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Production and characterization of microbial Poly-β-hydroxy butyrate(PHB) from marine bacterial isolates

A PROJECT REPORT

Submitted by

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BONAFIDE CERTIFICATE

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Abstract

Abstract

Considering the industrial interest of Poly-β-hydroxybutyrate (PHB), bacteria isolated from the various marine arenas were screened for their ability to accumulate PHB. Among the different isolates, the selected strain showed the maximum accumulation of PHB. A gram negative bacterium (designated strain SVS-7) that potentially accumulated PHB was isolated from marine water. To increase the productivity, steps were taken to evaluate the effect of carbon sources on PHB productivity by isolated marine organism. A four-factor central composite rotary design (CCRD) was employed to optimize the medium and to find out the interactive effects of four variables, viz. concentrations of cassava waste, sea water, ammonium sulphate and pH on PHB production. Using response surface methodology (RSM), a second-order polynomial equation was obtained by multiple regression analysis and a yield of 554 mg/L of PHB dry weight was achieved from the optimized medium at pH 9.The optimized conditions were further used for the fed-batch fermentation. The same medium was utilized for fermentor studies by fed-batch culture. The cassava waste is fed at two different time intervals at 0th, 18^{th} hour to keep the carbon source as excess and PHB production was checked for every 3 h. Maximum production of PHB (2.731 g/L) occurred at 48^{th} hour. The extracted polymer was compared with the authentic PHB and is confirmed to be PHB using FTIR analysis. Thus, the study highlights the potential of the use of marine organism in the commercial production of PHB.

Keywords: *SVS-7, Polyhydroxybutyrate, cassava waste, Sea water, RSM, Optimization, Fed-batch.*

List of symbols and abbreviations

μ	specific growth rate
$Y_{X/S}$	yield of biomass and substrate
$Y_{P/S}$	yield of product and substrate
PHA	Poly-hydroxyalkanoates
PHB	Poly-hydroxybutyrate
PHV	Poly-hydroxyvalerate
scl	Short chain length
Mcl	Medium chain length
Lcl	Long chain length
HA-coA	hydoxyalkyl-CoA
CCRD	Central composite rotary design
pH	Potential of Hydrogen
DO	Dissolved Oxygen
%	Percentage
^0C	Degree Celsius
RPM	Rotation per minute
mM	Millimolar
ml	millilitre
L	Litre
μl	Microlitre
g	Gram
mg	Milligram
h	hour

Introduction

1. Introduction

Plastic is one of a few new chemical materials which pose environmental problem. Polyethylene, polyvinylchloride, poly styrene are largely used in the manufacture of plastics. The invention of use and throw plastic items in the early twentieth century has changed the face of modern world. From computer to automobile parts, simple domestic appliances plastic has found its place almost everywhere. Plastic materials have molecular weight ranging from several 1000 to 150000. Excessive molecular size seems to be mainly responsible for the resistance of these chemicals to biodegradation and they persist in the soil environment.

Poly-hydroxyalkanoates (PHAs) have been intensively studied in the last two decades as possible substitutes for conventional polymers. They have mechanical properties which are similar to traditional thermoplastics and, additionally, present other important advantages: they are biodegradable, biocompatible and can be obtained from renewable resources (Schubert *et al.*, 1988).

Polyhydroxyalkanoates (PHAs) are a group of polyesters that are promising alternatives to conventional plastics due to their biodegradability and capability of being produced from renewable resources. Large efforts have been made to develop fermentation strategies and new bacterial strains for economic PHA production by pure cultures (Braunegg *et al.*, 1998). Two common PHA monomers are hydroxybutyrate (HB) and hydroxyvalerate (HV). Pure PHB is stiff and brittle but with introduction of other monomers in the polymer (as with the copolymer of PHB *co*-HV) the elasticity and flexibility increase providing a polymer with properties similar to polypropylene (Lee, 1996).

Current worldwide dependence on fossil fuels for plastics manufacture (appro.270 million metric tons of fossil fuels), the scarcity of space for disposal and growing environmental concerns for non-biodegradable synthetic plastics have fuelled research towards development of eco-friendly biopolymer materials (Grengross *et al.*, 2000). Considerable emphasis has been laid on the development of five different types of biopolymers which include fibre-reinforced composites, starch based materials, plant

and adhesives (Kolybaba *et al.*, 2004). Increasing awareness of environmental pollution has generated a resurgence of interest in biological methods for the production of biodegradable polymers (Patnaik, 2006).

Growth in the human population has led to the accumulation of huge amounts of non-degradable waste materials across our planet. The accumulation of plastic wastes has become a major concern in terms of the environment (Guillet, 2002; Derraik, 2002; Thompson *et al.*, 2004). Conventional plastics not only take many decades to be decomposed in nature, but also produce toxins during the process of degradation. For this reason, there is special interest in producing plastics from materials that can be readily eliminated from our biosphere in an "environmentally friendly" fashion (Gross and Kalra, 2002). The allure of bioplastic is also linked to diminishing petrochemical reserves. The industrialized world is currently highly dependent on fossil fuels as a source of energy for industrial processes and for the production of structural materials. Fossil fuels are, however, a finite resource and current evidence suggests, based on recent usage trends and the rate of discovery, that utilization rates will outstrip discovery from about 2010 (Zagar, 2000). This is a global problem as our economy is still very oil dependent. The world currently consumes approximately 140 million tons of plastics per annum. Processing of these plastics uses approximately 150 million tons of fossil fuels, which are difficult to substitute. All carbon based structural materials (e.g., plastics, foams, coating, and adhesives) owe their properties to long arrays of carbon–carbon bonds. The challenge to the world is whether we can substitute the source of these long carbon arrays from a non-sustainable source with a sustainable renewable one. The most widely produced microbial bio plastics are poly-hydroxyalkanoates (PHAs) and their derivatives (Madison and Huisman, 1999; Witholt and Kessler, 2002; Kim and Lenz, 2001). Beijerinck first observed lucent granules of PHA in bacterial cells in 1888 reported in (Chowdhury, 1963). The composition of PHAs was first described by Lemoigne as an unknown material in the form of a homopolyester of the 3-hydroxybutyric acids, called Poly-hydroxy butyrate (PHB) (Lemoigne, 1926, 1927). During the following 30 years, interest in this unknown material was negligible. The first report on functions of PHB appeared in (Macrae and Wilkinson, 1958). They reported the rapid biodegradability of PHB produced by *Bacillus megaterium*, by *B. cereus* and *B. megaterium* itself.

Since, PHB has properties similar to petroleum derived synthetic plastics like polypropylene (PP) and is completely biodegradable in the environment. Poly-hydroxy hlkanoates) (PHAs) have been intensively studied in the last two decades as possible substitutes for conventional polymers. The quality that sets PHAs apart from conventional plastics is their complete biodegradability in the environment both aerobically and anaerobically (Hankermeyer and Jieerde, 1998; Sudesh *et al.,* 2000). Until now, there are only few reports on marine PHAs-producing microorganisms (Chien *et al.,* 2007; Mavinkurve and Rawte, 2002; Weiner, 1997). The current review begins with a discussion of the chemical structure and properties of PHAs. This is followed by an examination of PHA synthesis in microorganisms.

They have mechanical properties which are similar to traditional thermoplastics (Galego *et al.,* 2000) and, additionally, present other important advantages: they are biodegradable, biocompatible and can be obtained from renewable resources (Steinbu¨chel and Fu¨chtenbusch, 1998). However, manufacturing costs still remain too high in comparison with polymers of petrochemical origin (Kahar *et al.,* 2004). Poly (3-hydroxy butyrate) (P(3HB)) is a biodegradable, biocompatible thermoplastic made by microorganisms (Byrom, 1987). The important properties of the biopolymer namely molecular weight, brittleness, stiffness, melting point, and glass transition temperature, are comparable to some of the most common petrochemical-derived thermoplastics, such as polypropylene (Barham, 1990). Therefore in certain applications, P(3HB) can directly replace some common traditional, non biodegradable polymers. P(3HB), in combination with other biocompatible and nontoxic polymers, would also have an enhanced scope in biomedical applications. Because P(3HB) is resistant to water and ultraviolet radiation, and it is impermeable to oxygen, it is especially suited for use as food packaging materials. P(3HB) has been used in surgical sutures (Braunegg *et al.,* 1998), and other applications are in the development and adaptation stage (Kim, *et al.,* 1998). The high cost of commercial grade P(3HB), about 15-fold greater than comparable synthetic plastics limits its application. One of the important factors deciding the cost of P(3HB) production on an industrial scale is the raw material cost. Among the substrates required, the carbon source is of primary concern in the case of P(3HB) production. The most common carbon sources are source, glucose, unrefined sugar sources such as cane molasses, beet molasses, etc. The contribution of the carbon source in the

1.1 Objectives:

- Isolation, identification and Characterization of PHB producing marine bacterial isolates from marine samples.
- To study the effect of different carbon source on production of PHB.
- To optimize the best substrate concentration and conditions using statistical media optimization.
- Synthesis of PHB using optimized condition by Fed-Batch cultivation.
- Characterization of PHB produced from the isolated bacterial strains.

Literature Review

2. Literature review

PHAs are among the most investigated biodegradable polymers in recent years. They are superior to other biodegradable polymers because of the large number of different monomer constituents that can be incorporated. At present, about 150 different hydroxyalkanoate units have been identified. The most common is Poly-β– hydroxybutyrate (PHB).

The PHA are non-toxic, biocompatible and biodegradable thermoplastics that can be produced from renewable resources. They have a high degree of polymerization; are highly crystalline, optically active, isotactic, piezoelectric and insoluble in water. These features make them highly competitive with polypropylene, the petrochemically derived plastics. PHAs are polyesters of hydroxy alkanoates with the general structural formula as shown in Fig 2.1.

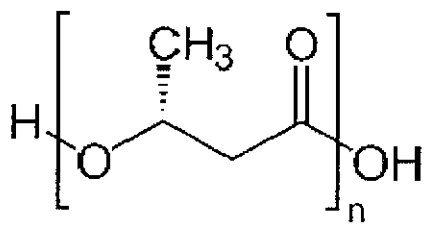


Fig 2.1. Structure of PHB

Approximately 40% of the manufacturing costs of PHAs are due to the raw materials employed. Furthermore, 40% of the costs are usually related to the downstream processing stages. The major problem associated with the industrial PHB production is its cultural optimization studies. Hence, different carbon sources, nitrogen sources, pH and salt concentration were tested to analyze the effect on PHB production by the higher yielding PHB producer. This has limited the use of PHB to specialized areas like surgery and medicine. Efforts on cost reduction have been directed towards increase in PHB content by choosing the cheaper carbon sources, developing better bacterial strains and efficient fermentation and recovery systems (Lee, 1996). Another approach involves the use of excess activated sludge from a wastewater treatment plant as a source of PHB and renewable carbon

addition to these approaches, researchers have prepared blends of starch and PHB (PHB:starch 70:30) which, in addition to reducing the final cost of product would result in a product having properties similar to that of pure PHB (Godbole, *et al.*, 2003).

2.1 Poly β-hydroxybutyrate (PHB):

The first and the best known member of PHA, the homopolymer poly(3-hydroxybutyrate) (PHB) was discovered by Maurice Lemoigne in 1925 (Jackson and Srienc, 1994). Approximately 150 different hydroxyalkanoic acids are at present known, as constituents of bacterial storage polyesters (Steinbüchel and Valentin, 1995). PHAs, synthesized by many Gram positive and Gram negative bacteria as storage compounds, are deposited as insoluble inclusions in the cytoplasm (Steinbüchel, 1991). These water insoluble PHAs exhibit rather high molecular weights, thermoplastic and/or elastomeric features and some other interesting physical and material properties. Plastics produced from PHAs have been reported to be truly, fully biodegradable (Page, 1995). Microbes belonging to more than 90 genera including aerobes, anaerobes, photosynthetic bacteria, archaebacteria and lower eukaryotes are able to accumulate and catabolise these polyesters. PHB is an aliphatic homopolymer of poly-β-hydroxybutyric acid with a melting point of 179 °C and is highly crystalline (80%). It can be degraded at the temperature above its melting point. PHB has some properties similar to polypropylene with three unique features: thermoplastic processability, 100% resistance to water, and 100% biodegradability (Hrabak, 1992).

(Byrom, 1990) summarized two major advantages of the PHB-PHV copolymer over the PHB homopolymer. Firstly, the copolymer has a lower melting point. However, HV content in the copolymer does not significantly decrease the temperature at which molecular weight degradation occurs. This important feature allows the copolymer to be processed with a larger range of temperature conditions. Secondly, it has a lower flexural modulus or level of crystallinity, which makes it tougher and more flexible. However, a disadvantage of having a low crystallization is that it takes a longer cycle time in the processing step, e.g., the injection moulding process (Hrabak, 1992). PHAs are a family of optically active biological polyesters, containing (R)-3HA monomer units (Anderson and Dawes, 1990). The 3-hydroxyalkanoic acids are all in the *R* configuration due to the stereo specificity of the

vary from methyl to tridecyl is positioned. This alkyl chain can be saturated, aromatic, unsaturated, halogenated or with branched monomers (Garcia *et al.*, 1999; Arkin *et al.*, 2000).

2.2 Classification of PHA:

PHAs are classified as scl – PHAs, mcl – PHAs and lcl – PHAs based on the length of the hydroxyalkanoic acid monomers (Steinbüchel and Pieper, 1992). Scl – PHAs are composed of C3 to C5, 3-hydroxy/4-hydroxy fatty acids. Poly(3-hydroxybutyrate) (PHB), the first of the PHAs studied extensively, falls in this group of PHAs. mcl – PHAs are composed of C6 to C16, 3-hydroxy fatty acids. This group includes polymers such as poly(3-hydroxyhexanoate) P(3HHx), poly(3-hydroxyheptanoate) P(3HHp), poly(3-hydroxyoctanoate) P(3HO), poly(3-hydroxydodecanoate) (3HDD) etc., The term lcl – PHAs is reserved for the PHAs composed of more than C163-hydroxy fatty acids. These classes are also referred to as scl-, mcl- and lcl-PHAs.

2.3 Importance of PHB to microorganisms:

PHB granules act as energy reserve materials when nutrients such as nitrogen and phosphorous sources are available in limiting concentrations in the presence of excess carbon source. (Senior and Dawes, 1971) proposed that PHAs could serve as a sink for reducing power and, therefore, be regarded as a redox regulator within the cell. Encystment of *Azotobacter* cells in lab conditions was linked to PHB accumulation (Stevenson and Socolofsky, 1973). However, later it was shown that in natural habitats, accumulation of large amounts of PHB was not an absolute requirement for encystment (Thompson and Skerman, 1979). (Anderson and Dawes, 1990) observed that the presence of PHB in a cell retarded the degradation of cellular components such as RNA and proteins during nutrient starvation frequently, but not universally. PHB enhanced survival of some but not all the bacteria and also served as a carbon and energy source for spore formation in *Bacillus* sp. (Steinbuchel, 1991) reported that PHAs were stored as intracellular cytoplasmic inclusions. And, these inclusions, as a reduced polymer, provided the advantage that they become unavailable as a carbon source for the competing organisms, and that they were osmotically

reported that the PHA biosynthesis in *Ralstonia eutropha* and *Rhodospirillum rubrum* included pyridine nucleotide dependent reduction of acetoacetyl-CoA.

Thus, PHB is a sink for reducing equivalents and can be considered to be a fermentation product. By contrast, in *Pseudomonas oleovorans* and *P. aeruginosa*, oxygen deficiency exerted a negative effect on the accumulation of PHA. Because in these organisms either reducing equivalents were formed or energy in the form of ATP was consumed.

In *Rhizobium* and *Bradyrhizobium japonicum*, during the bacteroid stage, the nitrogen fixation apparatus competed with PHB formation for reducing equivalents (Povolo *et al.*, 1994).

(Byrom, 1992) has discussed on the industrial production of PHA in ICI, UK. *Ralstonia* spp. was the organism of choice for ICI because it produced an easily extracted PHA with high molecular weight. PHA production from *Azotobacter* and methylotrophs were also investigated. However, PHA with low yield and molecular weight was produced from methylotrophs and was difficult to extract also.

Azotobacter was not an organism of interest because it used carbon substrate for polysaccharide synthesis rather than for PHA production. *R. eutropha* produced 70-80% polymer under phosphate limiting conditions. A good candidate for PHA production would be a culture that can store high PHA concentrations while growing on an inexpensive growth substrate. (Lee, 1996) also suggested that the growth rate of a microorganism and its polymer synthesis rate were factors that should be considered when selecting a potential candidate of PHA production.

(Yamane, 1996) studied the production of PHA by *A. latus* using sucrose as the feed substrate. Higher cell concentration (142 g/L) was obtained in a short culture time (18 h) and PHB content at the end of the culture time was 50%. They concluded that the inoculums size reduced the culture time. They compared the culture time required for the production of PHB by *R. eutropha* fed with glucose when the same technique (pH-stat fed-batch) was used. The culture time required for *R. eutropha* was longer (30 hours) to obtain high cell concentration

(122 g/L). However, a higher PHB production of 65 per cent was obtained. The average molecular weight of PHB obtained in this study was 3.16×10^5.

(Renner *et al.*, 1996) studied the production of copolymer by 13 bacteria from the rRNA superfamily III. They reported that different bacteria were able to produce PHAs with different PHV/PHB compositions when grown on the same substrate.

(Rawte and Mavinkurve, 1998) isolated PHB producing bacteria from mangroves along the Mandovi estuarine ecosystem. Preliminary screening of the isolates grown on tributyrin agar for accumulation of PHBs was done microscopically using the fluorescent dye Nile Blue A. out of 65 PHB producing isolates, 60 were able to fix atmospheric nitrogen and grew luxuriantly on nitrogen free media.

(Lokesh *et al.*, 2005) carried out the isolation of PHA producing bacteria and characterized the potent PHA producer as *Sphingomonas* spp. as confirmed by 16s rDNA sequencing. In their study, the strain was grown on different sugars and organic acids and its ability to accumulate PHA was analyzed. The strain could accumulate PHA when grown on disaccharides, aldohexose, sugar alcohols and some organic acids, but failed to assimilate ketoses, pentoses and starch. Among the sugars tested, PHA yield was found to be high with sucrose or mannose, contributing to 55-60 per cent of cell biomass.

(Pal and Paul, 2000) isolated aerobic free living nitrogen fixing bacteria from natural environments. Systematic screening of these isolates has indicated that nearly 70 per cent of isolates of the genus *Azotobacter* were capable of accumulating PHB. The PHB contents of majority of the strains ranged from 25-47 per cent of cell dry weight, while only 7 isolates accumulated PHB accounting to more than 50 per cent of their cell dry weight. One of the promising strains of *Azotobacter chroococcum* has been shown to accumulate the polymer accounting nearly to 70 per cent of cell dry weight, when grown under optimized conditions.

(Tajima *et al.*, 2003) isolated a gram-positive bacterium (designated as strain no INT005) from a field soil that accumulated poly hydroxyalkanoate (PHA) from a grass field soil. The PHA productivities of strain INT005 were higher than those of *Bacillus megaterium*

(Mukhopadhyay and Paul, 2003) reported the potentialities of phototropic purple non sulphur bacteria for PHB synthesis. They isolated 30 organisms from water and sludge samples collected from different water bodies of West Bengal by enriching under phototropic microaerophilic conditions. Systematic screening of these isolates for PHB production led to the identification of five strains with PHB content ranging from 10-15 per cent of cell dry weight, when grown in acetate containing medium under a light intensity of 10000 lux. Carbon sources like acetate and butyrate were most suitable for PHB accumulation. However, the presence of nitrogen source in the growth medium was found to be inhibitory for PHB accumulation although the growth was enhanced. Phosphate and sulphate limiting conditions enhanced the polymer accumulation by the isolates. They also evaluated the effects of physical factors like pH, light intensity on polymer accumulation.

(Ayub *et al.,* 2004) isolated *Pseudomonas* sp. 14-3, a strain from Antarctic environments that accumulated large quantities of polyhydroxybutyrate (PHB) when grown on octanoate. This isolate was characterized on the basis of phenotypic features and partial sequencing of its 16s ribosomal RNA gene.

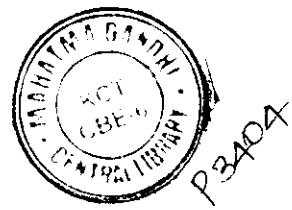
(Sujatha *et al.,* 2005) isolated PHB producing bacteria from different locations such as garden soil, tannery effluents, sewage sludge and field soil. They obtained higher PHB positive strains from sewage sludge and tannery effluents compared to other sources.

(Yilmaz *et al.,* 2005) isolated 29 strains of *Bacillus* from the grassland soils of Ankara, Turkey and were identified as *B. brevis*, *B. sphaericus*, *B. cereus*, *B. megaterium*, *B. circulans*, *B. subtilis*, *B. licheniformis* and *B. coagulans*. Poly-β-hydroxybutyrate (PHB) production by these strains was determined by the spectrophotometric method, and they found that PHB production ranged from 1.06–41.67% (w/v) depending on the dry cell dry weight. The highest PHB production and productivity percentage was found in *B. brevis* M6 (41.67% w/v).

(Sudesh *et al.,* 2001) observed that *Synechocystis* pcc6803 accumulated PHA. The PHA content was about 5% of cell dry weight. They showed that the biosynthesis could be improved by introducing multicopies of heterologus *pha* synthase gene. Nile blue A staining

cell cytplasam. The relatively low weight of PHA in the cyanobacterium when compared to other bacteria was probabaly due to the small size and mass. They also reported that PHA synthesizing ability of the cyanobacterium might, in fact, be quite similar to that was shown by most bacteria in nature.

(Safak *et al.*, 2002) isolated 15 strains of yeasts from Kombucha tea and identified them as *Saccharomyces cerevisiae, Candida krusei, Kloeckera apiculata* and *Kluyveromyces africans.* They were evaluated for PHB production. PHB accumulation in these strains were found to be between 0.50 and 16.67 per cent. They examined the effect of different carbon and nitrogen sources on the accumualtion of PHB in *Rhodotorula glutinus* Var. glutinus 60 and *S. diastaticus* 27 Different N sources tested did not have any influence on PHB production in *R. glutinus* Var. glutinus 60. On the other hand, when the strain was grown in mannitol medium as the carbon source, the PHB production was observed to be higher than the control and the PHB production was increased to 21.95 per cent.



2.4 PHB production in eukaryotic cells:

The production of bio plastic in bacteria is limited by its high cost compared to the costs associated with petroleum-derived plastics production. This aspect has been one of the driving forces in exploring eukaryotic systems, especially crops, as production hosts. Studies of PHA formation in yeast and insect cells can provide valuable information about how these pathways can be incorporated into plants. Synthesis of PHB has been demonstrated in *Saccharomyces cerevisiae* by expressing the PHB synthase gene from *R. eutropha* (Leaf *et al.*, 1996). PHB accumulation, however, was very low (0.5% of cell dwt), possibly because of insufficient endogenous β-ketoacyl-CoA-thiolase and acetoacetyl- CoA reductase activities. To improve the yield and to synthesize copolymers of PHAs, studies have focused on channeling the intermediates of β-oxidation pathway into PHA assembly. (Poirier *et al.*, 2001) introduced a modified phaC1 gene from *P. aeruginosa* into *S. cerevisiae*. Peroxisomal targeting (PTS1) of the gene product was achieved by developing a construct which resulted in the addition of a 34 amino acid stretch from the carboxylic end of *Brassica napus* isocitrate lyase.

When the recombinant yeast cells were grown in media containing fatty acids, they accumulated mcl- PHAs demonstrating that peroxisomal PHA synthase produces PHA in the peroxisomes using 3-hydroxyacyl-CoA intermediates of fatty acid oxidation. In contrast to *S. cerevisiae, Pichia pastoris* grows vigorously on fatty acids as a carbon source. (Poirier *et al.* 2002) introduced the above PTS1-modified *P aeruginosa.* phaC1 gene into *P. pastoris* and achieved mcl-PHA synthesis in this yeast system with fatty acids in the growth medium. The yield of PHA in the two described studies with yeast systems, however, was low, with accumulations lower than 1% cell dwt. (Marchesini *et al.,* 2003) have explored the possibilities of changing monomer composition of PHA in recombinant yeast cells. The investigators demonstrated that it was possible to alter the PHA monomer composition of mcl-PHAs produced in yeast from the intermediates of the β-oxidation of fatty acids by using a modified form of the peroxisomal multifunctional enzyme 2 (MFE-2, encoded by the fox2 gene). They transformed yeast cells with genes coding for two mutant forms of the 3-hydroxyacyl-CoA dehydrogenase domain of the MFE-2 of *S. cerevisiae.* The mutant MFE-2(aΔ) retain a broad activity towards short-, medium- and long-chain (R)-3-hydroxyacyl-CoAs, while the mutant MFE-2(bΔ), did not accept short chain (R)-3-hydroxyacyl-CoAs. Expression of MFE-2(bΔ), along with PHA synthase, resulted in a substantial increase in the proportion of the short-chain 3-hydroxyacid monomers at the expense of longer monomers. These transforming yeast cells were inefficient at using short-chain (R)-3-hydroxyacyl-CoAs generated by the β-oxidation cycle, leading to higher levels of these intermediates available to the PHA synthase. (Zhang *et al.,* 2006) engineered the synthesis of PHA polymers composed of monomers ranging from 4 to 14 carbon atoms in either the cytosol or the peroxisome of S. cerevisiae by harnessing intermediates of fatty acid metabolism and achieved accumulation of PHA up to approximately 7% of its cell dry weight. Insect cells have also been studied as a model for PHA production in eukaryotes. The phaC gene from *R. eutropha* was successfully expressed in cabbage looper cells and a soluble form of PHB synthase that could be rapidly purified was obtained (Williams *et al.,* 1996a). In a separate attempt, (Williams *et al.,* 1996b) transfected fall armyworm cells with a modified eukaryotic fatty acid synthase, which did not extend fatty acids beyond HB, along with the phaC gene from *R. eutropha.* PHB production was achieved in the transfected cells, although the yield was very low (b1% of cell dwt).

2.5 Biosynthesis of PHA:

PHA production starts in response to stress imposed on cells, usually by nitrogen or phosphorus limitation, although in the presence of abundant carbon source. Under these conditions (PHA accumulation phase), the cells do not grow or divide but instead divert their metabolites towards the biosynthesis of hydoxyalkyl-CoA (HA-coA). HA-CoA is an activated monomeric precursor that is polymerized by the enzymatic action of PHA synthase to form a PHA polyester. Being insoluble in water, PHA begins to form amorphous and nearly spherical granules that gradually fill the cells and force them to expand. The final number of PHA granules in a typical PHA producing cell of *Ralstonia eutropha* was ten (Anderson and Dawes, 1990; Ballard *et al.*, 1987) and the diameter was about 500 nm when growth ceased. Most of the organisms synthesize PHA using *R. eutropha* PHA biosynthetic pathway.

The biosynthesis pathways of *R. eutroph*a, *Zoogloea ramiger*a and *Azotobacter beijerinckii* are well established (Doi, 1990). Firstly, a substrate is condensed to acetyl-coenzyme A (acetyl-CoA). Two moles of acetyl-CoA are then used to synthesize one mole of PHB.

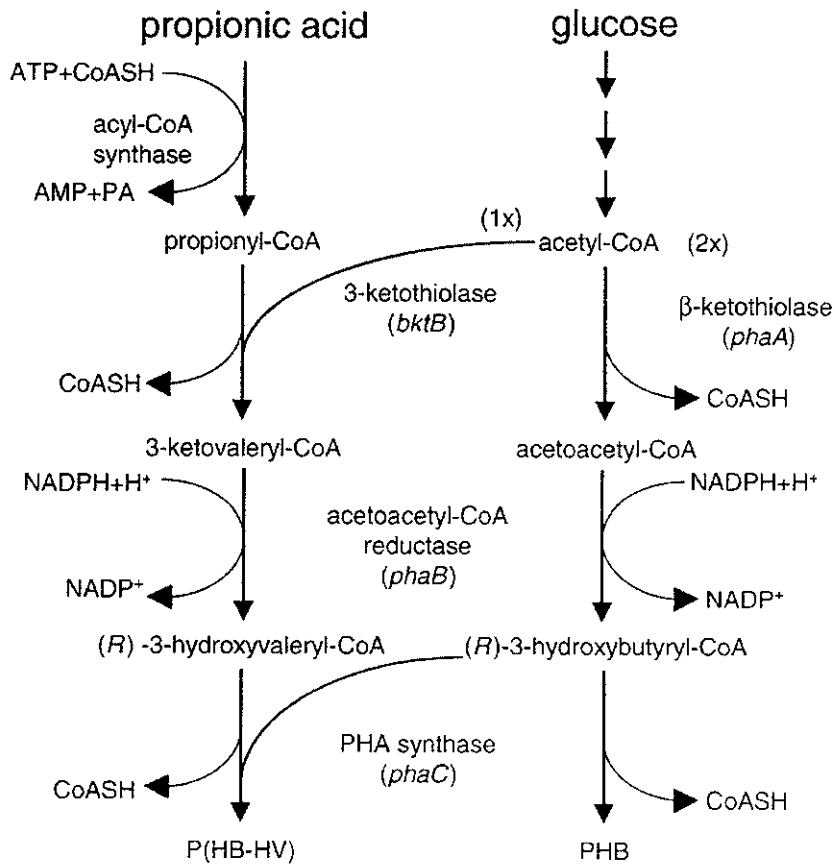
Rhodospirillum rubrum PHA biosynthetic pathway is similar to the *R. eutropha* pathway but two enoyl-CoA hydratases are involved in the second step of catalyzing the conversion of L-3-hydroxybutyryl-CoA to D-3-hydroxybutyryl-CoA via crotonyl-CoA (Anderson and Dawes, 1990; Doi, 1990; Lee, 1996).

Pseudomonas oleovorans PHA biosynthetic pathway is found in *P. oleovorans* and most *Pseudomonas* from the rRNA homology group I (Lee, 1996). They produce mediumchain- length (MCL) PHAs from MCL-alkanes, alcohols, or alkanoates. According to (Doi, 1990), production of short-chain-length (SCL) PHAs, i.e., PHB homopolymer and PHB-PHV copolymer could also be produced by these organisms but the production were less than 1.5%. This PHA biosynthesis involves the cyclic-ı-oxidation and thiolytic cleavage of fatty acids, *i.e.*, 3- hydroxyacyl-CoA, and intermediates of the β-oxidation pathways are used for PHA biosynthesis.

Most *pseudomonas* from the rRNA homology group I except *P. oleovorans* also

these organisms is called the *P. aeruginosa* PHA biosynthetic pathway. (Steinbuchel, 1996) reported that MCL-PHAs produced by this pathway were from unrelated substrates, e.g. gluconate or actetate. PHA was synthesized from acetyl-CoA via fatty acid synthetic pathways. (Wang and Lee, 1997) have classified bacteria that are used for the production of PHAs into two groups, based on the culture conditions required for PHA synthesis. The first group that includes *Ralstonia eutropha*, Methylotrophs, and *Pseudomonas*, require the limitation of an essential nutrient element in the presence of an excess carbon source for efficient synthesis of PHAs. The second group which includes *Alcaligenes latus*, *Azotobacter vinelandii* and recombinant *Escherichia coli*, does not require nutrient limitation for PHA synthesis and can accumulate PHA during growth. However, even in *Alcaligenes latus* PHB synthesis is enhanced by nitrogen limitation suggesting that this growth during PHA accumulation is not an efficient process.

2.6 Genes and enzymes involved in PHA synthesis:



Many species of bacteria, which are members of the family Halobactericeae of the Archaea, synthesize PHAs. The list of such microorganisms is growing and currently contains more than 300 organisms (Anderson and Dawes, 1990; Steinbuchel, 2001;Steinbüchel and Valentin, 1995; Braunegg *et al.,* 1998; Madison and Huisman, 1999; Zinn *et al.,* 2001; Ciesielski *et al.,* 2006; Berlanga *et al.,* 2006). The chemical diversity of PHAs is large; of which the most well-known and widely produced form is PHB (Hankermeyer and Tjeerdema, 1999; Kim and Lenz, 2001).

The synthesis of PHB is considered the simplest biosynthetic pathway. The process involves three enzymes and their encoding genes (Fig. 2.6.1) (Schübert *et al.,* 1988, 1991; Peoples and Sinskey, 1989a,b; Madison and Huisman, 1999; Steinbüchel and Hein, 2001; Reddy *et al.,* 2003). phaA gene encodes β-ketothiolase, the first enzyme for the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA. The next step is the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA catalyzed by the acetoacetyl-CoA reductase (Steinbüchel and Schlegel, 1991). The enzyme is encoded by the phaB gsene and is NADPH-dependent. The last reaction is the polymerization of (R)-3-hydroxybutyryl-CoA monomers catalyzed by PHA synthase, which is encoded by the phaC gene (Rehm, 2003; Stubbe and Tian, 2003). PHA synthase in *Ralstonia eutropha*, formerly known as *Alcaligenes eutrophus,* reacts with a narrow range of substrates, with chain length of C3–C5 and prefers C4-substrates (Steinbüchel and Schlegel, 1991). Therefore, PHAs obtained by this pathway contain short-chain-length monomers. All three enzymes for PHB synthesis are located in the cytosol of the cell where PHB accumulation takes place (Anderson and dawes, 1990). Apart from PHB, bacteria also synthesize a wide range of other PHAs (Kim and Lenz, 2001; Luengo *et al.,* 2003).

PHAs with different C3 to C5 monomers have been produced in several bacteria including *R. eutropha* through alterations in the type and relative quantity of the carbon sources in the growth media (Steinbüchel and Schlegel, 1991; Dias *et al.,* 2006). For example, addition of propionic acid or valeric acid in glucose media leads to the production of a random copolymer composed of HB and HV [P(HB– HV)]. In this pathway, the condensation of propionyl- CoA with acetyl-CoA is mediated by a distinct ketothiolase (3-ketothiolase, bktB;) (Slater *et al.,* 1998) (Fig.2.6.1). Reduction of 3-ketovaleryl-CoA to

by the same enzymes involved in PHB synthesis, namely acetoacetyl-CoA reductase and PHA synthase (Poirier *et al.*, 2002) (Fig.2.6.1). PHA synthases isolated from different bacteria are capable of using a wide range of hydroxyacyl-CoA thioesters as substrates. All known PHA synthases can be classified into four classes according to their substrate specificities and their subunit compositions (Rehm and Steinbüchel, 2002; Hai *et al.*, 2004). Genes encoding key enzymes involved in PHA synthesis have been cloned from several natural producers of the biopolymer.

These approaches have a dual advantage of saving cost on biomass generation and volume reduction of waste activated sludge by extracting PHB. The saving on waste activated sludge disposal cost following volume reduction could reduce PHB production cost thereby attributing economic advantage to the process. P(3HB), in combination with other biocompatible and nontoxic polymers, would also have an enhanced scope in biomedical applications. Because P(3HB) is resistant to water and ultraviolet radiation and it is impermeable to oxygen, it is especially suited for use as food packaging materials. P(3HB) has been used in surgical sutures (Braunegg *et al.*, 1998), and other applications are in the development and adaptation stage (Kim et al., 1998). One of the important factors deciding the cost of P(3HB) production on an industrial scale is the raw material cost. Among the substrates required, the carbon source is of primary concern in the case of P(3HB) production. The most common carbon sources are source, glucose, unrefined sugar sources such as cane molasses, beet molasses, etc. The PHB inclusion in the microbe was identified to be PHB in cassava waste from FTIR analysis by comparing with the authentic PHB. In conjunction with tackling the environmental problems PHAs can be used in the medical field, as they are biodegradable. The main advantage of using biopolymers in medicinal field is their biocompatibility and the product of degradation, 3-hydroxy butyric acid is normally present in the human blood at concentrations of $1.3 mmol L^{-1}$ (Zinn *et al.*, 2001). Moreover, PHB fibers are used to suture wounds in mouse and got thinner as the time proceeded as a result of biodegradability (Volova, 2004).

Poly-β-hydroxybutyrate (PHB) is a natural, biodegradable polymer accumulated in the form of intracellular granules by a large variety of bacteria. The polymer can be made in to films, fibers, sheets and even moulded in to the shape of a bag and bottle, besides special

large scale production and commercialization of PHB is its high cost of production. Use of less expensive substrates, improved cultivation strategies and easier downstream processing methods are required for reducing the cost. To achieve this condition, fermentation process have run under optimized condition.

2.7 Statistical Methods for Fermentation Optimization:

A common problem for a biochemical engineer is to be handed a microorganism and be told he has six months to design a plant to produce the new fermentation product. Although this seems to be a formidable task, with the proper approach this task can be reduced to a manageable level. There are many ways to approach the problem of optimization and design of a fermentation process, one could determine the nutritional requirements of the organism and design a medium based upon the optimum combination of each nutrient, i.e., glucose, amino acids, vitamins, minerals, etc. This approach has two drawbacks. First, it is very time consuming to study each nutrient and determine its optimum level, let alone its interaction with other nutrients. Secondly, although knowledge of the optimal nutritional requirements is useful in designing a media, this knowledge is difficult to apply when economics dictate the use of commercial substrates such as corn steep liquor, soy bean meal, etc., which are complex mixtures of many nutrients.

2.8 Traditional one-variable-at-a-time method:

The traditional approach to the optimization problem is the one variable- at-a-time method. In this process, all variables but one are held constant and the optimum level for this variable is determined. Using this optimum, the second variable's optimum is found, etc. This process works if, and only if, there is no interaction between variables. The optimum found using the one-variable-at-a-time approach was 85%, far from the real optimum of 90%. Because of the interaction between the two nutrients, the one-variable-at-a-time approach failed to find the true optimum. In order to find the optimum conditions, it would have been necessary to repeat the one-variable-at-a-time process at each step to verify that the true optimum was reached. This requires numerous sequential experimental runs, a time-consuming and ineffective strategy, especially when many variables need to be optimized. Because of the complexity of microbial metabolism, interaction between the variables is

The Taylor equation is named after the English mathematician Brook Taylor who proposed that any continuous function can be approximated by a power series. It is used in mathematics for approximating a wide variety of continuous functions. The RSM protocol, therefore, uses the Taylor equation to approximate the function which describes the response in nature, coupled with the special experimental designs for determining the coefficients of the Taylor equation.

The use of RSM requires that certain criteria must be met. These are,

1. The factors which are critical for the process are known.RSM programs are limited in the number of variables that they are designed to handle. As the number of variables increases the number of experiments required by the designs increases exponentially. Therefore, most RSM programs are limited to 4 to 5 variables. Fortunately for the scale up of most fermentations the number of variables to be optimized are limited. Some of the more important variables are listed in Table 2.9.1

Table 2.9.1 Typical Variables in a Fermentation

Aeration rate	Agitation rate
Temperature	Carbon/Nitrogen ratio
Phosphate level	Magnesium level
Back pressure	Sulfur level
Carbon Source	Nitrogen source
pH	Dissolved oxygen level
Power input	

2. The factors must vary continuously over the experimental range tested. For example, the variables of pH, aeration rate, and agitation rate are continuous and can be used in an RSM model. Variables such as carbon source (potato starch vs corn syrup) or nitrogen source (cotton seed meal vs soy bean meal) are non continuous and cannot be

many nutrients. Therefore, since it is both time-consuming and inefficient, the one-variable-at-a-time approach is not satisfactory for fermentation development. Fortunately, there are a number of statistical methods which will find the optimum quickly and efficiently.

2.9 Response surface methodology:

The best method for process optimization is response surface methodology (RSM). This process will not only determine optimum conditions, but also give the information necessary to design a process. Response surface methodology (RSM) is a method of optimization using statistical techniques based upon the special factorial designs of (Box and Wilson, 1951). It is a scientific approach to determining optimum conditions which combines special experimental designs with Taylor first and second order equations. The RSM process determines the surface of the Taylor expansion curve which describes the response (yield, impurity level, etc.) The Taylor equation, which is the heart of the RSM method, has the form:

$$\text{Response} = A + B.X_1 + C.A.X_2 + \ldots H\text{-}X_{12} + I.X_{22} + \ldots M * X_1 * X_2 + N*X_1*X_3 + \ldots$$

Where,

A, B, C, are the coefficients of the terms of the equation, and

X_1 = linear term for variable 1

X_2 = linear term for variable 2

X_1^2 = nonlinear squared term for variable 1

X_2^2 = nonlinear squared term for variable 2

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$X_1.X_2$ = interaction term for variable 1 and variable 2

$X_1.X_3$ = interaction term for variable 1 and variable 3

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.

optimized by RSM. However, level of corn syrup or level of soy bean meal are continuous and can be optimized.

3. There exists a mathematical function which relates the response to the factors.

For reviews on the RSM process see (Henika, 1972). For details on the calculation methods see (Cochran and Cox, 1957; Box *et al.*, 1978) or The difficult and time-consuming nature of these calculations have inhibited the wide spread use of RSM. Fortunately, numerous computer programs are available to perform this chore. They range from the expensive and sophisticated, such as SAS™, to inexpensive, PC based programs, SPSS-X™, E-Chip™, and X-STAT™. The availability of these programs, however, has led to a "black box" approach to RSM. This approach can lead to many problems if the user does not have a thorough understanding of the process or the meaning of the results.

2.10 Advantages and disadvantages of RSM:

The response surface methodology approach has many advantages over other optimization procedures.

Advantages of RSM:

1. Greatest amount of information from experiments.
2. Forces you to plan.
3. Know how long project will take.
4. Gives information about the interaction between variables.
5. Multiple responses at the same time.
6. Gives information necessary for design and optimization of a process.

Disadvantages of RSM:

1. Tells what happens, not why.
2. Notoriously poor for predicting outside the range of study.

2.11 Important statistical Terminology to predict the RSM output:

2.11.1 Correlation Coefficient:

The correlation coefficient is a measure of the relationship between the Taylor expansion term and the response obtained. The correlation coefficient can vary from 0 (absolutely no correlation) to 1 or -1 (perfect correlation). A correlation coefficient of 0.5 shows a weak but usual correlation. A positive sign for the correlation coefficient indicates that the response increases as the variable increases while a negative sign indicates that the response decreases as the variable increases.

2.11.2 Regression Coefficients:

The regression coefficients are the coefficients for the terms of the Taylor expansion equation. These coefficients can be determined either by using the actual values for the independent variables or coded values. Using the actual values makes it easy to calculate the response from the coefficients since it is not necessary to go through the coding process. However, there is a loss of important information. The reason for coding the variables is to eliminate the effect that the magnitude of the variable has upon the regression coefficient. When coded values are used in determining the regression coefficients, the importance of the variable in predicting the results can be determined from the absolute value of the coefficient. Using coded values for the independent variables, those variables which are important and must be closely controlled can readily be determined. The formula for coding values is,

Where;

Coded Value = (Value minus Midpoint value)/Step value

Value = The level of the variable used

Midpoint Value = Level of variable at the midpoint of the range

Step Value = Midpoint value minus next lowest value

2.11.3 Standard Error of the Regression Coefficient:

RSM determines the best estimate of the coefficients for the Taylor equation which explains the response. The estimated regression coefficient is not necessarily the exact value but rather an estimate for the coefficient. The advantage of statistical techniques is that from the standard error one has information about how valid is the estimate for the coefficient (The range with in which the exact value for the coefficient may be found). The greater the standard error, the larger the range within which the exact value for the coefficient may be, i.e., the larger the possible error in the value for the coefficient. The standard error of the regression coefficient should be as small as possible. A standard error which is 50% of the coefficient indicates a coefficient which is useful in predicting the response. Designing a process using coefficients with a large standard error can lead to serious difficulties.

2.11.4 Computed T Value:

The T test value is a measure of the regression coefficient's significance, i.e., does the coefficient have a real meaning or should it be zero. The larger the absolute value of T the greater the probability that the coefficient is real and should be used for predictions. A T test value 1.7 or higher indicates that there is a high probability that the coefficient is real and the variable has an important effect upon the response.

2.11.5 Standard Error of the Estimate:

The standard error of the estimate yields information concerning the reliability of the values predicted by the regression equation. The greater the standard error of the estimate, the less reliable the predicted values.

2.11.6 Analysis of Variance:

Three other statistical numbers which should be closely examined relate to the source of variation in the data. The variation attributable to the regression reflects the amount of variation in the data explained by the regression equation. The deviation from regression is a

the deviation from the regression should be very small in comparison to the amount of variation explained by the regression. If this is not the case, it means that the Taylor equation does not explain the data and the regression equation should not be used as a design basis. The third important factor is the relationship between the explained and unexplained variation. The greater the amount of variation explained by the regression equation, the greater the probability that the equation meaningfully explains the results.

2.12 Kinetic Parameter:

An engineering approach to tackle the problem like maximum PHB production under optimized condition would be to develop a mathematical model that will not only facilitate the understanding of the system but also help in designing the nutrient-limited fed-batch cultivation to improve the PHB productivity. This will significantly reduce the laborious experimentation to optimize the productivity. The prime requirement for such an effort is a robust (structured) mathematical model. In many cases, the objective of development of a mathematical model is explicitly aimed at providing the basis for controlling the performance of a bioreactor. Various metabolic structured model was reported, describing the observed kinetics of the P(3HB) formation and consumption (ShilpiKhanna and Srivastava, 2005; Dhanasekar, *et al.*, 2003).

Materials and Methods

3. Materials and Methods

3.1 Collection of soil samples from marine region:

Samples were collected from marine region of south India such as soil samples of Devipattinam at $9^0$28'52.20"N, $78^0$51'55.85" E.

3.2 Isolation and cultivation of marine bacteria:

Primary isolation of marine bacteria was done by serial dilution technique using Nutrient agar medium obtained from Hi media, India. The pH of the medium was about 7.2 and the media were sterilized before use. The plates were maintained in an aerobic condition at 37 ^{0}C for 24 h.

3.3 Identification and characterization of strain SVS-7:

The marine bacterial PHB producer was done in Nutrient Broth dissolved with marine water. The culture flasks were incubated at 37 ^{0}C for 48 h. Pure isolates containing lipophilic inclusions were identified based on their microscopic, morphological and biochemical characters (Holt et al., 1994) and by sequencing partial sequences of their 16S rRNA.

3.4 Media and growth conditions:

A loop of isolated strain SVS-7 was inoculated into 1.8 g of Nutrient Broth (NB) with marine water in a 100 ml Erlenmeyer flask, which was subsequently aerobically cultured at 37 ^{0}C for 24 h. Then, 2 ml of the cultured broth was inoculated to a 250 ml shake-flask containing 100 ml of NB medium. The flask was maintained at 37 ^{0}C for 24 h. Growth was monitored spectrophotometrically by measuring culture's absorbance at 600 nm and dry weight periodically. The cells were harvested by centrifugation, washed twice with phosphate-buffer and air dried. Weight of the dried mass was considered as the dry weight of the sample.

3.5 Extraction and estimation of PHB:

After 48 h incubation at 37 ^{0}C, 100 ml of culture was treated with ultrasonic crusher for 5 min. After, centrifuged at 10000 rpm for 15 min .The supernatant was discarded and the pellet was treated with 50 mL of sodium hypochlorite and the mixture was incubated at 30 ^{0}C for 1 h. After incubation, the mixture was centrifuged at 8000 rpm for 10 min and then washed with distilled water, acetone, methanol respectively for washing and extraction.

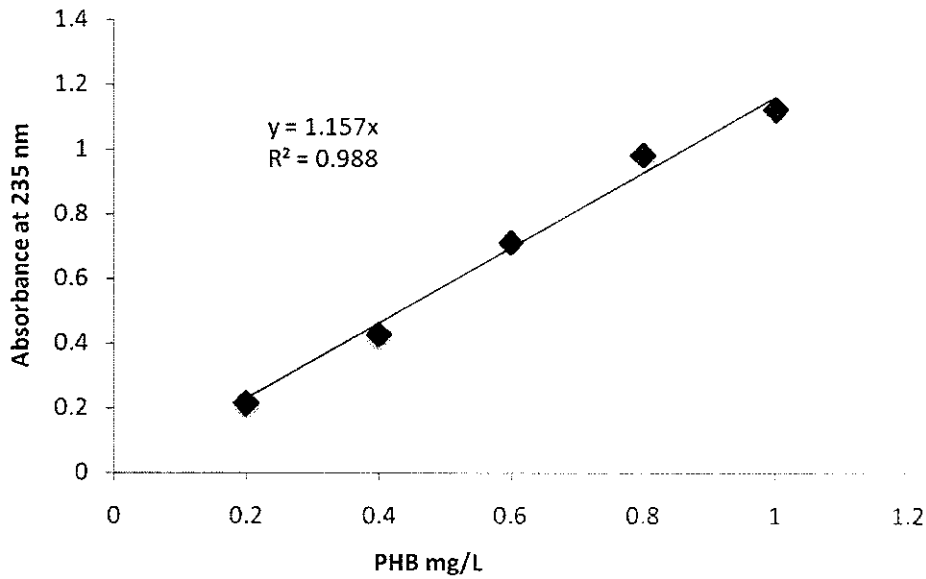


Fig 3.5.1 Standard graph for PHB

After washing, the pellet was dissolved in 25 ml of chloroform and was evaporated the chloroform by pouring the solution on sterile glass tray and kept at 4 ^{0}C. After evaporation the powder was collected.

PHB produced was extracted as described by the method of (Ramsay *et al.*, 1994). The amount of PHB in the extracted sample was determined spectrophotometrically at 235 nm (Lee and Choi, 1999) using ELICO SL 159, Hyderabad, UV Spectrophotometer.

3.6 Effect of carbon substrates on the production of PHBs:

Effect of various carbon substrates on the production of PHB was determined. The

glucose, sucrose, lactose, starch], ammonium sulphate (2 g/L) as a nitrogen source with made into marine water. After 48 h incubation the PHB was estimated.

3.7 Effect of cassava waste on the production of PHBs:

Effect of various substrates (Cassava waste, ammonium sulphate and sea water) on the production of PHAs was determined. The quantity of substrates was taken from low number to high number. The range of substrate concentration are, For cassava waste (100 g/L to 200 g/L), ammonium sulphate (1 g/L to 3 g/L), sea water (600 ml/L to 1000 ml/L) and the concentration of the substrates contributes for maximum production of PHBs was taken for Central composite Rotary Design (CCRD).

3.8 Effect of time and pH on PHBs production:

To find the effect of time on the growth and production of PHBs, the strain SVS-7 was inoculated into the production medium (Cassava waste (20% w/v), ammonium sulphate (2%) and sea water (80% v/v)) and samples were collected for every 6 h. The range of initial pH of the medium under consideration was 7–11. For determination of time and pH of maximum production of PHBs, the dry weight of PHBs was simultaneously checked.

3.9 Medium and condition optimization for the production of PHBs:

3.9.1 Experimental design

Central composite rotary design (CCRD) obtained by using the software Design-Expert (Stat-Ease, Inc., Minneapolis, USA, trial version) was applied to optimize and to ascertain the interactive effects of four variables, viz. concentrations of cassava waste, Ammonium sulphate, sea water and initial pH of the medium. Central composite design at the given range of the above parameters in terms of coded and actual terms is presented in Table 3.9.3.

3.9.2 Optimization of medium for PHAs production:

A two level four factorial central composite rotary design (CCRD) was used to optimize the medium composition. The four variables used were Cassava waste, Ammonium sulphate, sea water and pH. The actual level of variables for CCRD experiments were selected as a central point of experiments. An experimental design of 30 experiments with six trials for the central point was formulated using the Design-Expert software. Experiments were conducted in 250 ml Erlenmeyer flask containing 100 ml of media prepared according to the design inoculated with 3 ml culture. The flasks were kept in incubator shaker maintained at 37 ^{0}C and 150 rpm. Response studied was PHAs dry weights (g/L) at the end of 48 h. 3D graphs were created to understand the interaction of various factors. And the graphs were used to analyze the optimized components of the medium which influences the responses. The point prediction is a special feature of this software which was used to confirm the obtained optimum value.

3.9.3 Statistical analyses:

The soft ware Design-Expert, Stat-Ease, Inc., Minneapolis, USA was used for regression analysis of experimental data and to plot response surface. ANOVA was used to estimate the statistical parameters.

Table 3.9.3 Illustrates the coding levels involved in the optimizing the medium components.

Independent variables	**Symbols**	**Code levels**				
		-α	-1	0	+1	+α
Cassava Waste	A	50	100	150	200	250
Ammonium sulphate	B	0	1	2	3	4
Sea water	C	600	700	800	900	1000
pH	D	7	8	9	10	11

3.10 Fermentor studies-FED Batch:

The production of PHBs was carried out in a 2.5 L fermenter (Infors HT, Switzerland), with a working volume of 1.5 L using the optimized medium by RSM as a fedbatch process. Initially all the medium components (150g Cassava waste, 2 g Ammonium sulphate, 1.2 L sea water and 300 ml distilled water) were added into the fermentor, the pH was adjusted to 9.0 and the medium was sterilized ex situ. Dissolved oxygen was maintained at 100% air saturation at the start of the process. 100 ml of overnight culture (at log phase) was inoculated aseptically and the impeller speed was maintained at 300±3 rpm and temperature at 37 ^{0}C. The pH was maintained at 9.0 using 0.1 N NaOH / 0.1 N Hcl. The cassava waste dissolved with marine water was fed at two time intervals i.e. at 12th (150 ml) and 24th (100 ml) hour and the PHBs synthesis was monitored for every three hours. Antifoam (olive oil 1:10 v/v) at a concentration of 1:10 (v/v) in water was added at initially during the fermentation.

3.11 Fourier transform-infrared spectroscopy:

Purified PHBs (5 mg) was subjected to FT-IR spectrum using a Fourier Transform IR spectrophotometer. The spectrum obtained was compared with that of the commercially available PHB (Sigma, USA).

Results and Discussion

4. Results and Discussion

4.1 Strains isolation and identification:

The selected strain showed the maximum accumulation of PHB. A gram negative bacterium (designated strain SVS-7) that potentially accumulated PHB was isolated from marine water. From its morphological and physiological properties to be identified by 16S rRNA,

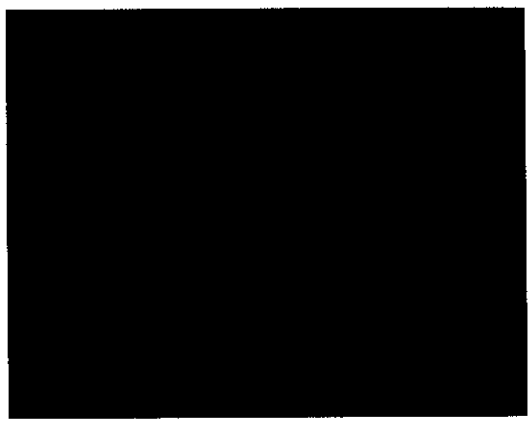


Fig 4.1.1 SVS-7 strain under UV illuminator

4.2 Effect of carbon substrate:

In order to find a better substrate, different carbon sources were tested for the productivity of PHB. The strain SVS-7, which exhibited the higher PHB productivity among the marine isolates, was selected for the more detailed analysis of the optimization process. The various conditions optimized for the intracellular accumulation of PHB by strain SVS-7 were carbon source–glucose, nitrogen source–ammonium sulphate, pH 9, and marine water (Fig 4.2.1). Thus, it is shown that depending upon the sources of carbon, nitrogen, pH and salts metabolized, PHB synthesis may be selectively induced in SVS-7 strain. The cheap source cassava waste was selected for the further studies.

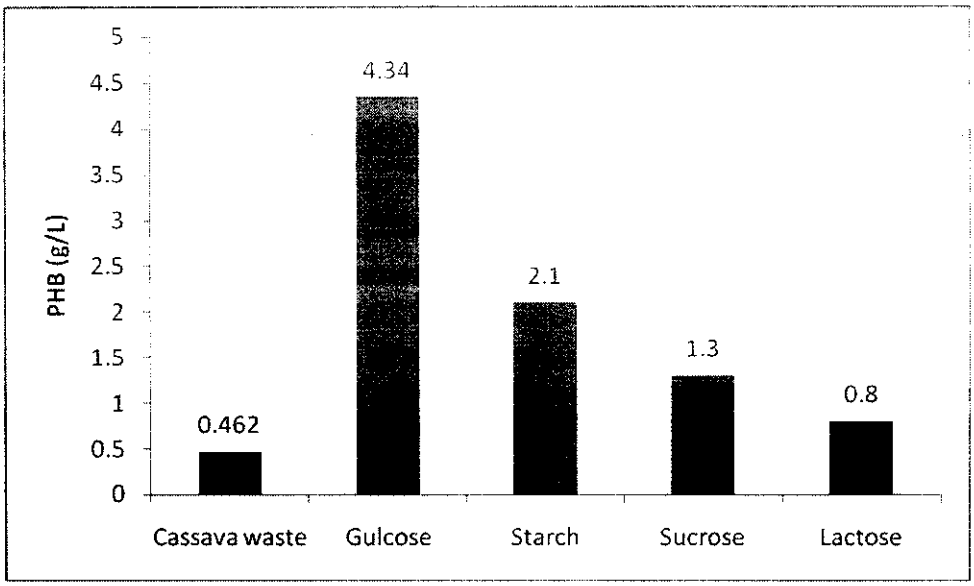


Fig 4.2.1 Effect of different carbon substrate on PHB production.

4.3 Effect of time on PHB production:

To assess the effect of time on the production of PHB, SVS-7 was inoculated into a production medium, where cassava waste is the sole carbon source. As expected the synthesis of PHB occurred in the stationary phase, with 48 h showing the maximum PHB production (462 mg/L). Although further incubation resulted in a slight increase in PHB production, the difference is almost insignificant. Therefore, at 48 h PHB production was found to be the maximum.

4.4 Effect of substrates, pH and time on the PHB production:

To assess the effect of substrates on the production of PHB, SVS-7 was inoculated into a production medium, where the cassava waste, ammonium sulphate and sea water were the nutrient sources. Considering the concentration of substrates optimized through conventional methods, 150 g/L of cassava waste, 2 g/L of ammonium sulphate, and 800 ml/L of sea water produced maximum amount PHB. Maximum PHB synthesis occurred at 48^{th} hour with the initial pH of the medium being 9.0. All these data were used to design the experiments for CCRD.

4.5 Response Surface Methodology:

The experimental results of PHB production by a complete four factor- two-level factorial experiment design with six replications of the central point and six axial points are shown in Table 4.5.1

$$PHB = +562.00 + 29.46 * A + 19.29 * B - 22.21 * C - 7.04 * D - 7.19 * A * B - 15.19 * A * C - 0.56 * A * D + 23.81 * B * C + 0.94 * B * D + 12.44 * C * D - 119.09 * A^2 - 83.34 * B^2 - 131.59 * C^2 - 114.97 * D^2 \longrightarrow (1)$$

The overall second-order polynomial equation for PHB production was given in Eq. (1) where, Y is PHB dry weight (w/v); A is waste from cassava industry (w/v); B is ammonium sulphate (w/v); C is sea water (v/v) and D is the initial pH of the medium. The statistical significance of the model equation was evaluated by the F-test for analysis of variance (ANOVA), which showed that the regression is statistically significant at 99% ($p < 0.05$) confidence level. Values less than 0.1000 indicate the model terms are significant (Table 4.5.1). In this case A, A^2, B^2, C^2,D^2 are significant model terms. The P-values are used as a tool to check the significance of each of the coefficients which, in turn, are necessary to understand the pattern of the mutual interactions between the best variables. The smaller the magnitude of the P, the more significant is the corresponding coefficient. ANOVA for PHB production showed that the model F-value of 24.90 for PHB production is statistically significant at Prob > F-value was < 0.0001. There is only a 0.01% chance that a "Model F-value" this large could occur due to noise. "Adeq Precision" measures the signal (response) to noise (deviation) ratio. A ratio greater than 4 is desirable. The ratio of 14.592 indicates an adequate signal in the case of medium optimization for PHB production. The coefficient of determination (R^2) was calculated to be 0.9588, indicating that the model could explain 95% of the variability in the production of PHB. The R^2 value was always between 0 and 1 and the closer the R^2 was to 1.0, the stronger the model and the better it predicted the response (Haaland, 1989). The effect of interaction of various nutrients on the PHB production (z axis) was studied by plotting three dimensional response surface curves against any two independent variables while keeping the other independent variable at their "0"

Table 4.5.1 Experimental design and results of CCD for Response Surface Methodology.

Standard	Run	Cassava waste (g/L)	Sea water (ml/L)	Ammonium sulphate (g/L)	pH	PHB (mg/L)
1	13	100	700	1	8	146
2	22	200	700	1	8	246
3	5	100	900	1	8	96
4	23	200	900	1	8	186
5	15	100	700	3	8	42
6	19	200	700	3	8	96
7	21	100	900	3	8	104
8	12	200	900	3	8	72
9	29	100	700	1	10	117
10	7	200	700	1	10	190
11	20	100	900	1	10	48
12	27	200	900	1	10	114
13	11	100	700	3	10	36
14	16	200	700	3	10	74
15	18	100	900	3	10	86
16	30	200	900	3	10	112
17	14	50	800	2	9	18
18	24	250	800	2	9	164
19	4	150	600	2	9	86
20	2	150	1000	2	9	382
21	10	150	800	0	9	44
22	28	150	800	4	9	38
23	17	150	800	2	7	97
24	25	150	800	2	11	118
25	9	150	800	2	9	562
26	3	150	800	2	9	562
27	6	150	800	2	9	562
28	8	150	800	2	9	562
29	1	150	800	2	9	562
30	26	150	800	2	9	562

Table 4.5.2 Analysis of variance (ANOVA) for the regression model of PHB production obtained from the experimental results.

Sources	Sum of squares	Df	Mean square	F value	p value prob > F
Model	1.067+006	14	76214.44	24.90	< 0.0001 significant
A-Cassava waste	20827.04	1	20827.04	6.81	0.0198
B-sea water	8932.04	1	8932.04	2.92	0.1080
C-Ammonium sulphate	11837.04	1	11837.04	3.87	0.0680
D-pH	1190.04	1	1190.04	0.39	0.5423
AB	826.56	1	826.56	0.27	0.6109
AC	3690.56	1	3690.56	1.21	0.2895
AD	5.06	1	5.06	1.654E-003	0.9681
BC	90725.06	1	90725.06	2.96	0.1057
BD	14.06	1	14.06	4.595E-003	0.9469
CD	2475.06	1	2475.06	0.81	0.3827
A^2	3.890E+005	1	3.890E+005	127.12	< 0.0001 significant
B^2	1.905E+005	1	1.905E+005	62.26	< 0.0001 significant
C^2	4.750E+005	1	4.750E+005	155.21	< 0.0001 significant
D^2	3.625E+005	1	3.625E+005	118.47	< 0.0001 significant
Residual	45904.58	15	3060.31		
Lack of fit	45904.58	10	4590.46		
Pure Error	0.00	5	0.000		
Core Total	1.113E+006	29			

Therefore three response surfaces were obtained by considering all three possible combinations. From the response surface (Fig. 4.5.1) it is obvious that all nutrients has significant effect on PHB production With the optimized medium the production of PHB

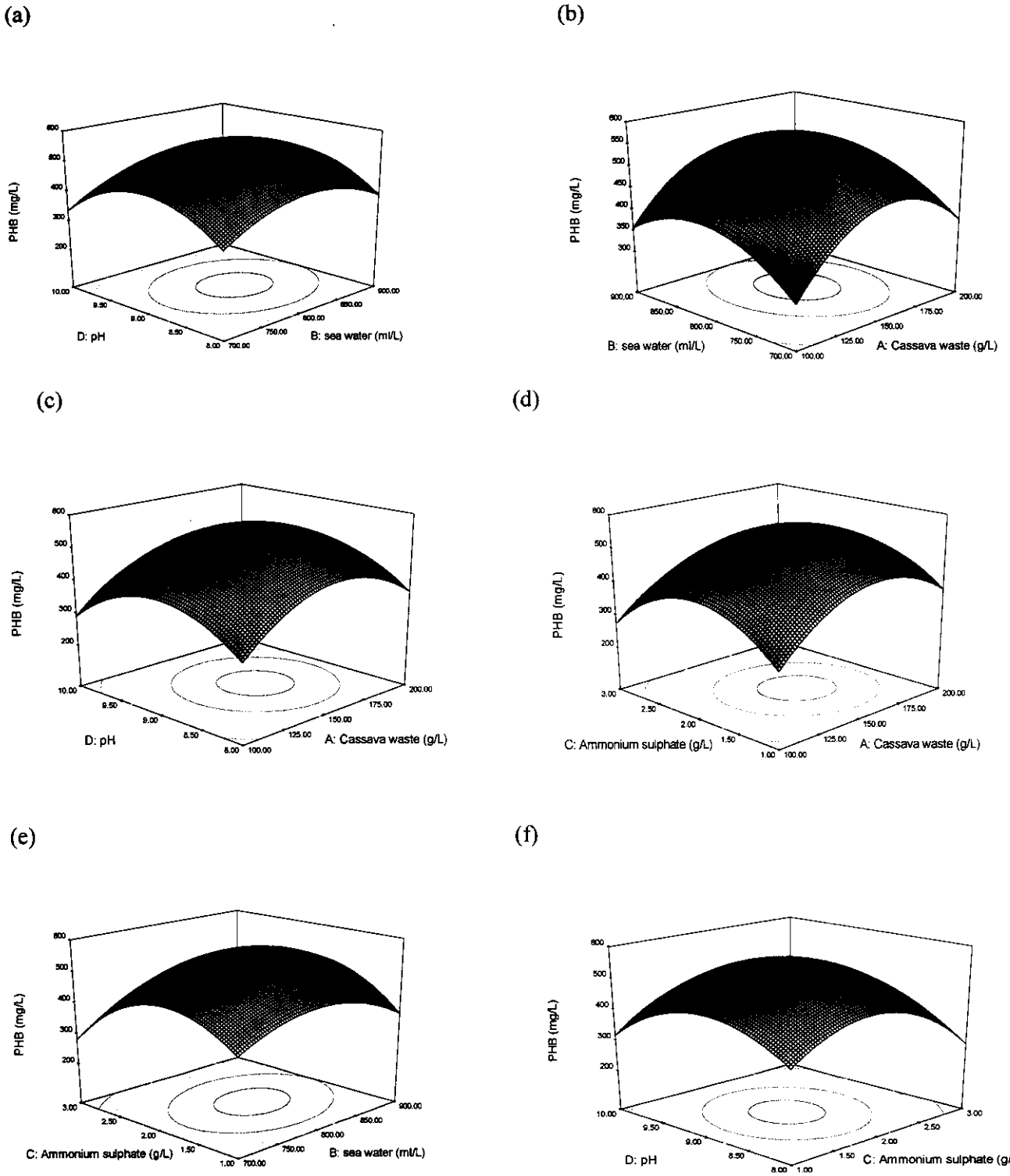


Fig 4.5.1 Three-dimensional curve showing the effect of (a) pH and sea water, (b) sea water and cassava waste, (c) pH and cassava waste, (d) ammonium sulphate and cassava waste (e) ammonium sulphate and sea water (f) pH and ammonium sulphate, on the production of PHB by the isolated strain SVS-7.

4.6 PHB production in 2.5 L Fermentor:

The medium optimized in shake flask was used to study the kinetics in a bench-top fermentor. Here the cassava waste was fed at 18th hour to maintain the carbon source level excess and the PHB synthesis is checked every three hours. Initially the synthesis was low and as the feed was given the accumulation of PHB was increased. During the accumulation of PHB, the pH of the medium was maintained constantly at 9.0. The synthesis of PHB decreased after the 48th hour. Fig. 4.6.1 shows the change in PHB production versus time. The time of maximum synthesis is similar to the RSM where we obtained the maximum synthesis at 48th hour.

Table 4.6.1 Amount of product formed and substrate consumed at different time interval, using cassava waste as the carbon source.

Time(h)	Biomass (g/L)	Glucose (g/L)	Ammonium (g/L)	PHB (g/L)
0	0	10.03	2	0
3	0.8	8.731	1.84	0.062
6	1.3	7.662	1.43	0.082
9	2.4	6.386	1.31	0.097
12	2.9	5.631	1.17	0.096
15	4.1	4.859	1.06	0.651
18	4.8	6.386	0.89	0.848
21	5.2	5.602	0.63	1.066
24	5.6	4.938	0.39	1.461
27	6.8	3.551	0.12	1.505
30	8.2	3.182	0.2	1.608
33	8.8	2.683	0.2	1.813
36	9	2.082	0.2	1.906
39	9.6	1.643	0.2	1.969
42	10.4	1.212	0.2	2.149
45	10.8	0.986	0.2	2.273
48	11	0.874	0.2	2.713

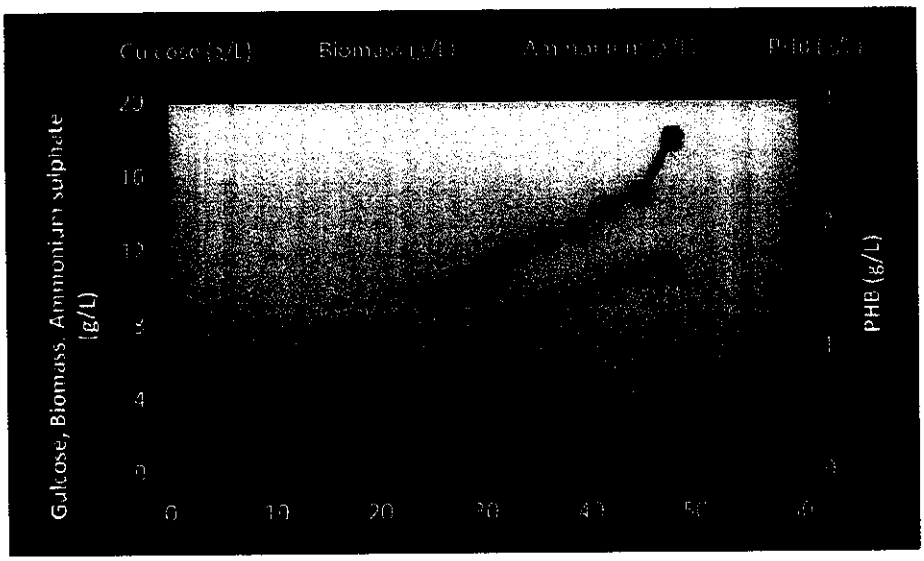


Fig 4.6.1 Kinetics of growth and PHB production of SVS-7 strain cultured on fed batch using cassava waste as carbon source

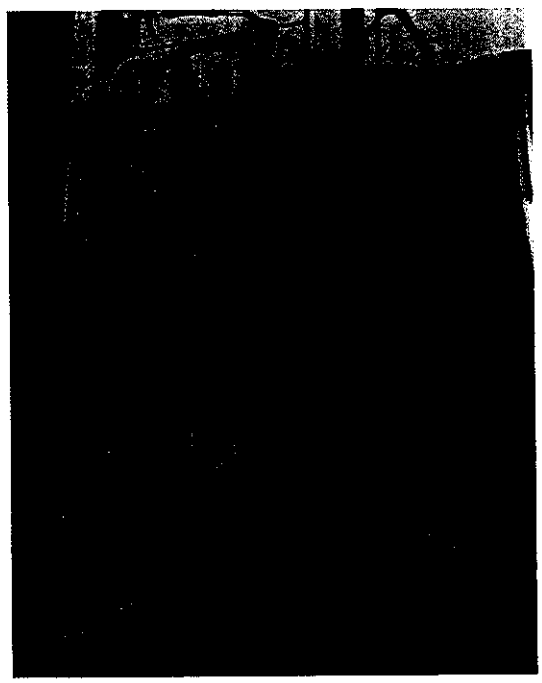


Fig 4.6.2 Batch fermenter inoculated with SVS-7
(Temp 37 ^{0}C, RPM-300±3, pH-9.0±0.3, DO-100 % air saturation)

4.6.1 Batch Growth Kinetics of *SVS-7* in Fed-batch cultivation:

Batch kinetics of the SVS-7 XXXX under nitrogen limitation was studied in a 2.5-L, bioreactor containing 1.5 L of media. The batch experiment was done and the values of the biomass, PHB, and residual nutrients are shown in Fig.4.6.2. A total of 55% PHB (P/X) was accumulated in the entire cultivation period (48 h). Although during the early exponential phase some PHB production was observed, mostly it was accumulated in the later stages of growth. After a lag phase of approximately 6^{th} h, the biomass increased to 11 g/L in 48 h. During this period, 1.8 g/L nitrogen was consumed out of an initial value of 0.2 g/L, whereas approximately 9.15 g/L of carbon source was metabolized during the entire period of cultivation. The synthesis of PHB, product of interest, started after 15^{th} h and reached a final concentration of 2.731 g/L toward the end (48h). The overall yield ($Y_{P/S}$) of PHB to cassava waste was 0.296 g/g and productivity of 0.056 g/L h was obtained. The maximum specific growth rate for the culture during the early exponential phase was found to be 0.093 h^{-1}, which then gradually decreased to zero during the later stages of fermentation when PHB production was at its maximum.

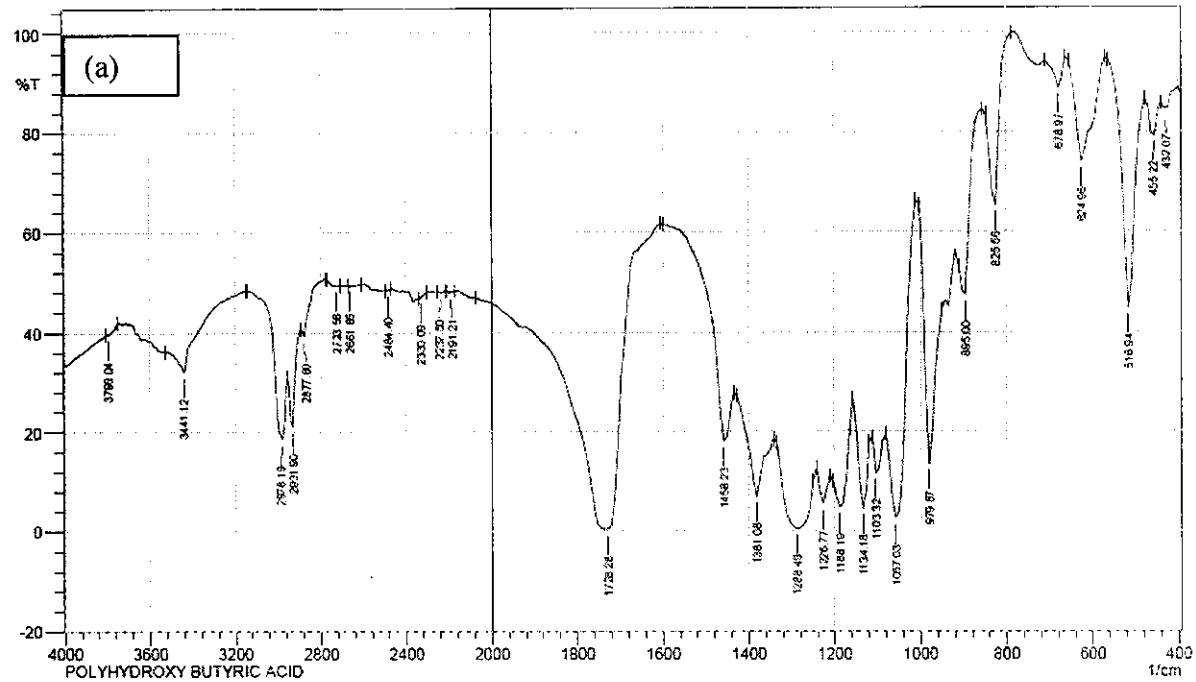
Table 4.7.1 Kinetics parameter of SVS-7 strain cultured on cassava for the production of PHBs

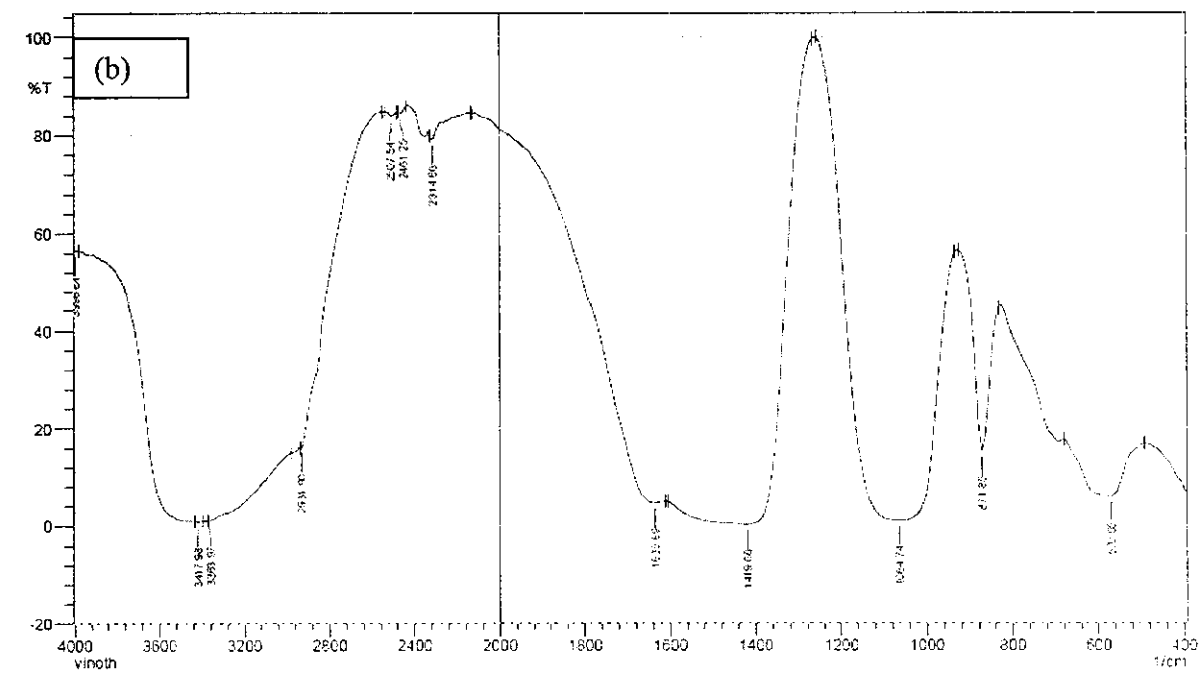
Parameter	Cassava waste	
	Culture step	
	Batch	**Fed batch**
μ (h^{-1})	0.084	0.093
$Y_{X/S}$	1.317	1.124
$Y_{P/S}$	0.232	0.339
PHB accumulation (% DWC)	17.66	24.66
PHB productivity (g/L h)	0.047	0.093

4.8 Qualitative analysis of PHB:

PHB produced by the strain SVS-7 were subjected to FTIRs analysis. The band found at 2931.90 cm^{-1} corresponds to the C – H bond. The bands found at 1635 cm^{-1} correspond to the asymmetrical C–H bending vibration in CH_3 group, while the one found at 1120 cm^{-1} is

C–O–H bond. The bands at 1064.74 indicate the presence of C–O band. The series of bands located at 1000–1200 cm^{-1} correspond to the stretching of the C–O bond of the ester group. The bands at 3417.98 cm^{-1} indicate the presence of O–H bond. The obtained IR absorption peaks correlated with the literature value (Silverstein *et al.*, 1991) and with the spectrum of pure PHB. Thus, it was identified that the compound was PHB.





Conclusion

5. Conclusion

This study shows that the marine isolate SVS-7 strain is able to accumulate a high level of PHB. A significantly higher biomass of 11 g/L with a PHB content of 2.713 g/L was obtained when fed batch cultivation was conducted in 2.5 L laboratory-scale fermentor, thus giving a productivity of 0.093 g/L/h by utilizing 91.6% and 100% of cassava waste and ammonium sulphate, respectively. This type of approach will improve our ability to identify the unexploited industrially important microorganisms present in the environment.

In this study we demonstrate the production of PHB using cheap nutrient and mineral sources. For industrial and large scale productions, this medium will be very useful and also this process can act as a key for the disposal of cassava waste in a cheap and efficient way. Also the synthesis of PHB was increased in fed-batch culture. Finally, our results suggest that SVS-7 strain is a good candidate for the production of this biopolymer by fermentation, since the medium containing cheap and local sources of carbon as the main substrate for PHB accumulation.

Appendices

1. Appendix I

I. Composition of media

1. Nutrient Agar:

Peptide digest of animal tissue	- 5g
Sodium chloride	- 5g
Beef extract	- 1.5g
Yeast extract	- 1.5g
Agar	- 15g
Distilled water	- 1000ml

2. Nutrient Broth:

Peptide digest of animal tissue	- 5g
Sodium chloride	- 5g
Beef extract	- 1.5g
Yeast extract	- 1.5g
Distilled water	- 1000ml

2. Appendix II

II. Formula for kinetic parameter calculation

μ (g / L) = $K^{'}$ = ln (X_{t2} / X_{t1}) / (t_2 - t_1); $t_2 > t_1$

$Y_{X/S}$ (g / g) = (X_2 - X_1) / (S_1 - S_2)

$Y_{P/S}$ (g / g) = (P_2 - P_1) / (S_1 - S_2)

where X_t are biomasses at the different time points (t_1 and t_2) respectively (g).

S - Substrate concentration (g).

P - Product concentration (g).

PHB Accumulation (%) = (PHB dry weight / biomass) * 100

PHB productivity (g / L h) = PHB dry weight / Time

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