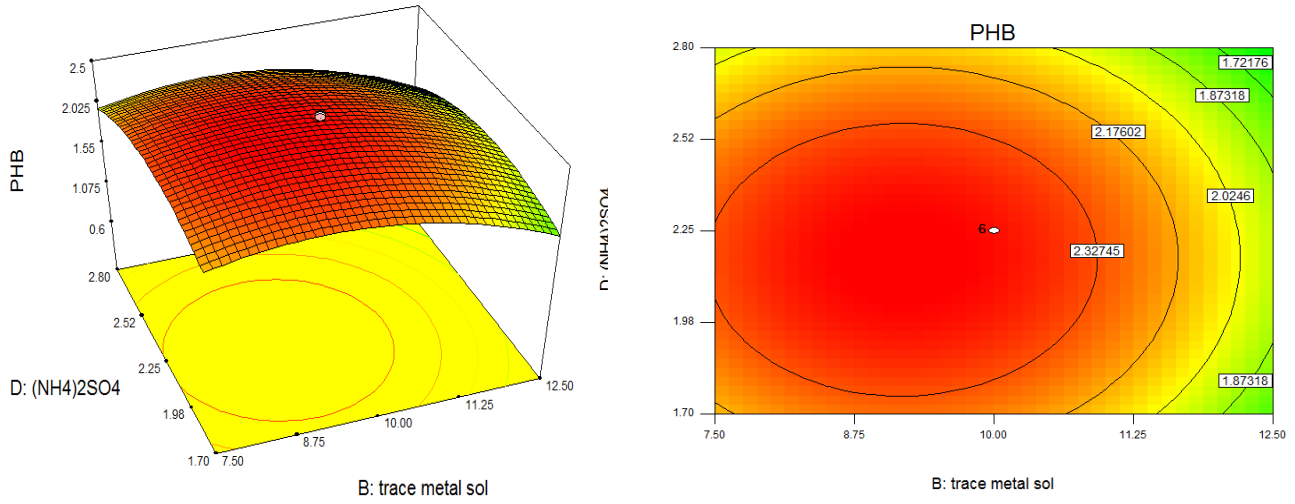

Figure 17: Response surface plot showing the effect of $(NH_4)_2SO_4$ and trace metal solution in PHB production

In the design, criteria selected for optimization were as follows: peptone (A) - minimize, trace metal solution (B) – in range, yeast extract (C) - minimize, $(NH_4)_2\ SO_4$ (D) – in range and PHB concentration at maximum level. A number of optimized solutions with different concentrations were proposed by the software. From those proposed solutions, two acceptable solutions were taken as follows:

(1) Peptone – 2g/L
Trace metal solution – 7.5 ml/L
Yeast extract – 4.09g/L
$(NH_4)_2\ SO_4$ – 1.91 g/L
Expected PHB concentration – 2.02 g/L

(2) Peptone – 2g/L
Trace metal solution – 9.15 ml/L
Yeast extract – 3.10g/L
$(NH_4)_2\ SO_4$ – 2.18 g/L
Expected PHB concentration – 1.93g/L

Experiments with selected optimum criteria were performed in shake flask. In the first case PHB concentration obtained was 1.61 g/L and in the second case it was 1.86g/L. The predicted response was in close agreement with the actual experimental values in the second case. Thus, the second solution was selected as the final optimized solution.

5.2 Scale up of PHB production to fermenter level (Batch process)

5.2.1 Determination of mixing time in fermenter

For assessing the mixing efficiency in reactors and fermenter, mixing time is calculated. It is the time required to attain a specified level of homogeneity starting from the totally segregated state.

For calculating the mixing time (T_m), the fermenter was filled with water and the impeller speed was set at 60 rpm. Initial pH was recorded. 1 mL of 2M H_2SO_4 was added and the change in pH after every 2 seconds was noted. After some time, pH was observed to be stable. The time required to achieve this stable pH is the mixing time. The same procedure was followed to calculate the mixing time at different rpm, viz. 100, 200 and 250.

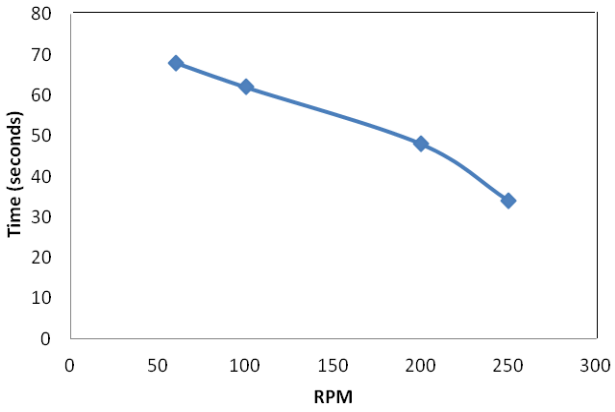

Figure 18: variation of mixing time (T_m) with rotation speed of impeller

It was observed that the mixing time (T_m) is inversely proportional to the rotation speed of the impeller. High mixing time is required at low rotation speed of the impeller and as the rotation speed increases, the mixing time decreases.

5.2.2 Estimation of k_La by static method

Gas-liquid mass transfer is very important in bioprocess because oxygen is required in aerobic fermentations. Thus, providing sufficient gas exchange is among the most crucial factors in fermenter operation. The volumetric oxygen mass transfer coefficient, k_La, is used to characterize the mass transfer of oxygen from gas to liquid.

For estimation of k_La, static gassing out method was used which is based on unsteady- state mass balance for oxygen. The fermenter was initially filled with water. The water was deoxygenated by sparging nitrogen into the fermenter. The decrease in dissolved-oxygen concentration C, is recorded as a function of time. Air is then sparged into the vessel at a constant flow rate along with agitaion. The dissolved-oxygen concentration C, which now increases with time, is recorded. The oxygen concentration should remain above C_{crit}. The dissolved-oxygen concentration soon reaches a steady state value C*. The equation for calculating k_La is as follows:

$$dC / dt = k_La (C^* - C) – q_ox \qquad \text{…..(i)}$$

where q_ox is rate of oxygen consumption which is zero in this case as there are no microbes present. Integrating eqution (i) we have

$$\ln(C^* - C) = - k_La \cdot t \qquad \ldots.(ii)$$

A graph was plotted between ln (C* - C) and time during re-oxygenation period. The slope of this graph gives the value of $-k_La$. From the figure below it can be seen that the value of k_La was found to be 0.0374 s^{-1}.

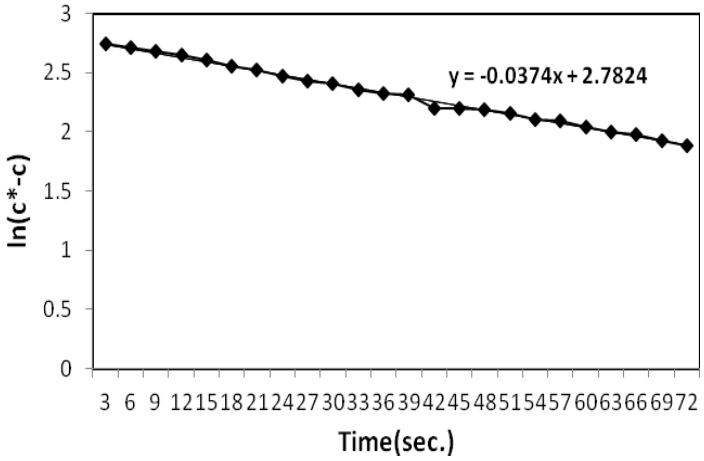

Figure 19: Variation of ln (C* - C) with time during re-oxygenation

5.2.3 Determination of kinetic parameters

Scale up of PHB production from shake flask level to fermenter level was done to establish the batch growth and product kinetics in a 3L bioreactor under controlled conditions. This would also yield the basic kinetic parameters.

Figure 20: 3 L fermenter (Bio–Age)

Figure 21 shows the growth curve for batch cultivation of *C. necator* in the bioreactor. It was observed that the lag phase was considerably minimized in the bioreactor. This was because inoculation was done with actively growing culture in a shake flask and controlled environmental conditions. Maximum specific growth rate was 0.324 hr^{-1} at 12 hrs.

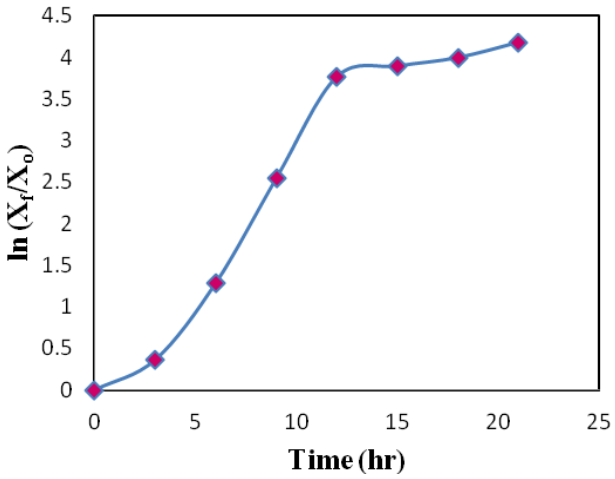

Figure 21: Growth curve for batch cultivation of *C. necator* in bioreactor

The figure 22 below elucidates the variation in concentration of sucrose, biomass, PHB with time during batch cultivation of *C. necator* for PHB production in a 3L bioreactor.

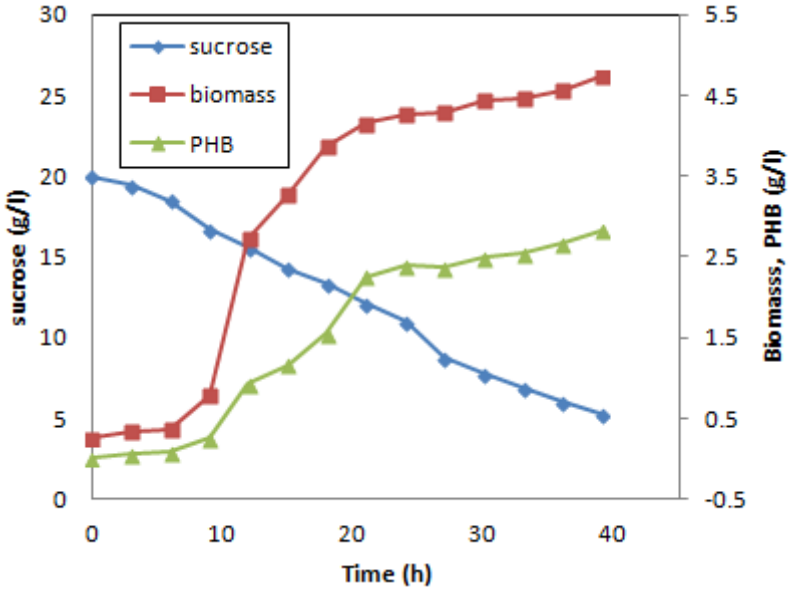

Figure 22: Batch kinetics of cell growth, nutrient consumption and PHB production for *C. necator* in a 3L bioreactor

During the 39 hr cultivation, the maximum value of biomass and PHB achieved was 4.724 g/L and 2.824 g/L respectively. 14.75 g/L sucrose was metabolised from an initial value of 20 g/L with only 5.25 g/L left unconsumed at the end of cultivation. The biomass yield ($Y_{X/S}$) was observed to be 0.320 g/g and the product yield ($Y_{P/S}$) was found to be 0.20g/g with an overall PHB productivity of 0.0724 g L^{-1} hr^{-1}. Dissolved oxygen concentration was also monitored. Its variation with time is shown in figure 23 below. It was observed that the concentration falls suddenly after 6 hr as the culture enters the log phase and is actively growing thus oxygen consumption increases.

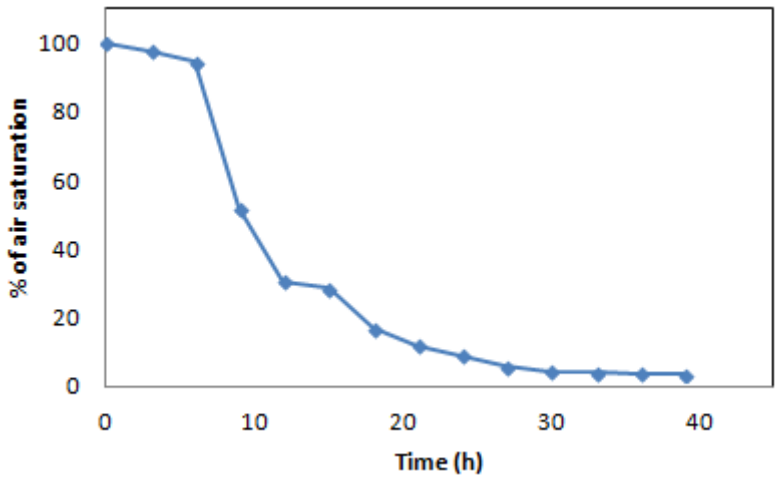

Figure 23: Variation of dissolved oxygen concentration with time

Table 6: Comparison of concentration and productivity data obtained in shake flask and in bioreactor using optimized media

Kinetic parameters	**Optimized Media (Shake Flask)**	**Optimized Media (Fermenter)**
Maximum biomass concentration	3.85 g/L	4.724 g/L
Maximum PHB concentration	2.01 g/L	2.824 g/L
PHB yield	0.13 g/g	0.20 g/g
PHB productivity	0.0502 g/L-h	0.0724 g/L-h

5.3 Fed batch fermentation process to enhance PHB production

To enhance the production of PHB fed-batch fermentation studies were done in 5 L fermenter. The concentration of sucrose and nitrogen maintained in the feed was 100 g/L and 1.2 g/L respectively. 1.2 g/L nitrogen translates to 5.65 g/L ammonium sulphate which was used as the nitrogen source. The feeding was initiated at two different feed rates – 70 mL/h and 30 mL/h and the results obtained were then compared.

Initially the fermentation was started as batch cultivation with 3 L media. Samples were collected after every 3 h for analyses. After 20 h fermentation, 1.5 L culture was removed from the fermenter and stored at 4ºC for further studies. To the remaining 1.5 L culture, nutrient feeding was given at a flow rate of 70 mL/h. The nutrient feeding was initiated at 20 h as it was seen from the batch cultivation studies that the culture is in actively growing state at 20 h. The feeding was continued for 20 h (from 20 to 40 h). After 40 h, fermenter was once more operated in batch mode up to 44 h for the consumption of remaining substrates.

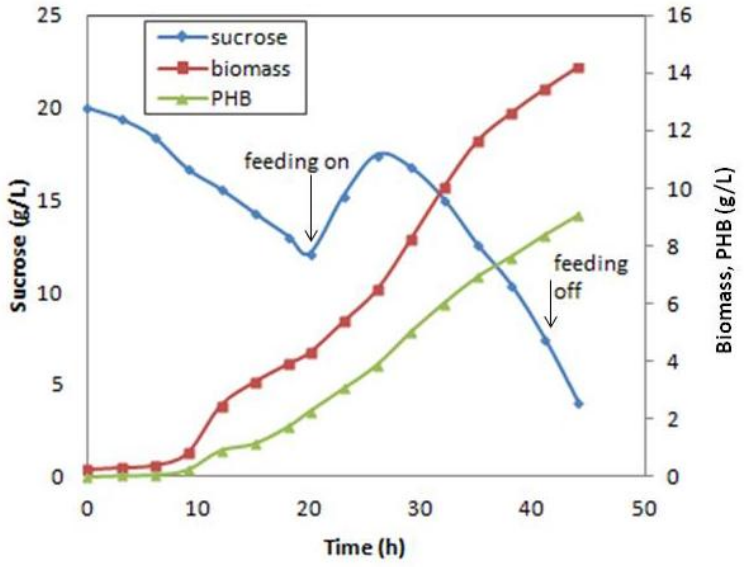

Figure 24: Fed-batch fermentation at flow rate of 70 mL/h.

Maximum concentration of biomass and PHB obtained was 14.182 g/L and 9.15 g/L respectively. Thus, 2.88-fold increase in PHB production was achieved. PHB productivity was improved significantly (0.322 g/L-h) as compared to batch production (0.0724 g/L-h).

After this the fermenter was cleaned and prepared for next fermentation. The 20 h grown culture which was previously preserved at 4ºC was now pumped into fermenter. Nutrient feeding was then given at flow rate of 30 mL/h for 20 h (from 20-40 h). After 40 h, the fermenter was operated in batch mode up to 44 h for consumption of remaining nutrients. The samples were collected at intervals of 3 h for analyses.

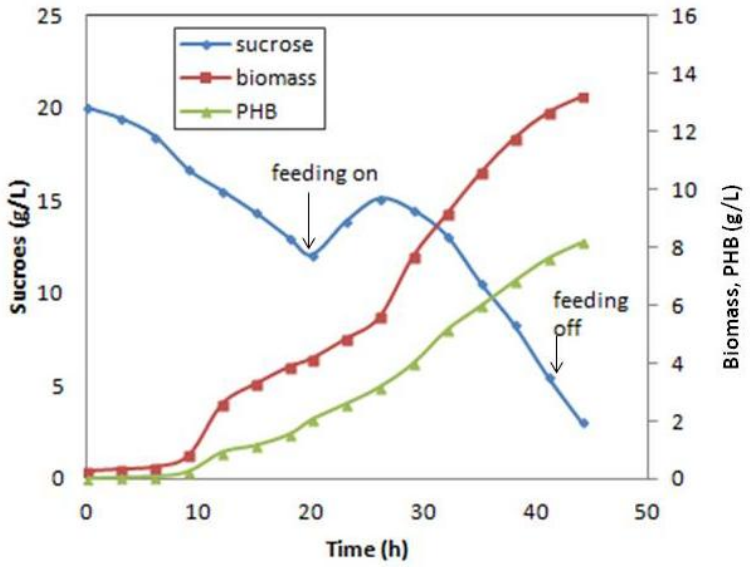

Figure 25: Fed-batch fermentation at flow rate of 30 mL/h.

In this experiment, the maximum concentration of biomass and PHB obtained were 13.201 g/L and 8.17 g/L respectively. PHB productivity was increased to 0.185 g/L-h. Table 7 shows the comparison of data obtained in batch and fed-batch fermentations.

Table 7: comparison of batch and fed-batch fermentation data

Cultivation strategy	**Feeding flow rate**	**Total fermentation time (h)**	**Maximum biomass (g/L)**	**Maximum PHB (g/L)**	**PHB Productivity (g/L-h)**
Batch	No feeding	39	4.72	2.82	0.072
Fed-batch	70 mL/h	44	14.18	9.15	0.208
Fed-batch	30 mL/h	44	13.20	8.17	0.185

It can be seen that fed-batch fermentation with high flow rate (70 mL/h) gave better results in terms of maximum biomass, maximum PHB and PHB productivity. The reason behind is that

during fed- batch cultivation, exponential growth of culture and rates of biomass and PHB accumulation are maintained at high values due to feeding of nutrients while in batch cultivation due to limiting nutrient availability rate of biomass and PHB accumulation decreases eventually. At high flow rate (70 mL/h), higher amount of substrate is being provided per hour as compared to low flow rate (30 mL/h), thus, PHB production is high at high flow rate.

Scanning electron microscopy and energy-dispersive X-ray spectroscopy of the PHB obtained after fed-batch fermentation was carried out. EDS technique is used for elemental analysis of a sample. The figure below shows PHB in granular form. The EDS spectrum shows that the sample mainly consists of carbon and oxygen which is the case with PHB as it is a polymer of hydroxybutyric acid.

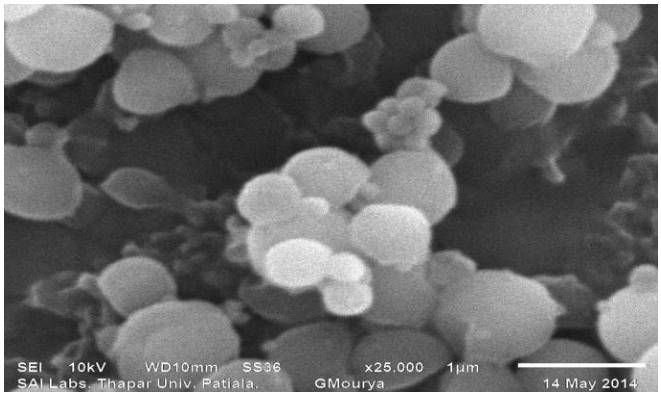

Figure 26: SEM image of PHB obtained after fed-batch fermentation

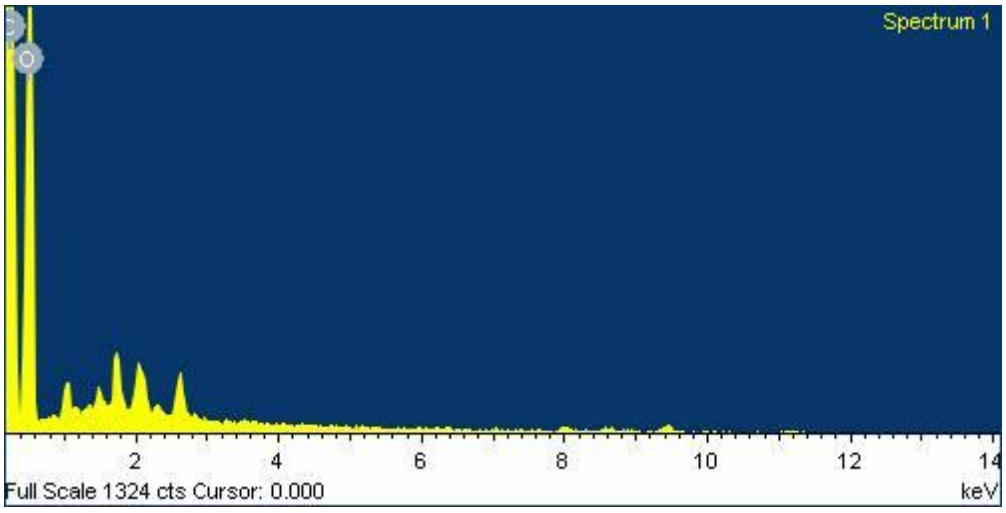

Figure 27: EDS spectrum of PHB obtained after fed-batch fermentation

X-ray diffraction studies of the PHB obtained were also carried out. The pattern was recorded from 0- 70 of 2theta. It can be observed from the figure that the characteristic 2 theta peak values of 13.4, 16.8, 20.0, 22.4 and 25.4 that are found in pure PHB (S.Roy *et.al* 2007) were also found for PHB extracted from *C. necator* after fed-batch fermentation. The sharp peaks and high peak intensities imply that the PHB obtained has high crystallinity.

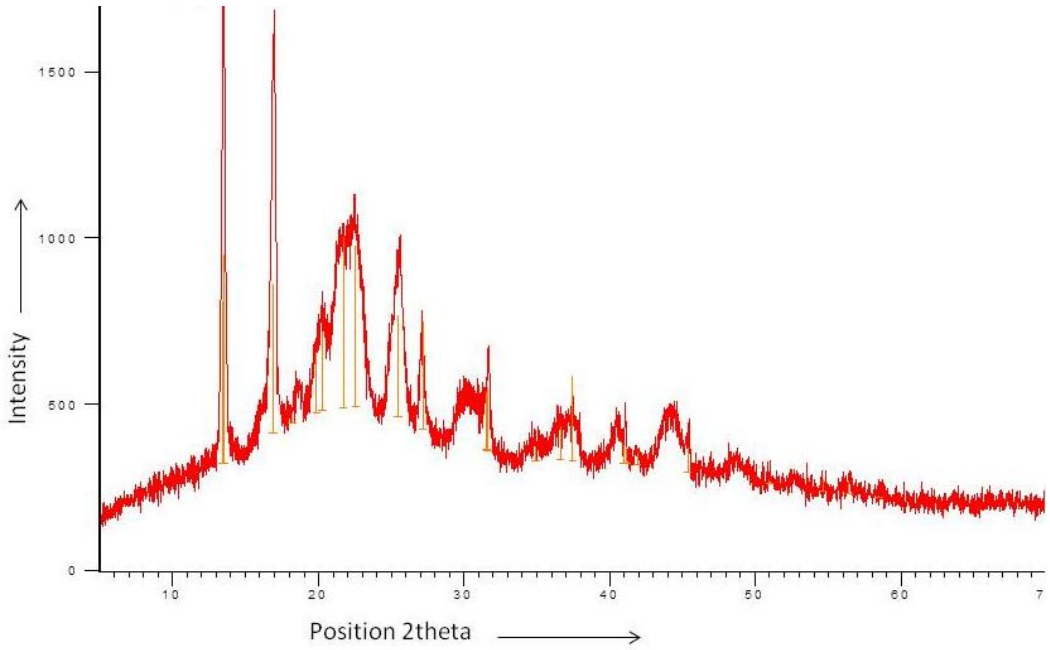

Figure 28: XRD peak profile of PHB obtained after fed-batch fermentation

5.4 Extraction efficiency of different methods

The figure below shows a comparison of amount of PHB extract obtained from *C. necator* by using different methods. It can clearly be observed that surfactant hypochlorite method is the most efficient method as it gives the maximum PHB extract per gram biomass. This method is also advantageous as its operating cost is low and degradation of PHB is limited, as compared to other methods. Thus, surfactant-hypochlorite digestion method was used in the present study for extraction of PHB from the bacterial cells.

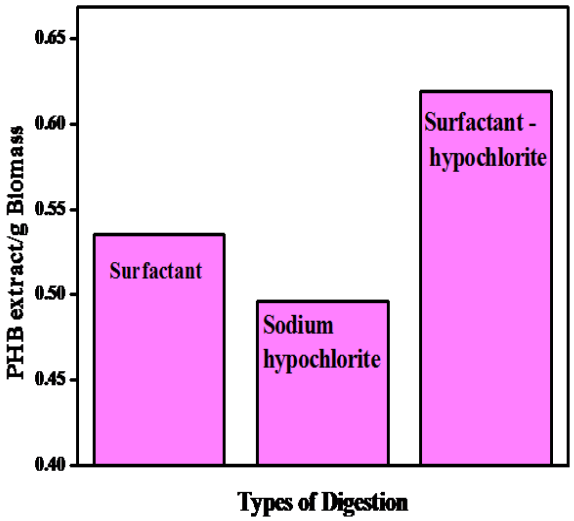

Figure 29: Comparison of PHB extract obtained by different methods

5.5 Discussion

According to the literature available regarding PHB production by *A. eutrophas,* nobody used cheap sucrose based media like table sugar. Concentration of PHB produced in batch fermentation itself is comparatively better than some studies like 2 g/L (Young *et al*., 1994), 1.55 g/L (Ramsay *et al*., 1995), 1.28 g/L (Waranya *et al*., 2011). In terms of biomass concentration, produced biomass concentration is comparatively better than the study like 1.17 g/L (Kim, 2000) and productivity is also relatively high. In fed-batch fermentation, the maximum PHB obtained was 9.15g/L only but this value can possibly be further improved by better control of feeding. During fed-batch fermentation, feeding is required to be controlled so that microbial growth is not limited, yet the concentration of substrate in the broth remains low or increases slowly to a low upper limit so that the substrate inhibition of PHB synthesis is minimal.

6. CONCLUSION

This study established that statistical optimization of medium components for fermentative production of PHB plays an important role in increasing the concentration of biomass and PHB with number of experiments minimised. Also fed-batch fermentation gives high cell density cultures and leads to significant improvement in the production of PHB. A 3.2-fold increase in PHB production was achieved by fed-batch fermentation strategy as compared to batch. Thus, it can be extremely useful for the production of PHB even in industrial scale with similar quality and quantity. Surfactant-hypochlorite digestion method proved as a most efficient separation process to extract maximum PHB from PHB containing cells. Adopted green approach for biopolymer production is fully sustainable and based on fully renewable resource.

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