# BACTERIAL PRODUCTION AND SCREENING OF POLYHYDROXYALKANOATES BY USING A NOVEL SPECTROFLUOROMETRIC METHOD

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## ABSTRACT

### BACTERIAL PRODUCTION AND SCREENING OF POLYHYDROXYALKANOATES BY USING A NOVEL SPECTROFLUOROMETRIC METHOD

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In this study, polyhydroxyalkanoate production capacities of producer microorganisms were monitored by using a newly established method employing fluorescent Nile red as a probe. A detailed study, involving growth parameters was performed first with four strains of *Ralstonia eutropha, Alcaligenes latus, Pseudomonas aeruginosa* and *Escherichia coli.* Next the behaviour of the strains during the polymer production was followed and extraction of these polymers from cells were optimised. Nile red applied cells were examined microscopically and spectrofluorimetrically leading to the construction of a calibration curve which linked the concentration of the polymer present to the florescence intensity after Nile red staining. By this way the polymer production could be quantified within the cells without destruction. A colony screening method, based on these findings, was also developed to monitor potential polymer producing strains in nature.



Keywords : Nile red, Polyhyrdoxyalkanoates, *Ralstonia eutropha, Alcaligenes latus,* MTT, Screening

# ÖZ

## POLİHİDROKSİALKANOATLARIN BAKTERİYEL ÜRETİMİ VE YENİ SPEKTROFLOROMETRİK BİR METOD OLARAK NİL KIRMIZISI KULLANILARAK İZLENMESİ

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Bu çalışmada füloresan Nil kırmızısı probu kullanılarak üretici mikroorganizmaların polihidroksialkanoat üretim kapasiteleri izlenmiştir. Öncelikle kullanılan dört farklı mikroorganizma olan *Ralstonia eutropha, Alcaligenes latus, Pseudomonas aeruginosa* ve *Escherichia coli*'nin üreme parametrelerini inceleyen ayrıntılı bir çalışma yapılmıştır.Daha sonra üretici suşların polimer üretimi sırasındaki davranışları incelenmiş ve bu polimerlerin hücrelerden elde edilmesi için gerekli şartlar optimize edilmiştir.Nil kırmızısı uygulanan hücreler mikroskopik olarak incelenmiş ve spektroflorometre ile üretilen polimer miktarını Nil kırmızısı boyaması sonucunda oluşan floresans ile bağlayan kalibrasyon eğrileri çizilmiştir. Bu yöntem ile hücre içindeki polimer konsantrasyonu hücre parçalanmadan belirlenebilmektedir. Bu sonuçlara bağlı olarak doğadaki potansiyel polimer üreticilerini belirleyebilmek için bir koloni tarama metodu da geliştirilmiştir.

Anahtar kelimeler : Nil kırmızısı, Polihidroksialkanoatlar, *Ralstonia eutropha, Alcaligenes latus*, MTT, İzleme

To my family whom I owe my life

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# CHAPTER 1

# INTRODUCTION

## 1.1. Polyhydroxyalkanoates (PHA), biopolymers of interest

PHA's were simple macromolecules synthesized by many bacteria in nature. Their production was a strategy as a sink for reducing equivalents When there was an unbalanced condition for the growth, the storage of excess nutrients was an advantage . These polimeric structures were stored intracellularly and the cell will not undergo osmotic stress, moreover the loss of these valuable compounds was prevented.

When we extract PHA's from the bacterial cells, they show desirable properties, similar to common plastics. But their natural origin makes these polyesters available to many microorganisms for degradation. This availability makes them biodegradable and recyclable unlike petrochemical based plastics.

Due to their thermoplastic properties and being natural compounds, PHA's are expected to replace petrochemical based plastics.

### 1.1.1. General properties of PHAs:

#### 1.1.1.1.Chemical structure of PHAs :

Polyhydroxyalkanoate (PHA)'s are a class of optically active polyesters which contain 3-hydroxy fatty acid monomers. Polimerization takes place when a carboxyl group of one monomer forms an ester bond with the hydroxyl group of the other monomer. They were discovered by Lemoigne *et al.* [1] in 1926. Traditionally the most extensively studied and commercially produced polymer was poly-β-hydroxybutyrate. Copolymers of P(3HB) can be formed by co-feeding of the producer microorganisms with various substrates. With such strategies polymers with (3HV) or (4HB) can be produced.

PHAs schematical representation is as follows ;

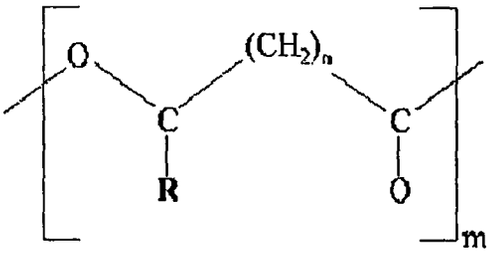



**Figure 1** : Schematical representation of PHA's

n=0 not present as stored polymers

n=1 Mostly present e.g. 3HB (R=$CH_3$)

n=2 only 4HB (R=H)

n=3 only 4HB (R=H)

n=4 not present

The R group can be variable, as shown below ;

**R** = pendant group (always in R configuration)

*β*--hydroxybutyrate where R= $CH_3$ (HB)

*β*--hydroxyvalerate where R= $CH_2CH_3$ (HV)

*β*--hydroxycaproate where R= propyl (HC)

*β*--hydroxyheptanoate where R= butyl (HH)

*β*--hydroxyoctanoate where R= pentyl (HO)

*β*--hydroxynonanoate where R= hexyl (HN)

*β*--hydroxydecanoate where R= heptyl (HD)

*β*--hydroxyundecanoate where R= octyl

*β*--hydroxydodecanoate where R= nonyl

From $C_1$ (methyl) to $C_6$ (hexyl) for R group, they were grouped as short chain length (scl) or short side chain (ssc) polymers, poly-β-hydroxybutyrate is an example. When chain length is more than $C_6$ up to $C_{16}$ , they mentioned as medium

### 1.1.1.2. Physical characteristics of PHA's :

Molecular weight of PHA's lies within the range of 50 kDA to 1000 kDa. As their molecular weight increases, their properties approaches to other conventional plastics like polypropylene. Unlike polypropylene, PHB is resistant to UV weathering, stiffer and more brittle.

Marchessault and coworkers [5] stated that PHB was a compact right-handed helix with a twofold screw axis and a fiber repeat of 0.596 nm, two repeating unit volume was found as 0.11 $nm^3$. The crystalline chain conformation of poly-3-hydroxyvalerate have similar to PHB with fiber repeat of 0.556 nm. and two repeating unit volume is 0.13 $nm^3$[73].

According to Bloembergen and coworkers [6], rate of crystallization decreases with increasing HV content, and it can be increased with incorporation of nucleating agents, which also reduces the brittleness, increases elongation at break. Decrease in stifness (Young's modulus) and increase in toughness are desirable properties for commercial applications.

When the polymers are within the cell, they exist in an amorphous fluid state, however after extraction they become highly crystalline, stiff and brittle

When the polymers are within the cell, they exist in an amorphous fluid state, however after extraction they become highly crystalline, stiff and brittle material. The melting temperature ($T_m$) and decomposition temperature of homopolymer is very close, so it is a drawback for thermal processing. 3HV monomer incorporation to the polymer results in copolymer formation of P(3HV-3HB), which is less brittle and can be used to cast films. Unlike scl PHA's like P(3HB) and P(3HB-co-3HV), mcl PHA's are more elastic and less crystalline.

As a quantitative example , Keller et al. [4] found that the degree of crystallinity of PHB samples varied generally between 60-80%, however with the incorporation of 3HV monomers up to 30 mol%, the degree of crystallinity decreases drasticallty This drastic change could also be observed in $T_m$; with homopolymer it was 174°C and decreases to 143°C with the copolymer.

**1.1.1.3. PHA's biological importance :**

Due to many monomeric units that can be incorporated to a PHA molecule with a biological polymerization system, enormous number of high molecular weight polymers can be produced in an environmentally friendly manner.



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One of the desirable properties of PHA's is the biodegradability. Naturally, numerous microorganisms can degrade and use PHA's as energy sources [6]. The PHA depolymerases and hydrolases are activated according to the environmental conditions and the structure and properties of the polymer. Typically a homopolymer rod mostly degrades on a scale of months.

Another important feature is the renewability of the resources that PHA's are produced. Main raw materials for polymer production are sugars and fatty acids, which are derived from $CO_2$ and water. After their breakdown, the end products are also $CO_2$ and water. These two properties are in fact coinciding, biodegradibility brings out the renewable nature of these polymers.

### 1.1.2. Production of PHA's:

#### 1.1.2.1. Natural producers of PHA's :

Although numerous natural producers have been reported, Below shown a summary of mostly referred species in recent studies (Table 1). It seems that there are different strategies and necessities, and every different species produces PHA's under different stress conditions and with different metabolic pathways. These pathways can be grouped into some main strategies, and will be discussed later. As

here. [14,15].

**Table 1:** Producer species of PHA's

| Genus Name | Species name | Notes |
|---|---|---|
| *Acinetobacter[21]* | *spp.* | |
| *Actinobacillus* | *spp.* | Unlike other producers , there is no need for nutrient limitation |
| *Aeromonas[11]* | *Caviae* | Unique PHA biosynthetic enzymes |
| *Agrobacterium* | *spp.* | |
| *Alcaligenes[38,39]* | *latus* | Another producer strain preferred in commercial production, growth associated producer. |
| *Azosprillum* | *spp.* | |
| *Azotobacter* | *vinelandii[13], salinestris* | Coproduction of alginate with PHA's |
| *Bacillus* | *megaterium[20], thuringiensis* | *B. megaterium* is the first bacterium that PHB is observed |
| *Chromatium* | *vinosum* | |
| *Clostridium* | *spp.* | |
| *Escherichia [24,-28,72]* | *coli* | Wild type is not producing PHA's except PHB/Ca-Polyphosphate channels in plasma membrane, |
| *Halobacterium* | *spp.* | |
| *Methylobacterium* | *extorquens[17], rhodesenium* | |
| *Methylocystis* | *spp.* | |
| *Micrococcus* | *spp.* | |
| *Nocardia[18]* | *corralina* | |
| *Pseudomonas[10]* | *acidophila, denitrificans, aeroginosa, oleovorans, putida, syringae, fluorescens, resinovorans, cepacia, lemoignei* | All *Pseudomonas* (except *P. stutzeri*) virtually produces PHA's, unlike *R. eutropha* this genus mainly produces mcl-PHA's. |

| | | |
|---|---|---|
| *Paracocccus[17]* | *denitrificans* | |
| *Rhizobium* | *meliloti, etli[19],ruber* | |
| *Rhodobacter* | *spheroides* | Photosynthetic |
| *Rhodococcus[23]* | *spp.* | |
| *Rhodosprillum* | *spp.* | |
| *Ralstonia[9]* | *eutropha* | Previously known as *Alcaligenes eutrophus,* most widely examined biosynthetic pathway, producer strain for commercial production. |
| *Sphaerotilus [12]* | *natans* | New promising candidate for PHB and PHBV production |
| *Staphylococci[16]* | | Pathogenic |
| *Syntrophomonas* | *spp.* | |
| *Synechocystis* | *spp.* | |
| *Thiocystis* | *violacea* | |
| *Zooglea[8]* | *ramigera* | A widely studied microorganism |

### 1.1.2.2. Recombinant producers :

PHA's stands for highly economic aspects and in that the production by natural producers have limited production capacities due to their long generation time with low optimum temperature for growth, problems in extraction, natural pathways for degradation and usually expensive substrate cost, very promising recombinant systems have been developed. Among them, *E. coli* has been the choice of organism for its being a powerful tool in molecular modification for economic production. First studies were made in 1988 by two groups (Dennis *et al* in USA and Steinbüchel in Germany [24,25]).

Cloning of *R. eutropha* PHB synthesis genes in *E. coli* resulted in polymer production as high as 95% (dry cell weight) with a genetically mediated lysis system for isolation of PHB granules [26]. This achievement was a result of a high copy number of a plasmid (500 copies in stationary phase). Lysis was done with T7 bacteriophage lysozyme gene with a strategy that caused 99% burst in stationary phase where PHA production was maximum.

Without a lysis technique, very high concentrations of PHB (88.8 g/l) were still achieved in a recombinant *E. coli* study by Lee *et al.* [27]

Copolymer (3HB-co-3HV) production in a recombinant *E. coli* producer has been also achieved [28]. The ratio of 3HB to 3HV solely depends on the ratios of carbon sources used as glucose and propionate.

A number of recombinant bacteria other that *E. coli* have also been developed during cloning studies and examined for the synthesis of various PHA's. When *R. eutropha* PHA synthesis operon was cloned to *P. oleovorans* , recombinant strain accumulated a blend of P(3HB) and P(3HH-co-3HO) which were stored as separate granules, but in the same cell with octanoic acid. [29] An interesting point was the observation that huge P(3HB) granules (3.5μm diameter)

appeared when *R. eutropha* harbored the PHA synthase genes of *Rhodospirillum rubrum*.[30]

Progress was still required to produce a variety of PHA's with single carbon and cofeeding strategies. Also optimization of the fermentation systems is a challenge as the responses of high cell density cultures with *E. coli* under nutrient limitation was unpredictable and caused the loss of plasmids. Therefore in the near future new fermentation strategies have to be developed.

### 1.1.2.3. Eucaryotic systems for PHA production :

For the large scale production, to overcome the massive needs of the world's demand on plastics as an alternative to petrochemical based ones, production of PHA's in plants is an ultimate solution that can reduce the costs. Somerville *et al.* tried to produce PHB in transgenic plants, [32]. They used the genes from *R. eutropha* that encoded two genes for the enzymes required to convert acetoacetyl CoA to PHB, which were placed under the transcriptional control of cauliflower mosaic virus 35S promoter which was introduced to *Arabidopsis thaliana.* In the cells of mature leaves, cotyledons and roots, PHB was detected in

granular form within nucleus, vacuole and cytoplasm. However expression of large amounts of acetoacetyl CoA reductase in plants cause significant reduction in growth and seed production with respect to wild types. This is more extensive in hybrids having also a PHB synthase together with acetoacetyl CoA reductase. A reason can be found by explaining the depletion of acetyl CoA or noxious effects of PHB.

An improved plant production mechanism was developed thereafter by the same group where all three genes of PHB synthesis were expressed in plastids of *A. thaliana.* The result were succesful, due to high content of acetyl CoA in plastids, as the production rised to 14% PHB in dry weight basis with a uniform mass distribution around 500 kDa[33].

In commercial cotton plant (*Gossypium hirsutum*), expression of PHB genes was achieved and improvements in characteristics of cotton fiber was reported. According to their studies only 3.4 mg of PHB per gram of fiber drastically improved the heat capacity. Further improvements were also expected after arrangements of compositions in fibers with PHA granules[34].

Another eukaryotic producer for PHA's is *Saccharomyces cerevisae*. With the construction of a yeast plasmid, enabling the expression of a functional synthase

enzyme, results in production of PHB around 0.5% of cell dry weight [35]. Eventhough the production is far below the aimed limits for production of polymers, this study gives us a new insight for understanding the production mechanism of PHA in yeasts.

Finally, two studies summarizes the expression and production of PHA synthase genes in insect cells. First study only expresses PHA synthase from *R. eutropha* by using bacalovirus *Autographa californica* nuclear polyhedrosis virus in *Trichoplusia ni* (cabbage looper) [36]. The aim of this study was not the production of PHA, but only to examine the expression of synthase in insect cells. However in the second one [37], a mutant rat fatty acid synthase was transfected to *Spodeptera frugiperda* cells again with a baculovirus system. This fatty acid synthase expression resulted in 3HB production which polymerized in P(3HB) with a polymerase from *R. eutropha.* Only visible granules were resulted with a yield of 0.16 % of dry cell weight, which is far more lower than any kind of production purpose.

Although successful P(3HB) synthesis has been achieved in plants, there is still much to be done for significant production schemes. Unlike microorganisms, plants and other eucaryotic systems possess a compartmentalized metabolism,

making the problem more complex. Although potential seems to be great, the limits of transgenic PHA production by plants is still unknown.

### 1.1.3. PHA's for industry :

It was the beginning of 1970's when the first interest of industry arose for the biodegradable polymers with Imperial Chemical Industries (ICI) in Britain. PHB was then marketed under the tradename of Biopol®, by Zeneca Bioproducts (formerly ICI), under two molecular weight forms (standard; 423 kDa and low 67.7 kDa). Due to its biocompatibility and biodegradability, it attracts medical attention [56]. The bacteria chosen for production is *R. eutropha*, on a scale of 1000 tonnes per year for P(3HB-co-3HV) under phosphate limiting conditions. The cost was about $5-15/kg depending on homopolymer or heteropolymer, with respect to $1/kg for synthetic plastics from petroleum.[57]

Another commercial producer for PHB was Chemie Linz GmbH from Austria. They started investigation of PHB in early 80's, with the basis of fermentation process by *A. latus* DSM 1124, in a single step unlike *R. eutropha*'s double step production. The preferred carbon source was sucrose, with respect to higher cost of glucose for Biopol. According to Hrabak [39], the production was

1000 kg of PHB per week in a 15 $m^3$ fermenter. However this process is no longer operational.

The main reason for high productivity costs is the carbon source and co-substrates, as well as the maintenance of a large fermentation facility and purification cost for the polymer. The limit for Biopol's usage is high cost and low production volume, nevertheless the potential market is in million ton range.

The applications on industrial and medical scale of PHA was summarized in the following Table 2.[44]

**Table 2**: Applications of PHA's:

| **Medical Applications:** |
|---|
| • Biodegradable drug carrier systems |
| • Bone growth stimulants due to piezoelectric properties |
| • Bone replacements and plates |
| • Artificial blood vessels |
| • Wound dressings |
| • Sutures, swabs, staples, syringes and pins |
| **Industrial Applications:** |
| • Diapers and femine hygenie |

| |
|---|
| • Containers , coffee cups, bottles, wrappings and bags |
| • Biodegradable long term carrier systems for fertilizers, insecticides, herbicides, fungicides etc. |
| • Disposable food service-ware |
| • Razors, pens, golf tees |
| • Non-woven fabrics |
| • Hot melt and pressure sensitive adhesives |
| • Toner and developer formulations |
| • Dairy cream substitues and flavor delivery agents |
| • Monomers can be used for synthesis of bioactive agents due to stereospecifity |
| • Paper coating applications |

### 1.1.4. PHA biosynthetic pathways :

#### 1.1.4.1. Three step (scl) pathway :

P(3HB) and more generally scl-PHA biosynthetic pathway consists of three enzymatic reactions catalyzed by three distinct enzymes. The first reaction consists of condensation of two acetyl Coenzyme A molecules to acetoacetyl CoA under

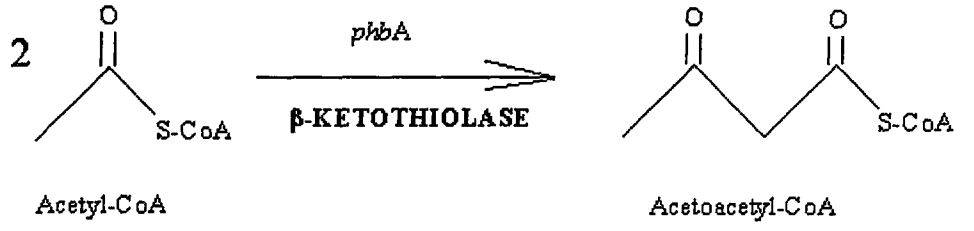



**Figure 2:** Action of β-ketothiolase in the first step of scl-PHA biosynthesis

Second reaction is the reduction of this acetoacetyl CoA to (R)-3-hydroxybutyryl CoA, by an NADPH dependent acetoacetyl-CoA dehydrogenase (hydroxybutyryl-CoA dehydrogenase EC 1.1.1.36). This enzyme is encoded by gene *phb*B.(Fig 3)

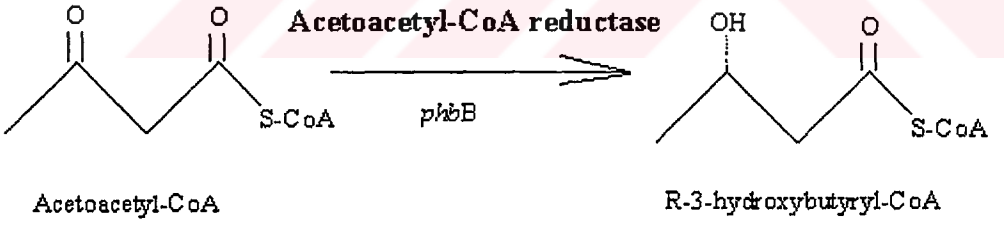



**Figure 3** : Action of acetoacetyl-CoA reductase in the second step of scl-PHA biosynthesis

Finally these monomers of (R)-3-hydroxybutyryl CoA are polymerized into P(3HB) by PHB polymerase (unclassified by EC) encoded by *phb*C.(Fig 4)

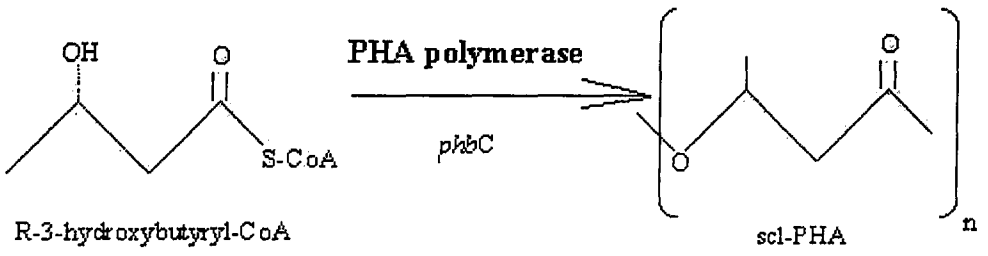



**Figure 4** : Action of PHA polymerase on the last step of scl-PHA biosynthetic pathway.

These three enzymes are encoded by a *phb*CAB operon with a promoter upstream of phbC as shown below for *R. eutropha* and *A. latus*. (Fig5)

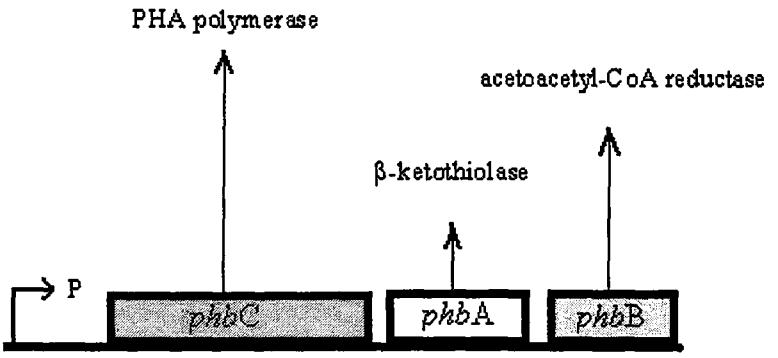



**Figure 5** : Genes that were transcribed to biosynthetic genes. P: promoter.

### 1.1.4.2. Properties of enzymes of three step pathway :

- **β-ketothiolase:** In nature β-ketothiolases are subdivided into two groups due to their substrate specificity. First group is mainly involved in degradation of fatty acids. Our interest is in the second group which are considered biosynthetic and have a narrow specifity from $C_3$ to $C_5$. Their roles are various, mainly P(3HB) synthesis, steroid and isoprenoid synthesis and ketone body formation. The specifity of these enzymes is primarily for acetoacetyl-CoA. *R. eutropha* has two β-ketothiolases (A and B). Their difference is in their substrate specificities, but both are involved in polymer synthesis [58]. The enzymatic mechanism was also

examined by Masamune *et al* in *Zooglea ramigera* [59], in which β-ketothiolase was shown to be the enzyme controlling the PHB biosynthesis in *R. eutropha*, with CoA as the key effector metabolite.

- **Acetoacetyl-CoA reductase:** The enzyme is an NADPH dependent reductase, up to date only a single NADH dependent reductase has been found in *C. vinosum*. Therefore the availibility of reducing equivalents in the form of NADPH is a driving force for P(3HB) synthesis.[60] After the activity of acetoacetyl- CoA reductase, the monomers for P(3HB) is ready. However this thiolase-reductase monomer supply pathway is not necessary for polymer synthesis, there are other strategies that can supply monomers. Again there are two acetoacetyl-CoA reductase present in *Z. ramigera* and *R. eutropha,* possessing different substrate and coenzyme specifities.[61,62]
- **P(3HB) polymerase:** The enzyme was first thought to be located on the surface of granules. Today it is known that there are also soluble polymerases together with granule bound one. These soluble (hydrophilic) and granule-bound (hydrophobic) states may be a result of cells adaptation to problematic protein-hydrophobic surface interactions. It was proposed that the concentration of these fractions were altering when cell shifted from carbon limiting to nitrogen limiting conditions in *R. eutropha* [63] and similiarly in Z. ramigera [64]. This is a hypothesis

for explaining the PHB accumulation with nitrogen limitation, with the activity of granule associated PHB polymerase, and disappereance of soluble polymerase. PHB polymerase of *R. eutropha* is specific for D-(-) substrates. Single cysteine residues were found to be important in the activity of PHB polymerase, Cys 319 , which was conserved in all PHA polymerases. Later it was found that P(3HB) polymer was covalently bound to PHB polymerase through this residue, which was in its active site. As PHB polymerase is forming ester bonds, it is natural that active site Cys319 is present within a lipase box. The catalytic activity of nucleophile Cys319 is enhanced by an aspartate and a histidine residues forming a catalytic triad.[65] Another post translational mechanism is also proposed for the activity, given that PHB polymerase may have two active site thiols [66].

Post translational mechanisms again seems to control the PHB polymerase in cyanobacteria *Synechococcus* sp.[67]

### 1.1.4.3. Pathways for mcl-PHAs :

PHA having various medium chain length 3-hydroxyalkanoic acids were first detected in cells of *P. oleovorans* grown on n-octane by Witholt and

coworkers. [10] Later it was found that all pseudomonads belonging to RNA homology group I were able to synthesize $PHA_{mcl}$. [68] It was suggested that the monomers of $PHA_{mcl}$ were derived from the fatty acid β-oxidation pathway when $C_6$-$C_{10}$ alkane, alkanol and alkanoic acids were used as substrates.(Fig 6)

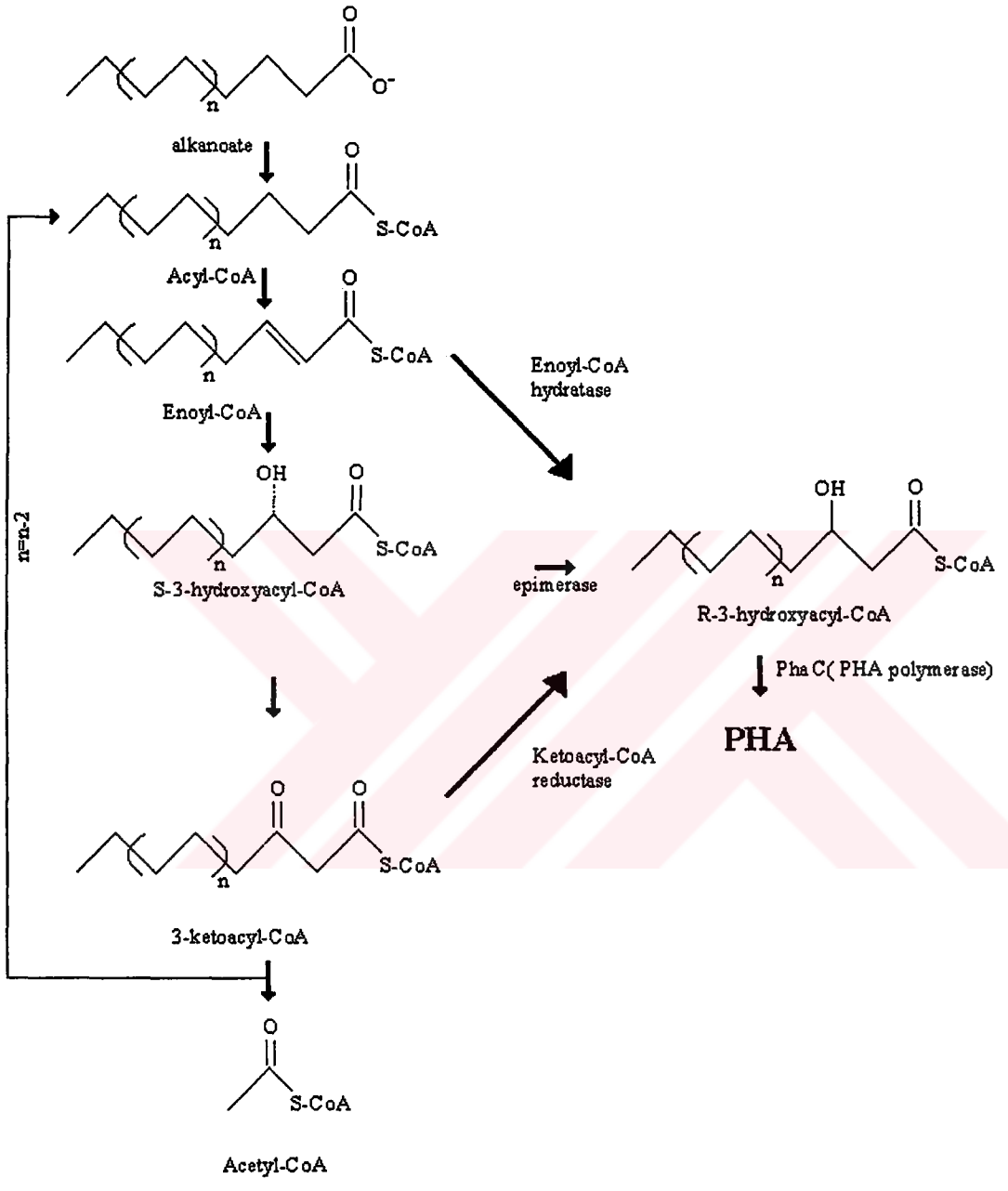



**Figure 6**: Biosynthetic pathway for mcl-PHA from alkanes, alcohols or alkanoic acids ($C_6$-$C_{10}$) [76]



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When carbohydrates were used, then *de novo* fatty acid synthesis pathway provided the required monomers.(Fig 7)

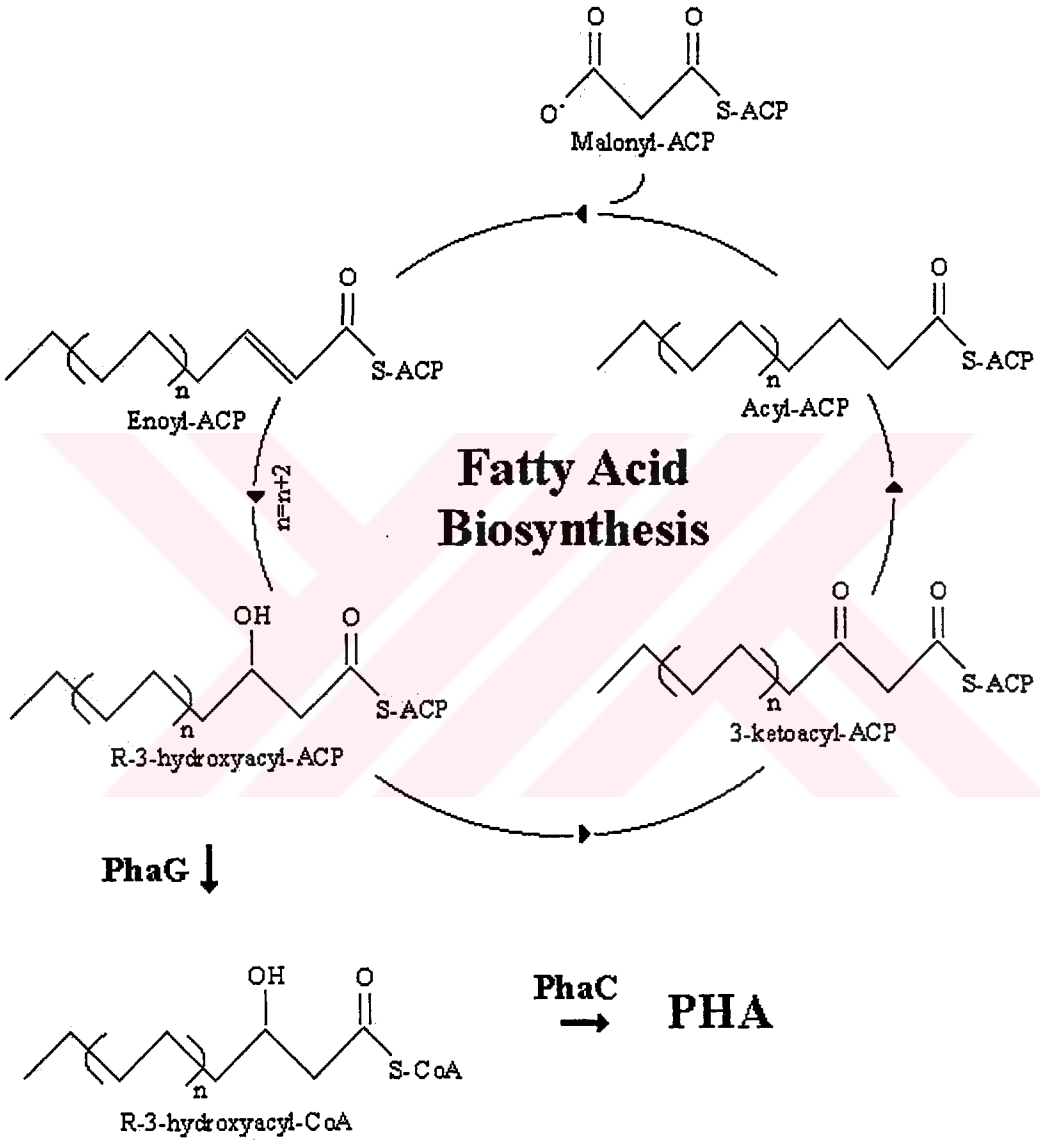



**Figure 7**: Biosynthetic pathway for mcl-PHA from carbohydrates

These two pathways are acting independently in PHA biosynthesis.[69] Moreover the PHA polymerases acting on $PHA_{scl}$ and $PHA_{mcl}$ synthesis are hypothesized to be mutually different, when both are present in a single organism, a blend instead of a copolymer is produced.

### 1.1.5. Roles of PHA's in nature :

#### 1.1.5.1. PHA's as carbon and energy reserve materials :

The role of PHA's as a carbon and energy reserve material is previously mentioned. This role will be explained in more detail here. The main advantage of storing these high molecular weight polymers as granules within the cytoplasm is the ability to deposit large amounts of reduced carbon source without significantly affecting the osmotic balance of the cell. This was shown to be important in sporulation of *Bacillus* species and encystment of azotobacters. In *Rhizobium* species, an interesting role of PHB other than being energy and carbon source is hypothesized, which is to protect nitrogenase within the nodule, when oxygen concentration in the nodule rises.[22]

### 1.1.5.2. PHB in Plasma Membranes :

Reusch and Sadoff [2] extracted a poly-β-hydroxybutyrate complex from the plasma membranes of competent *E. coli* , together with polyphosphate and calcium. They propose a formation of exolipophilic-endopolarophilic helix for PHB around an inner framework of calcium polyphosphate. According to their results this symmetrical structure forms a channel through the membrane and can play a role for calcium, phosphate and most importantly DNA transfer, as close correlation is observed between the concentration of membrane complex and the transformability of the cells. Despite the general belief of PHB is specific for procaryotes, PHB-Ca-polyphosphate was found to be universal eventhough the storage molecules were absent in wild type eucaryotes. The chain length of eucaryotic PHB was similar to procaryotes, only poly(P) is rather longer.

### 1.1.6. Biodegradation of PHA's :

Generally a series of parameters can influence the rate of extracellular biodegradation of the polimeric material in nature, including environmental type, presence of microbial activity and population, water availability, temperature,

thickness of the material, surface structure, porosity and if present additives in the matrix such as fillers.

It is accepted that there are two general mechanisms for the extracellular degradation of PHA. First is the hydrolytic mechanism, which mainly depends on physical factors like pH and temperature. Next is the enzymatic degradation of the polymer with depolymerases which hydrolyze the polymer to water soluble products, secreted by widely distributed natural environmental consumers.

Extracellular enzymes capable of depolymerizing PHB have been found in a many organisms (Table 3), one of them is depolymerase form *Alcaligenes faecalis* [40]. These structural depolymerase genes have been also cloned into *E. coli* and sequenced by Saito *et al.*[41] The genes are highly expressed and enzyme is excreted. Isoenzymes of this PHB depolymerase was also found in *P. lemoignei.*[42,43] In contrast to PHB, PHA with longer pendant chains have slower biodegradation rates mainly because the increase in hydrophobicity with the increasing chain lenghts, which prevents the habitation of the polymer surface with microbial population.

**Table 3**: Microorganisms with extracellular depolymerases

| Genus | Species | Polymer Specifity | References |
|---|---|---|---|
| *Pseudomonas* | *Lemoignei* | Scl-PHA | 46, 47 |
| *Alcaligenes* | *Faecalis* | Scl-PHA | 40 |
| *Comamonas* | *Testosteroni* | Scl-PHA | 48 |
| *Pseudomonas* | *Pickettii* | Scl-PHA | 49 |
| *Fungi* | *spp.* | Scl-PHA | 50, 51 |
| *Pseudomonas* | *Fluorescens* | Mcl-PHA | 52 |

In homopolymers , the effect of molecular weight seems not to be correlated with the biodegradation, solely because high molecular weight (800 kDa) PHB homopolymer degrades as rapidly as a low molecular weight (150 kDa) one [44].

For the examination of polymer composition on degradation, Doi and colleagues examined degradation of films (0.07 mm thick) in soil, sludge and buffers. They found that 4HB monomer units greatly enhances degradation in all conditions, 3HB-co-4HB films decomposition are much more higher than either PHB or P(3HB-co-3HV) copolymers. Their conclusion summarizes hydrolytic degradation by homogenous erosion in two ways. First is the random hydrolytic scission of ester groups resulting in the molecular weight loss and secondly the

erosion and weight loss when molecular weight decreases to 13 kDa. They found also that the rate of chain scission is increased proportionally by the presence of 4HB monomers.[45]

In an elegant study by Jendrossek and coworkers[46], *P. lemoignei* PHA depolymerase system was examined in detail. They found that *P. lemoignei* had five different depolymerase genes (phaZ1 to phaZ5) encoding for 4 different depolymerases of PHB (PHB depolymerase A,B,C,D) and one PHV depolymerase. All these enzymes were located extracellularly. PHA depolymerases were sequenced and Pha Z1 was cloned to *A. eutrophus* H16 followed by observation of growth supplemented by extracellular PHA, which was not present in wild type. They also found that PHB hydrolysis depended on divalent cations like $Ca^{2+}$ and inhibited by EDTA.

Further studies on depolymerase effect on bacterial and synthetic crystals of PHB can be found in studies of Fuller *et al* [53] and Jendrossek *et al* [54]. Also a scaning electron microscopic study is present, which explains the different pattern of erosion in PHA sheets between different pure cultures of degrading bacteria. According to this study degradation of amorphous regions of the polymer are more rapidly than the crystalline regions. [55]

## 1.2. Nile red as a novel specific stain for PHA's :

### 1.2.1 Previous considerations with Nile blue A :

Ostle and Holt [3] are the first for proposing a fluorescent detection method by fluorescent staining of PHB. In their study they heat fixed and stained the cells with 1% aqueous solution of Nile blue A and observed PHB as orange fluorescent bodies, but glycogen and polyphosphate did not stain. Despite their finding that the fluorescence was directly proportional to the PHB concentration, it was concluded that the assay of PHB with Nile blue A was not sensitive enough to be of further use. However the staining technique was still emphasized to be much more sensitive than the previous staining and detection method with Sudan black B. [75]

### 1.2.2. Structure of Nile red :

Lysochromes are dyes used to stain tissues. An ideal lysochrome should have a strong color, being very soluble in the target material, showing no interaction with the tissues except with solution and especially without unwanted

interaction with other constituents in the environment . Nile red was chosen to be used in the present investigation since it meets these conditions exceptionally well.

Nile red ; Nile blue A oxazone, 9-diethylamino-5H-benzo[α] phenoxazine-5-one, is a non-ionic dye giving bright red to yellow-gold fluorescence with specifity to neutral lipid droplets.As can be seen below (Fig 8), it has a heterocyclic nature, and hence should be cared as a possible carcinogen.

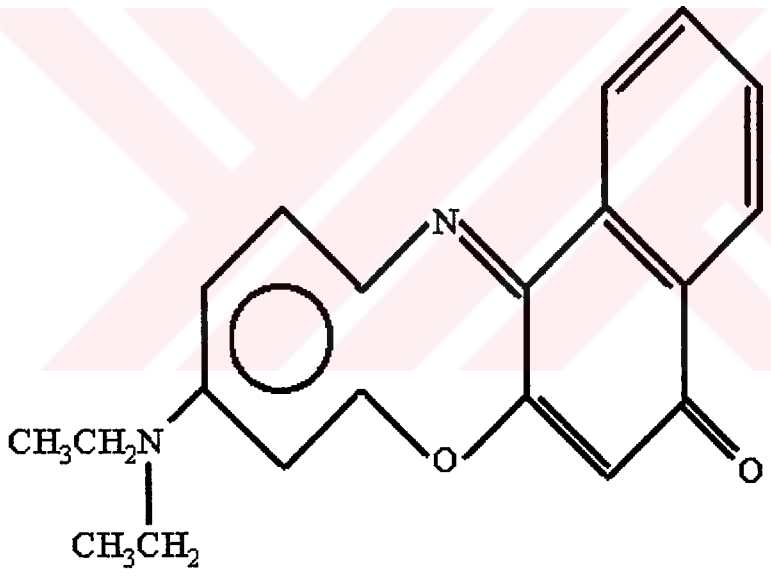



**Figure 8.** Structure of Nile red.

Nile red is poorly soluble in water, but it dissolves well and intensly fluoresces in many organic solvents like n-heptane, chloroform, acetone and ethanol.

Its fluorescence quickly quenches in water. However it has been reported that Nile red's fluorescence depends on a lipid hydrophobic environment, which can be observed even in an aqueous medium.[74] Therefore it can be accepted as a good hydrophobic fluorescent probe for cellular polymer inclusions, since polymer granules are known to have an hydrophobic environment together with a cytoplasmic aqueous medium. Moreover the dye can be applied to the cells in an aqueous solution but it dissolves preferentially in hydrophobic environments. Any solubilization in aqueous medium quenches, enabling the stained material to be observed .

**1.3. Aim of the Study:**

In this study our aim is to show that, for the detection of intracellular polymer presence Nile red staining with fluorometric measurements was an alternative to the traditional gravimetric or spectrophotometric measurements.

# CHAPTER 2

# MATERIALS AND METHODS :

## 2.1. Materials :

All chemicals were of analytical grade and their suppliers were listed in Appendix A on page 108.

## 2.2. Microorganisms :

In this study the following microorganisms were used. *Ralstonia eutropha* ATCC 17699 (previously known *as Alcaligenes eutrophus*), *Alcaligenes latus* ATCC 29713, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* V517.

Growth medium for each bacterial strain was different depending on the experiments run (general properties of the producer species and a list of growth media are presented in Appendix B p.110).

## 2.3. Storage and maintenance of the microorganisms :

### 2.3.1 Storage and maintenance of *R. eutropha* :

*R. eutropha* cells were routinely streaked on nutrient agar plus yeast extract plates at two weeks intervals. Pure cultures were stored at 4°C. For the long-term storage, log-phased liquid cultures of *R. eutropha* were frozen with liquid nitrogen after the addition of glycerol (20% v/v) in sterile Eppendorf tubes and stored at

-20°C in deep freeze. The stocks were monitored every six months for purity and renewed if required

### 2.3.2. Storage and maintenance of *A. latus* :

*A. latus* cells were subcultured to minimal sucrose agar plates every two weeks and stored at 4°C. For long term storage same procedure was followed as in 2.3.1.

### 2.3.3. Storage and maintenance of *P. aeruginosa* :

Stab cultures of *P. aeruginosa* were made on Pseudomonas agar P in test tubes and stored at 4°C. The cells were lyophilized for long-term storage.

### 2.3.4. Storage and maintenance of *E. coli* :

*E. coli* cultures were made in nutrient agar plates and renewed every two weeks.

### 2.4. Tests of Purity :

Routine checks of colonial morphology (shape, diameter, color and smell), and Gram staining were done every six months. Polymer production capacity of the cultures was checked by staining with Sudan black B and examination under microscope as a conventional method.

## 2.5. Growth curves :

### 2.5.1. Growth curve of *R. eutropha* :

*R. eutropha* cells were inoculated from a single colony of stock plate to nutrient rich liquid medium (2x5 ml), and grown for overnight at 30°C at 150 rpm. This seed culture was used to inoculate the experimental culture of 2x100 ml on 4% v/v basis and incubated at optimal conditions. Optical densities of samples were measured at two different wavelenghts (550 and 610 nm.) initially, every hour for 12 hours and then with two hour intervals for 28 hours. The results were plotted as absorbances versus time.

### 2.5.2. Growth curve of *A. latus* :

Growth curve of *A. latus*, was constructed in a similar manner with *R. eutropha.* A seed culture was prepared with inoculation of 2x5 ml of minimal sucrose medium from the stock streak plate. The culture was incubated overnight at 30°C at 150 rpm. and used for inoculation of 2x100 ml of minimal sucrose medium on 4% v/v basis. Sampling was done as above.

Absorbances at 550 and 610nm were plotted against time for construction of the growth curve.

### 2.5.3. Growth curve of *P. aeruginosa* :

Growth curve of *P. aeruginosa* is plotted simultaneously with the metabolic activity studies. To do this, a seed culture of 2x5 ml was prepared by inoculating the Lauria-Bertani broth with a single colony from the stock plate. After incubation

at 30°C for overnight at 150 rpm, the seed culture was used for inoculation of 2x100 ml of Lauria-Bertani broths in 500 ml flasks.

1 ml of samples were taken from the flasks up to 72 hours with two hour intervals for measurement of absorbances at 550 nm.

## 2.6. Polymer production :

### 2.6.1. Polymer production by *R. eutropha* :

For production of polymer, laboratory scale fermentations were done by using *R. eutropha*. The volume varies with the amount of polymer required, but generally 1L of growth medium is used in 5L flasks. Firstly a seed culture is prepared by inoculating 2x5 ml of inoculum medium containing 20g/L fructose, from the stock culture plate and incubated overnight at 30°C at 150 rpm. The culture was used to inoculate 2x20 ml culture of growth medium containing again 20g/L fructose, which would be the inoculum for 1L culture containing variable ratios of carbon source depending on the kind of polymer. After overnight incubation, inoculation of 1L culture of growth medium was done on 4% v/v basis. The fermentation was carried at 30°C at 150 rpm for 25 hours and was centrifuged in a Sorvall RC5B refrigerated superspeed centrifuge (Du Pont Instruments USA). The supernatant was collected and the pellet was dissolved in equal volume of nitrogen free medium. A further incubation for 48 hours for enhancing the polymer production was allowed and then the cells were harvested. They were processed and extracted as explained in further sections.

### 2.6.2. Polymer production by *A. latus* :

Unlike *R. eutropha*, polymer production by *A. latus* occurs in growth-associated manner and hence, said to be a single step production.

For this purpose, a seed culture was prepared by inoculating 2x5 ml of minimal sucrose solution with 20g/l sucrose, with a single colony from the stock plate of *A. latus*. The culture was incubated overnight in 30°C at 150 rpm. and used for inoculation of 2x20 ml minimal sucrose medium.

After the same incubation conditions, 1L minimal sucrose medium was inoculated with these cultures on 4% v/v basis. Incubation continued for 19 hours, (this is the time of carbon depletion according to our experience), then an extra carbon source was supplied to the culture . This extra source determines the nature of the polymer, therefore if a copolymer is wanted to be produced, a co carbon-source (like γ-hydroxybutyric acid) has to be introduced together with the main carbon source sucrose.

A further incubation for 24 hours was done prior to extraction of polymer .

### 2.7. Harvesting of cells :

For the cultures to be harvested over 50 ml, harvesting was done by centrifugation for 15 minutes at 5500 rpm.

Supernatant was discarded and the pellet was weighed and stored frozen at –20 °C in a deep freeze.

## 2.8. Extraction of polymers :

### 2.8.1. Preparation for extraction :

The frozen cells were lyophilized by using a freeze-dryer (Freezone 6, Labconco USA) at –85 °C and 0-5 $10^{-3}$ mBar pressure.

### 2.8.2. Extraction procedures :

#### 2.8.2.1. Sodium hypochloride extraction :

Cells have been usually extracted by employing sodium hypochloride (NaOCl) with a modified procedure of Williamson and Wilkinson [71], for the destruction of cell membrane and release of polymer granules. For this purpose a known amount of dry cell pellet was dissolved in glass tubes with 10 ml of 1% commercial NaOCl solution and incubated in a water bath at 55°C , after vigorous shaking for 2 hours. Then the cells were centrifuged with Hettich EBA III Benchtop Centrifuge (Hettich Germany) for 15 minutes at 8000 rpm. Supernatant was discarded carefully and the pellet was washed with distilled water. After centrifugation under the same conditions, the cells were washed twice with methanol and once with acetone.

Finally after drying of the pellet in oven at 50°C for 2 hours, the polymer was weighed and recorded. By dividing the polymer weight to the dry cell weight of the microorganisms, percent yield is calculated.

### 2.8.2.2. Chloroform extraction :

When molecular weight of the extracted polymer is important, NaOCl extraction stated above cannot be used due to the decrease of molecular weight, when this procedure was applied. In our study, the polymers were extracted by using a modified Soxhlet apparatus. This apparatus is composed of an air and a water cooling units vertically attached to a round bottom flask immersed in a thermostated waterbath with a magnetic stirrer. All units are attached to each other with Pyrex Quickfit fittings. For our extraction procedure, minimum 2 g of dry cells were ground in mortar and added to round bottom flask with a magnet and chloroform of ratio 20 ml to 1 g of cells. This solution was mixed well manually and introduced to the apparatus. Chloroform extraction with refluxing was carried out for 6 hours.

The solution was cooled and filtered through Whatman No. 1. and the volume was recorded. Polymer was seperated from chloroform by addition of 10 volumes of cold ethanol over the solution with a fast mixing on a magnetic stirrer for 20 minutes.

This solution was refiltered and dried in vacuum oven at 50°C for two hours.

## 2.9. Determination of polymer content :

After extraction of polymers, polymer content could be determined in two ways. First the polymer amount can be calculated gravimetrically by weighing the polymer.



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However there is a spectrophotometric way of determination of polymer content providing the polymer is solely composed of PHB. This method is named crotonic acid assay and depends on the conversion of PHB to crotonic acid with the action of sulfuric acid. The final product gives an absorbance at 235 nm and amount of formed crotonic acid is directly proportional to absorbance, hence PHB amount.

For this purpose known amounts of polymer is added to glass tubes and all solvents were evaporated in a boiling waterbath. 10 ml of sulfuric acid ($H_2SO_4$) is added to the tubes and tubes are heated in boiling waterbath for 10 minutes. Solutions are cooled, mixed well and absorbances are detected at 235 nm against a sulfuric acid blank. The amount of PHB in the sample is detected with the help of a previously constructed calibration curve.

## 2.10. Construction of polymer deposition profiles :

### 2.10.1 Polymer deposition profile of *R. eutropha* :

In order to observe deposition of polymer by the producer microorganisms under standard growth conditions, the amount of polymer with respect to the cell dry weight should be detected. To observe this relationship, the following procedure was followed.

*R. eutropha* seed cultures were prepared as explained before to inoculate 2x500 ml of appropriate growth medium with optimal temperature and aeration for 25 hours. As described before, these cells were transferred to 2x500 ml nitrogen

free production medium and during further cultivation sampling was done in every two hours for 2x40 ml in duplicates. Cells were centrifuged and frozen thereafter.

For the observation of deposition, the frozen cells were freeze-dried, and their dry cell weights were recorded. Polymer extraction was achieved with sodium hypochloride method, and a deposition profile showing the percentage of polymer present with respect to the dry weight was plotted. The results were independent of increase in cell weight because polymer mass was demonstrated on percent dry weight basis. Standard deviation was calculated and shown on the graph as error bars.

### 2.11. Determination of properties of Nile red :

#### 2.11.1. Spectrofluorometric analysis of Nile red :

In spectrofluorometric studies, the properties of the fluorescent probe used should be determined.. This is the excitation and emission wavelenghts of the dye. However, there is a very broad range of wavelengths to be scanned (200 nm to 1000 nm) and therefore a multispectral measurement is a must if we have no idea on the probe. This is a general trial and error method to roughly estimate the excitation and emission wavelengths.

For this purpose 3.625 ng/ml Nile red in 70%v/v ethanol is prepared and checked for excitation and emission measurements on two dimensions, first dimension (x-axis) scanning the emission wavelengths from 550 nm. to 700 nm, whereas second dimension (y-axis) scans for excitation wavelenghts between 550 nm. and 590 nm. with a bandwidth switch of 10 nm. Excitation and emission slits

were 5 nm. each. Results were plotted and the emission peak wavelength was detected.

### 2.11.2. Excitation wavelength detection of Nile red :

From the multispectral measurement , approximate excitation wavelength can be deduced from the maximum emission peak length corresponding excitation wavelength. However this value should be further checked for accuracy.

Therefore excitation spectrum of 3.625 ng/ml Nile red in 70% v/v ethanol was measured and plotted. This operation requires manual entering of the emission wavelength detected in multispectral measurement and lets a more sensitive detection of this wavelength. An excitation wavelength range was scanned between 550 nm and 650 nm with 5 nm slit width. From the graph plotted, emission wavelength was detected on central display unit , by finding the corresponding wavelength to the excitation peak.

### 2.11.3. Emission wavelength detection of Nile red :

After finding the proper excitation wavelength, the emission wavelength obtained through multi spectral measurement was verified by giving this value manually.

Once again, the emission spectrum of 3.625 ng/ml Nile red in 70% v/v ethanol was measured and plotted. Emission wavelength was scanned starting from 550 nm to 700 nm with 5 nm slit width. The emission wavelength was detected, on the central display unit finding the corresponding wavelength to the emission peak.

## 2.12. Deteremination of Nile red activity with different solvents:

### 2.12.1. Nile red in ethanol:

Nile red solubility and linearity of Nile red fluorescence in ethanol was detected as follows. First solubility of the Nile red was checked in the solvent to be sure about the safe concentration range to be worked. For this purpose, 5 ml of the solvent was placed in a screw capped vial and gradual addition of the Nile red was carried out. After addition the vial was vigurously mixed with vortex and heated in a water bath with a temperature below the boiling point of the used solvent. Vial was examined qualitatively for the presence of any undissolved Nile red crystals in the solution.

A suitable concentration was next chosen for further studies which was half or two third of the saturation concentration. A serial dilution of this concentration was made to measure the linearity of the fluorescence with the increasing concentration of the dye. A graph was sketched for showing the linearity.

### 2.12.2. Nile red in acetone:

Same procedures described above was used for Nile red in acetone. The value of solubility was very high in acetone than ethanol therefore excitation and emission wavelengths were detected and the graph showing the linearity was measured with a proper concentration selected.

### 2.12.3. Nile red in chloroform:

As in acetone, Nile red easily dissolves in chloroform even in high concentrations with a very high fluorescence value. Though respectively lower concentration is used in chlorofom for detection of excitation and emission wavelengths and linearity of the fluorescence.

## 2.13. The relationship between fluorescence intensity and Nile red concentration :

For the fluorescent probe to be used safely in quantification, a linear increase in fluorescence measurements is expected against increased concentrations.

To show this, wide range of Nile red concentrations were prepared, to see any deviations from the linearity if present. The concentrations prepared were as follows; 0.05 μg/ml, 0.1 μg/ml, 0.25 μg/ml, 0.5 μg/ml and 1 μg/ml. The fluorescence values corresponding to these concentrations were measured and a calibration curve was constructed.

## 2.14. Fluorescence microscopic examinations :

### 2.14.1 Preparation of cells for qualitative fluorescence microscope examination:

Cells grown under optimal conditions for polymer production had to be examined under fluorescence microscope for the presence of Nile red stained polymer granules.

To achieve this, two methods were used. Cells were either sampled from the culture and stained separately in small scale or Nile red was directly added to the culture medium for on-line staining.

In the first method , 1 ml of culture was sampled and centrifuged by using an Eppendorf benchtop centrifuge. It was washed once with distilled water and incubated with 0.2 ml of 1 μg/ml of Nile red for 10 minutes. After the staining, excess dye was removed by washing with water , centrifugation and the cells were resuspended in distilled water. Cells were transferred to slides and heat fixed , while 10 μl amounts were tranferred to slides for examination of alive cells under fluorescence microscope.

In the second method, Nile red was directly added to the culture medium. 1% v/v filter sterilized Nile red was added to the culture medium prior to cell harvest. The cells were then sampled as usual (1ml) and after washing and centrifugation, resuspended in distilled water.

**2.14.2. Examination of cells with fluorescent microscope :**

The cells, transferred to pre-cleaned glass slides, and covered with a coverslip were examined under inverted phase contrast-fluorescent microscope (Olympus IX70, Japan) equipped with a normal and CCD camera with a computer for image analysis. The excitation filters of the microscope show a wide band selectivity, ranging from 330-385 nm ( U cube ) with a 420 nm barrier filter, 470-490 nm ( B cube ) with a barrier filter 515 nm and 530-550 nm ( G cube) with a 590 nm barrier filter.

For the observations , generally 40X and 60X magnification were chosen with B cube selection to yield orange fluorescence of the intracellular polymers . Microscopic observations were either saved as images or microphotographed (Kodak 400 ASA color films).

## 2.15. Construction of calibration curves

### 2.15.1. Calibration curve of Nile red stained *R. eutropha :*

*R. eutropha* cells were grown under optimal conditions as discussed before. Experimental set consists of a duplicate sets of culture medium (2x500ml). During the polymer production step (starting at 25 $^{th}$ hour), three samplings were done from each flask (2x1ml and 40 ml). The first two were used for staining, where they were washed once with distilled water and stained with Nile red, and the third was used for determination of dry cell weight (with centrifugation and lyophilization) followed by polymer extraction with sodium hypochloride assay. Sampling was done on various intervals (see Figure 9)

Several trials were done according to the same procedure and the collected data was averaged. If the polymer produced was homopolymer PHB, the content was also checked with crotonic acid assay.

To show the linearity of binding within a single sampling time, another calibration curve is plotted. This time the sample at 38$^{th}$ hour was diluted in 1/10 fold , 5 times and the variation in fluorescence was observed.

All calibration curves were plotted on fluorescence intensity vs dry cell weight basis , and fluorescence intensity vs produced polymer basis.

### 2.15.2. Construction of calibration curve of Nile red with *A. latus* :

*A. latus* cells were grown under optimal conditions as discussed before. Experimental set consists of a duplicate sets of culture medium (2x500ml). During the cultivation, three samplings were done from each flask.(2x1ml and 40 ml) First two was used for staining, where they were washed once with distilled water and stained with Nile red, and the third was used for determination of dry cell weight (with centrifugation and lyophilization) followed by polymer extraction with sodium hypochloride treatment. Sampling was done at varying intervals as shown in the Figure 10. At the $19^{th}$ hour extra carbon-source was added. Several trials were done according to the same procedure and the data were used for calculation of mean and the standard deviation. . When the polymer produced was PHB, crotonic acid assay was used to check the results.

A calibration curve was plotted also for *A. latus*. This time the sample at $36^{th}$ hour was diluted in 1/10 folds, 5 times and the variation in fluorescence was observed.

All calibration curves were plotted on fluorescence intensity vs dry cell weight basis, and fluorescence intensity vs produced polymer basis.

### 2.15.3. Construction of calibration curve of Nile red with *E. coli* :

For the cultivation of *E. coli* V517 cells, nutrient broth was used. 5 ml of nutrient broth was inoculated with a loopful of cells from the pure culture plate and

ml ... at 2 ...

These samples were stained like the producer strains and their fluorescence intensities were detected. These values were plotted in a calibration curve. From the 10 ml sample their dry cell weight values weredetermined gravimetrically.

From the 18$^{th}$ hour, serial dilutions were carried out starting from 1/10 up to 1/50 and these values were sketched for a calibration curve that corresponded to possible background fluorescence staining.

**2.16. Colony staining Procedure :**

A colony screening method was developed in order to be able to detect potential polymer producer strains from the nature during isolation procedures.

Cellulose nitrate and polycarbonate filters (Millipore Ireland) were employed in the screening method as a base for growing the colonies of known producer strains together with control strains. The filters were first divided into four compartments with a marker or pencil. Then they were sterilized in glass petri dishes with a few drops of distilled water to avoid drying. They were asceptically transferred onto proper solid growth medium (for *A. latus* minimal base and sucrose, for *R. eutropha* nutrient rich medium with fructose). Colonies from the pure cultures of the strains to be tested were toothpicked onto the filters. Four compartments were used; first for known producer strain , second for the control strain and the rest for the new strains. These plates were incubated until colonies became visible at 30°C.

In the screening process, for the colonies belonging to *A. latus* were checked right away for their polymer production capacity, whereas for *R. eutropha* colonies, filter had to be transferred to a nutrient deficient media for production of polymers.

Both the filters were removed later from the plates and dipped into saturated Nile red solution in 70% ethanol for 10 seconds and then to distilled water for removing excess dye. The filters were then examined under daylight or UV and photographed.

## 2.17. Monitoring of metabolic activities in terms of polymer production:

A new approach in monitoring the polymer production potential of the strains is to measure their metabolic activities. For this purpose, *R. eutropha* and *A. latus* and *P. aeruginosa* were employed as representative strains. The former two strains have a different PHA production pathway as they produce short chain length PHAs, whereas the latter strain produces medium chain length PHA. The polymer production in both groups can be measured through monitoring changes in oxidative metabolism.

For metabolic activity measurements, three indicators were correlated. These were absorbance, Nile red fluorescence and optical density after reduction of MTT ( 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide or Thiazoyl Blue). The first two indicators were explained before.

MTT is a chemical used for measuring cellular metabolic activity ( it is an extremely toxic compound and should be handled with care!). For preparation, 1 mg of MTT was dissolved in 10 ml of distilled water as a stock solution. It was filtered through a 0.22 μm filter and kept cold in dark. The solution was 5 fold diluted in PBS prior to use.

MTT procedure requires the disruption of cells after reduction of MTT. For this purpose acidic isopropanol should be applied. It was prepared by addition of 2ml 37 % v/v HCl to 198 ml of isopropanol. The solution was kept cold in dark bottles.

Equal volumes of cell culture (0.2 ml) was mixed with 1:5 diluted MTT (0.2ml) in Eppendorf tubes and incubated at 30°C for three hours. Then 0.6 ml of acidic isopropanol was added to disrupt the cells and the solution was incubated at 30°C for 20 minutes with shaking at 300 rpm. The absorbance of this solution was measured at two wavelengths of 570 nm and 680 nm against a blank composing of 0.4 ml of MTT and 0.6 ml of acidic isopropanol. The absolute value after subtraction was accepted as the metabolic value of the culture.

*A. latus* study continues for 42 hours in a 500 ml culture with sampling at two hour intervals upto 26$^{th}$ hour, and the incubation was terminated at 42$^{nd}$ hour. Extra (20 g/l) sucrose was added to the culture at 19$^{th}$ hour. (Data not shown)

*R. eutropha* and *P. aeruginosa* cells were similarly cultured, accept that the cultivation time was 72 hours, with three extra samplings at 47$^{th}$, 65$^{th}$ and 72$^{nd}$ hours.(Figures 44 and 46)

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1. Microorganisms:

The characteristics of the microorganisms used in this study are summarized below. The ones with bold characters denotes the characteristics checked during the experiments.

**Table 4**: Microorganisms used in this study and their characteristics

| **Characteristics** | ***R. eutropha*** | ***A. latus*** | ***P. aeruginosa*** |
|---|---|---|---|
| **Shape of the cells** | Rods | Coccoid rods | Rods |
| **Size of the cells (Diameter, length, μm)** | 0.7, 1.8-2.6 | >1.2, 1.6-2.4 | 0.5-0.7, 1.5-3 |
| **Gram reaction** | Negative | Negative | Negative |
| Motility | Motile with peritrichous flagella | Motile with peritrichous flagella | Motile with single flagella |
| **Metabolic mode** | Obligate aerobic | Obligate aerobic | Aerobic |
| **Colony morphology** | Round, opaque, cream colored, non-pigmented | Round, opaque, greyish pink, non-pigmented | Large , smooth Elevated center "fried egg", |

| | | | |
|---|---|---|---|
| **Opt. Growth Temperature (°C)** | 30 | 30-35 | 37 |
| Catalase | Positive | Positive | Positive |
| **Granulation** | Granulated | Granulated | Non-granulated |
| **Nitrogen source** | Ammonium salts or nitrate | Ammonium salts or nitrate | Various |
| **Utilization of Fructose** | Positive | Positive | Positive |
| **Glucose** | Only mutants | Positive | Positive |
| **Maltose, Sucrose** | Negative | Positive | Negative |
| Glycerol | Negative | Positive | Positive |
| L-Serine | Negative | Positive | Positive |
| L-Threonine | Negative | Positive | Negative |
| L-Histidine | Positive | Negative | Positive |
| L-Phenylalanine | Positive | Negative | Positive |
| **Liquefaction of Gelatin** | Negative | Positive | Negative |

### 3.2. Growth curves :

The growth curves of the three microorganisms used in the production studies of PHA are presented in Figures 9,10 and 11. Spectrophotometric measurements for *R. eutropha* and *A. latus* were made at both 550 and 610 nm. For *P. aeruginosa* only 550 nm was employed.

### 3.2.1. Growth curve of *R. eutropha :*

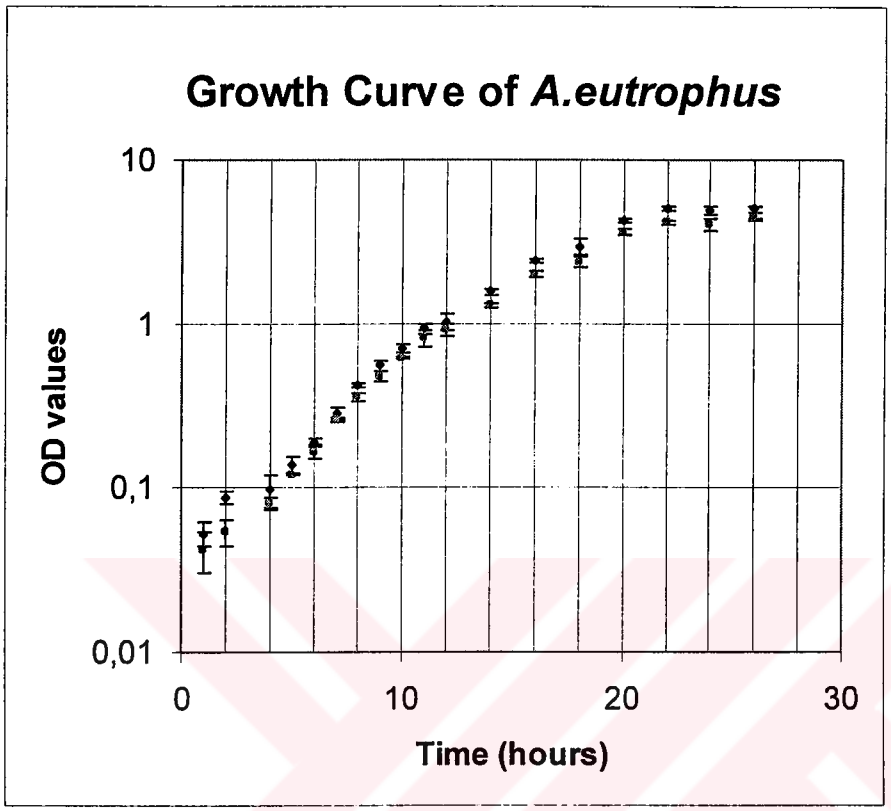



**Figure 9 :** Growth curve of *R. eutropha* at $OD_{550}$ ♦ and $OD_{610}$ •

As can be seen from the graph, the lag period of a typical *R. eutropha* culture lasts for 6-7 hours. The culture enters a sharp logarithmic phase (between $8^{th}$ and $20^{th}$ hours) where cell number greatly increases. As seen, the culture enters the stationary phase around at $22^{nd}$- $24^{th}$ hours, suggesting that the cells should be transferred to a starvation medium for enhanced polymer production.

### 3.2.2. Growth curve of *A. latus* :

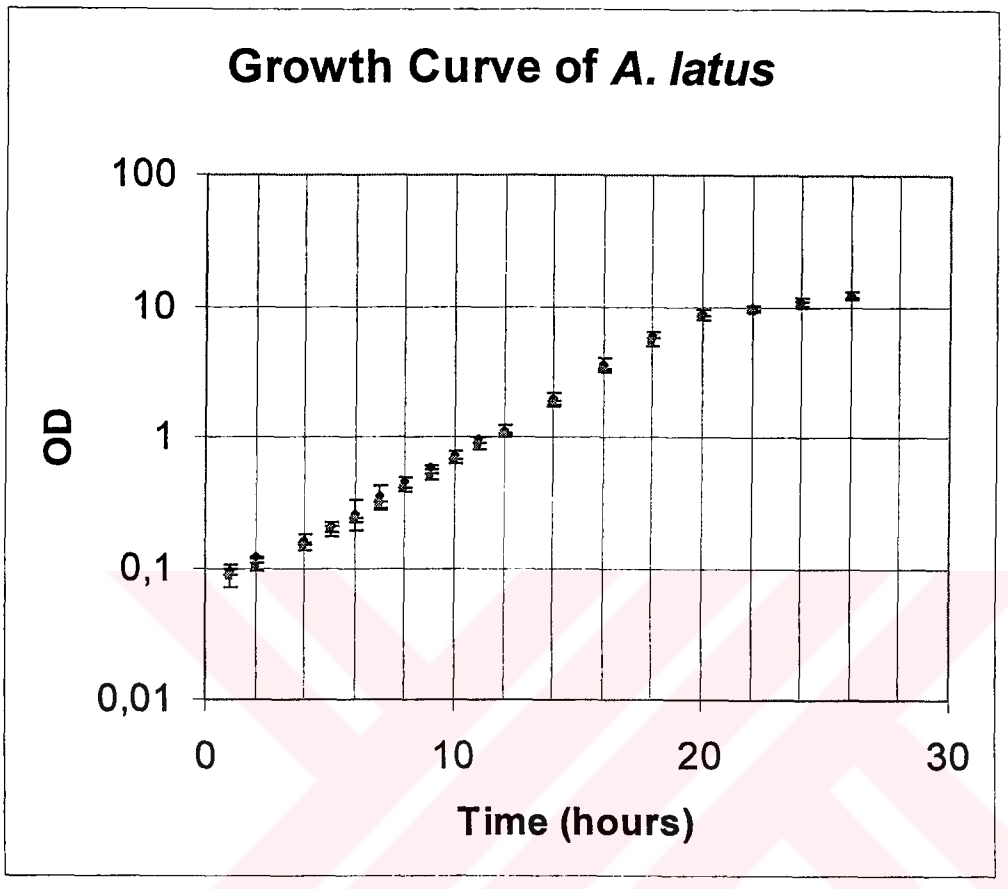



**Figure 10:** Growth curve of *A. latus* at $OD_{550}$ ♦ and $OD_{610}$ •

As seen in Figure 10 , *A. latus* had a longer lag period (generally lasting for 10 hours), probably because of supplemented sucrose as the carbon source. However after adapting to the conditions, *A. latus* culture showed a faster metobolic activity reaching higher concentrations of cells. The carbon source was depleted at the

19th hour. As *A. latus* cells were growth- associated producers, it was necessary to maintain the maximum number of cells with the highest polymer yield.

### 3.2.3. Growth curve for *P. aeruginosa* :

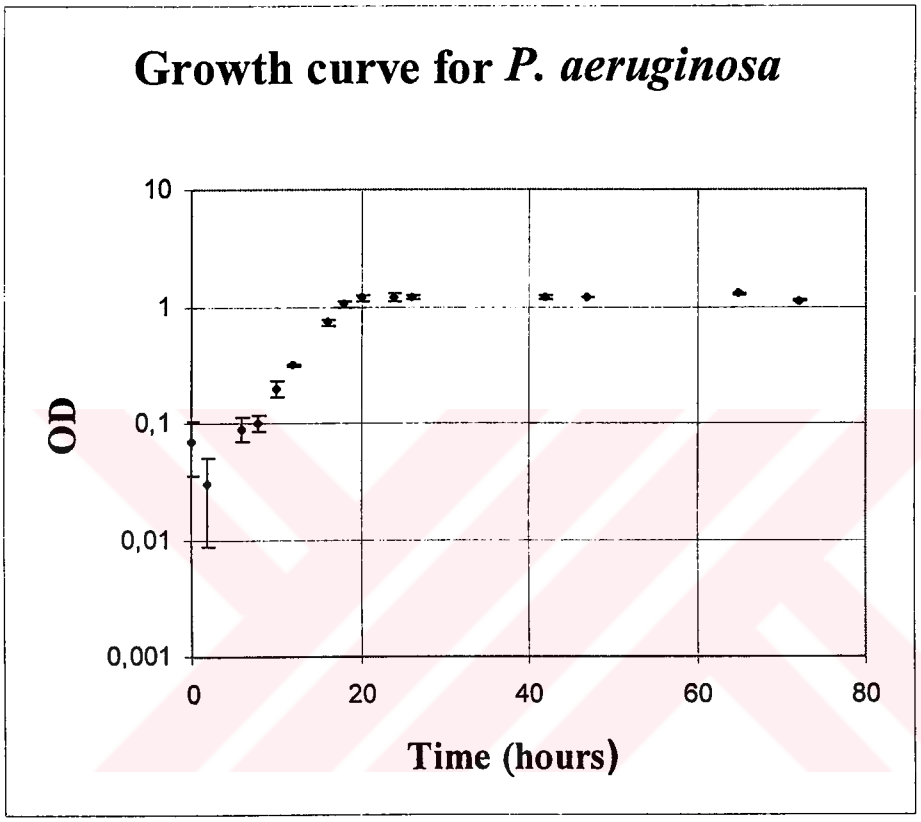



**Figure 11:** Growth curve of *P. aeruginosa* as measured at 550 nm.

Unlike the other two, growth curve of *P. aeruginosa* was constructed in a single study as a duplicate sampling, therefore the error bars sketched shows the standard deviation in this study. As seen in the figure above, the lag phase ended by

$10^{th}$ hour followed by the logarithmic phase lasting about a 10 hours. This phase sharply ended by $20^{th}$ hour as culture entered to a long (40 hour) stationary phase.

### 3.3 Construction of polymer deposition profiles :

Polymer deposition profiles for producer microorganisms, are shown below.

#### 3.3.1. Polymer deposition profile of *R. eutropha*

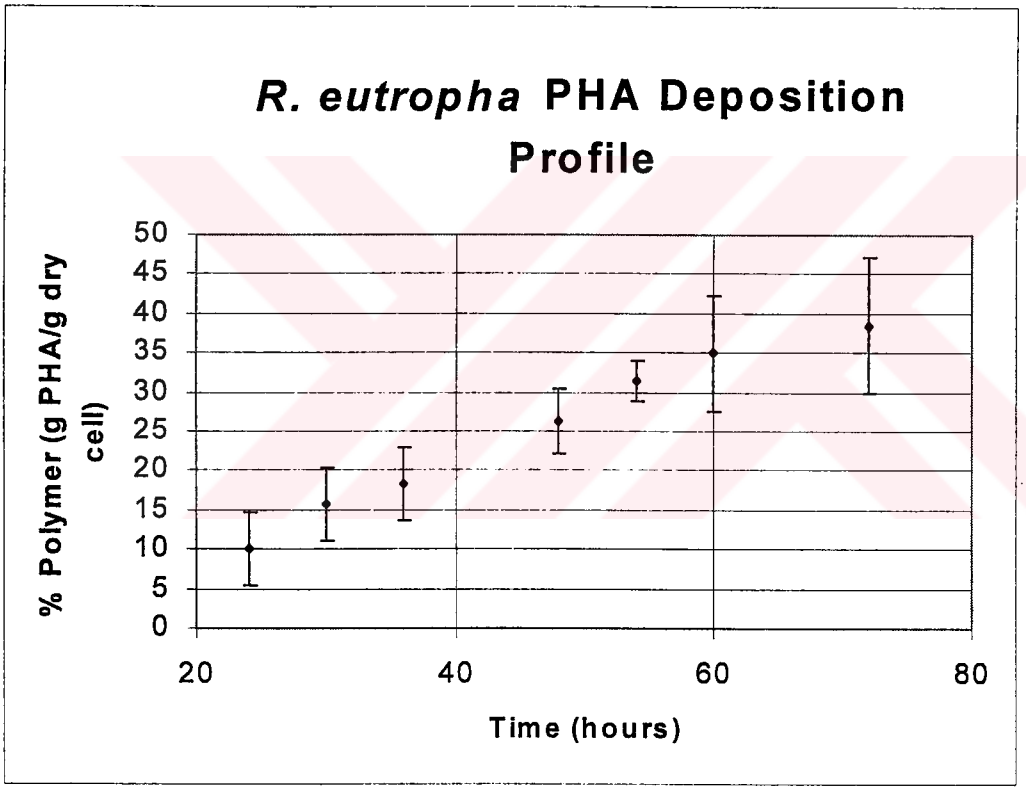



**Figure 12:** Polymer deposition profile of *R. eutropha* started after transfer to nutrient deficient medium (20th hour)

Deposition profile of *R. eutropha* showed a gradual increase in the polymer amount after it was transferred to nitrogen deficient medium. This graph summarises the several production experiments. Even though the growth conditions were standard, the reason for such a large deviation could be the method of extraction. Success of the experiments usually varies depending on the method and procedure.

The decrease of slope at the last two sampling hours denotes the probability of depolymerisation. In other words, if the culture time was further continued beyond $72^{nd}$ hour, cellular depolymerases can be used to consume the deposited polymers within the cell. This would significantly influence the recovery, so the harvest time should be carefully selected.

### 3.3.2. Polymer deposition profile of *A. latus* :

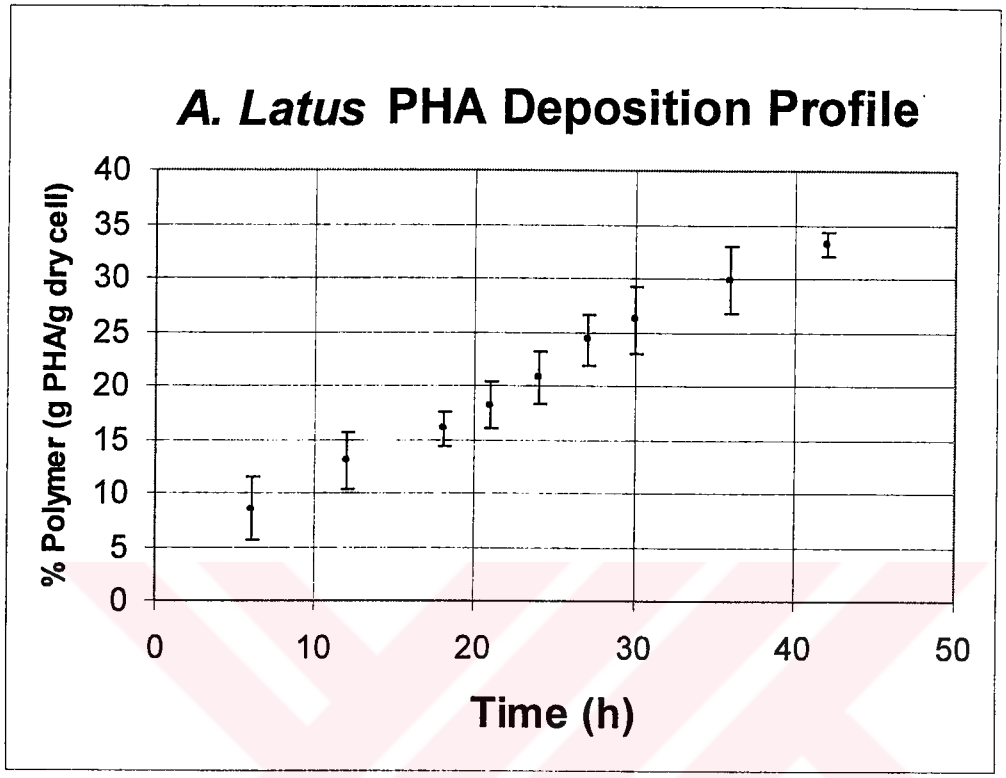



**Figure 13:** Profile of PHA deposition in *A. latus*.

In Figure 13 , polymer deposition profile of *A. latus*, in association with cell mass increase, can be seen. The first sample was taken 6 hours after inoculation, before the logarithmic phase The results indicated that 5-12% polymer production was already present at the initial stages of growth.The production gradually increased through the 42$^{nd}$ hour prior to cell harvest.

### 3.4. Detection of excitation and emission wavelengths of Nile red in ethanol:

Multispectral measurements and later the excitation and emission wavelength detection measurements were done by using 0.36 μg/ml of Nile red in ethanol. As seen below, the excitation and emission wavelengths were detected as 585.6 nm, and 624 nm respectively.

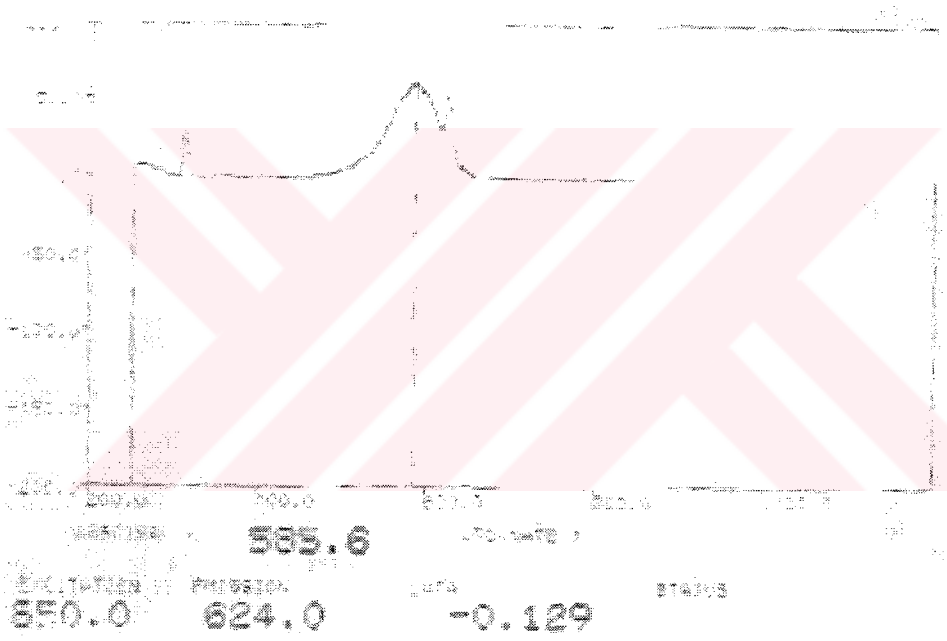



**Figure 14:** Excitation and emission wavelength detection of Nile Red in ethanol

### 3.5. Detection of excitation and emission wavelengths of Nile red in acetone

The methods used were the same as in the previous sections. . Due to high fluorescence, this time the concentration of the solution was 22 μg/ml with again using the low sensitivity. Excitation and emission wavelengths were found as 582.4 nm and 601.6 nm respectively.

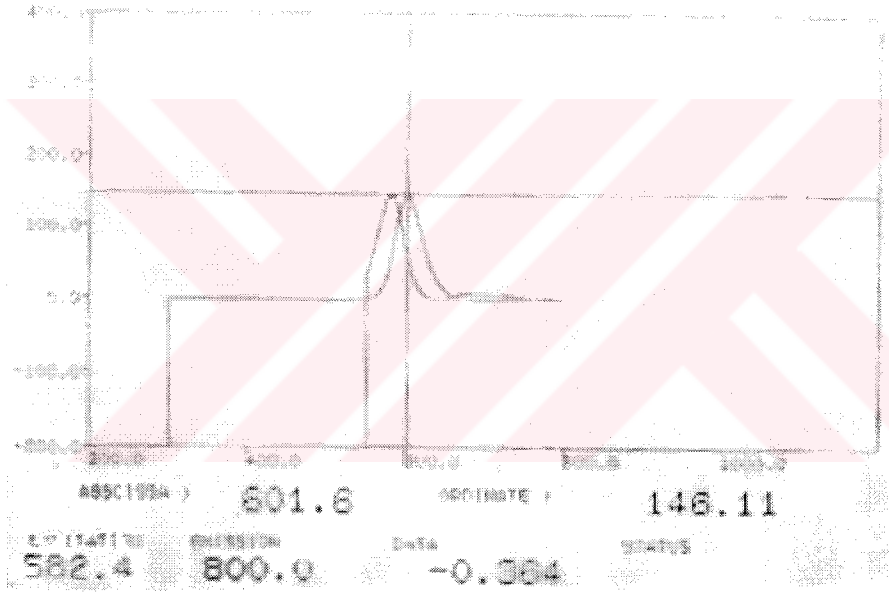



**Figure 15:** Excitation and emission wavelength detection of the Nile Red in acetone

### 3.6. Detection of excitation and emission wavelengths of Nile red in chloroform

To detect the excitation and emission wavelengths of Nile red in chloroform, a concentration of 0.2 ng/ml was used. When the usual procedures were applied, the excitation and emission wavelength were detected as 556.8 nm and 585.6 nm respectively. (Figures below). These sets of experiments clearly showed that the fluorescence properties of the same fluorophore can be greatly influenced by the solvent used for dissolution. It is interesting that both excitation and emisson wavelengths increased as the solvents changed in the following order; chloroform, acetone and ethanol. This relation was also valid for water miscibility and the polarity of the solvents.

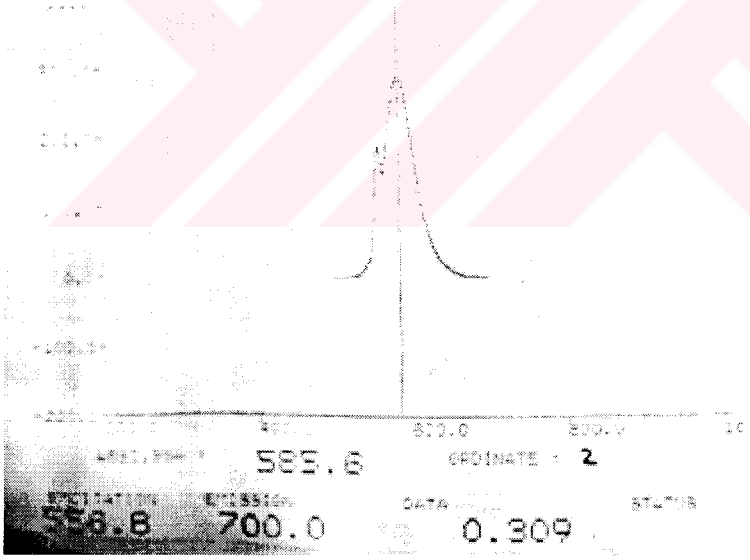



**Figure 16:** Excitation and emission wavelengths of Nile Red in chloroform

**3.7. Showing linearity of fluorescence increase with increasing Nile red concentration in ethanol:**

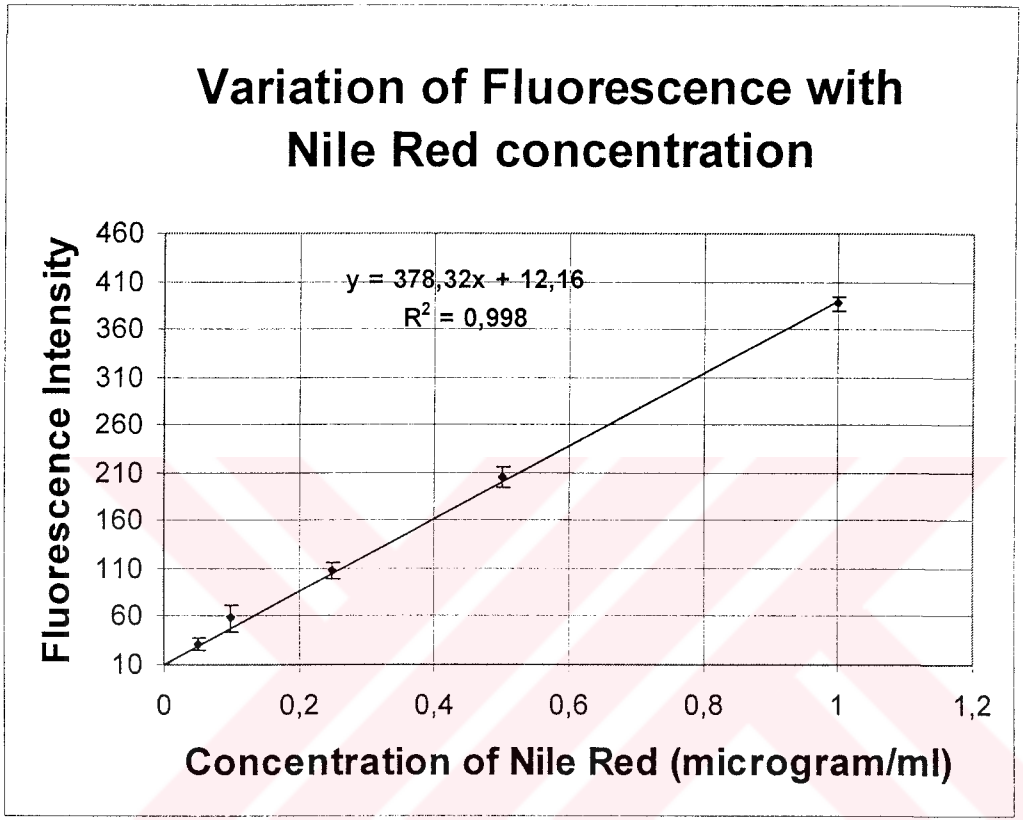



**Figure 17**: Fluorescence intensity vs Nile red concentration relation in ethanol

In this graph, it was shown that there was nearly a perfectly linear relationship between the concentration of the Nile red dissolved in ethanol and fluorescence intensity. The relationship was examined within the range of 0 to 1 μg/ml Nile Red that was later used in the staining experiments. The excitation and

emission wavelengths used in this graph is 585.6 nm and 624 nm respectively as detected before. Note that the fluorescence intensity measured in high sensitivity could reach up to 400 units.

### 3.8. Linearity of fluorescence intensity with incresing Nile red concentration in acetone :

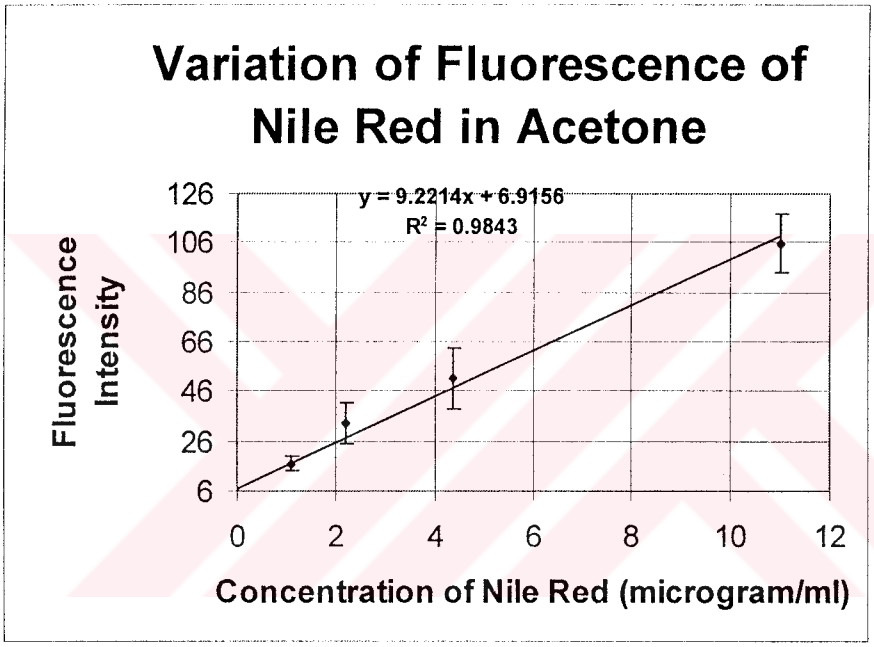



**Figure 18:** Fluorescence intensity vs Nile red concentration relation in acetone.

In this experiment, the solvent used was acetone. The excitation and emission wavelengths were adjusted to 582.4 nm and 601.6 nm respectively as detected earlier.

The concentrations used for this study was 0 to 11 μg/ml, where the error bars showed the standard deviations after several consequent measurements. The fluorescence and the solubility of Nile red was higher in acetone than in ethanol.

### 3.9. Linearity of fluorescence intensity with increasing Nile red concentration in chloroform :

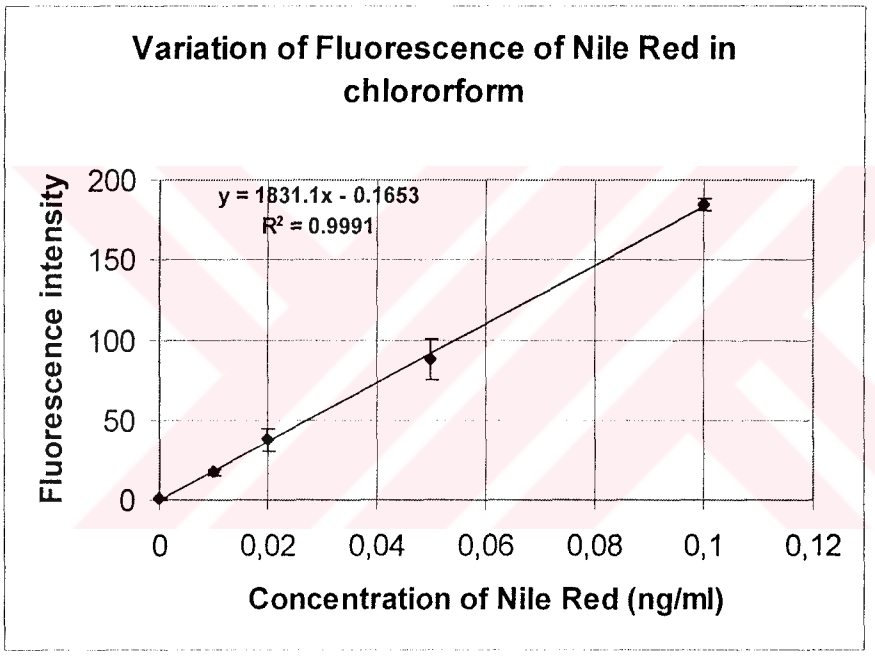



**Figure 19:** Fluorescence intensity vs Nile red concentration relation in chloroform.

A linear increase in the fluorescence intensity was followed in high sensitivity. However, this time concentrations were 1/10000 times less than ethanol, within the range of 0 to 0.1 ng/ml (0 to 1 μg/ml in ethanol). This clearly

indicated that the fluorescence of Nile red was greatly enhanced with less polar solvents. But our application on producer cells requires water-miscible and partially polar solvents, rather than chloroform for application in aqueous media and penetration through lipid bilayer cell membrane.

### 3.10. Examination of cells with fluorescence microscope:



**Figure 20:** Microphotograph of *R. eutropha* cells (400 X)

Note the typical morphology of *R. eutropha* cells in this phase contrast microphotograph. The cell size is considerably longer than the other cells which makes them easily distinguished under microscope.



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**Figure 21:** The same cells under UV (600X)

This microphotograph is targeted and magnified to note the obvious fluorescent orange colored polymer inclusions within *R. eutropha* cells. Cell membrane was also stained due to the activity of Nile red towards cell membrane lipids but it is far more lower than the fluorescence originating from the inclusions.

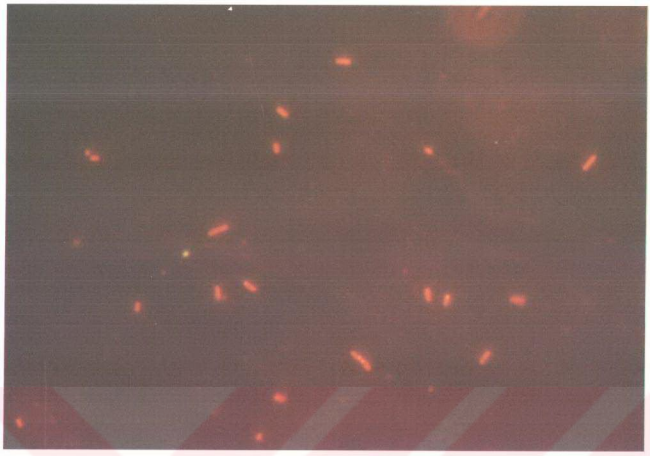


**Figure 22:** A detail of *P. aeruginosa* cells under UV (600 X)

Both the morphology and the presence of very tiny inclusions can be traced from this photograph. *P. aeruginosa* cells generally were smaller than both *A. latus* and *R. eutropha* cells in diameter. Note the presence of fluorescent bodies within cells in much lower sizes with respect to the cell size as compared the width of other microorganisms.

### 3.11. Construction of calibration curves :

#### 3.11.1. Calibration curve of Nile red with *R. eutropha*:

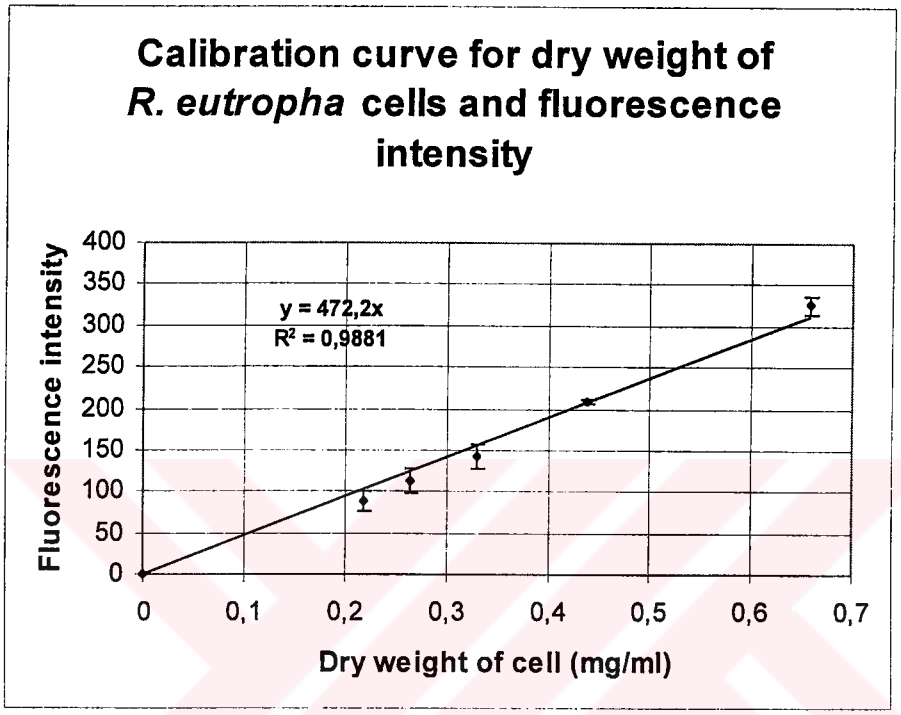



**Figure 23:** Calibration curve of *R. eutropha* ( fluorescence intensities vs dry cell weights)

This calibration curve shows us the fluorescence intensity of *R. eutropha* cells after staining with Nile red in ethanol. The concentration of cells increases to 0.65 mg/ml corresponding to the fluorescence intensity around 350 units which arises mainly from the inclusions within the cells. The fluorescence intensities can

easily be converted to the polymer amount as the polymer amount in unit mass of cells was known. The used concentrations are serial dilutions from a single cell source after optimal growth conditions, so the amount of polymers in all is assumed to be the same on cell basis. Error bars in the graph corresponds to standard deviations after several studies with the same method.

### 3.11.2. Calibration curve of Nile red with *A. latus* :

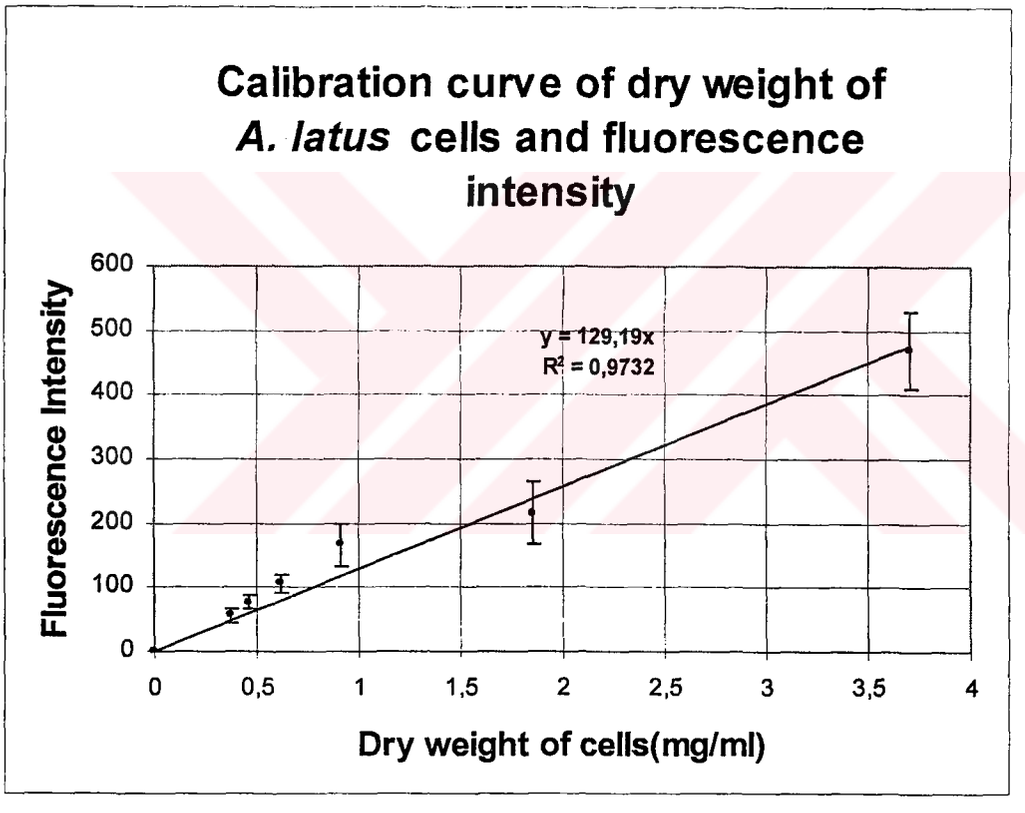



**Figure 24:** Calibration curve of *A. latus* cells dry weights and their fluorescence after staining.

Same calibration curve is sketched for *A. latus* cells within the cell concentration range of 0 to 3.7 mg/ml cells. Also this graph is sketched with serial dilutions of a single sample, so the polymer amount can be easily related to the cell weight. Standard deviations of several studies have been presented as error bars in the graph.

When we compare the fluorescence intensities of both the *R. eutropha* and *A. latus* cells, eventhough the cell dimensions were greately different it is seen that *R. eutropha* cells show 3.5 fold increase in fluorescence than *A.latus* with the same cell concentration (0.65 mg/ml).

Upon these findings a calibration curve of *A. latus* with a broader range of cell concentration was also constructed to observe whether the relationship was still valid.

### 3.11.3. Calibration curve of Nile red stained *P.aeruginosa* cells :

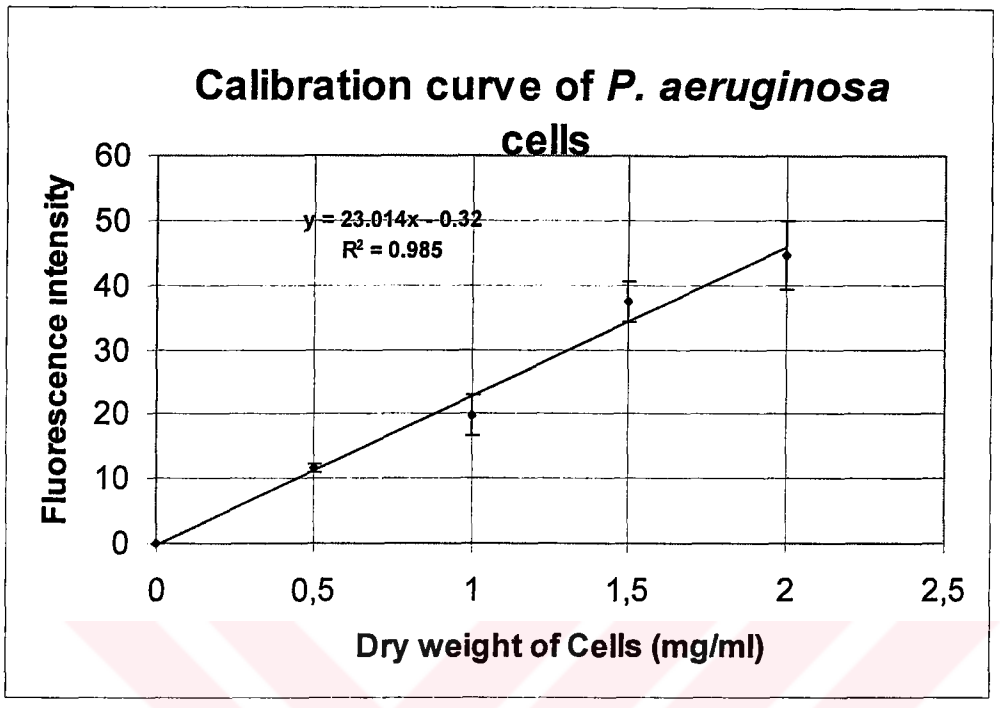



**Figure 25:** Calibration curve of *P. aeruginosa* cells after staining with Nile red

Calibration curve of *P. aeruginosa* cells shows us the relation of fluorescence intensity within the cell concentration in the range of 0 to 2 mg/ml. Standard deviations were given as error bars in the graph originating from two studies with *P. aeruginosa*. Relative fluorescence of *P. aeruginosa* cells were quite low with respect to previous two microorganisms producing scl PHA's. *P. aeruginosa* is known to deposit lower amounts of polymer with respect to its cell mass and also the polymer characteristics are quite different in mcl PHA's, as the

number of carbons in the main backbone of the polymer is more than six. This number is 1 to 5 in scl PHA's.

When the fluorescence of 0.65 mg/ml cell mass was compared with the other two microorganisms, it was found to be around 15 units. This intensity was 22 fold lower than *R. eutropha* and 6.7 fold lower than *A. latus*. However it was still higher than that of the *E. coli* cells.. This clearly indicated that the Nile red method was reliable even in low polymer concentrations. We were unable to extract polymer from *P. aeruginosa* probably due to its low concentration, but the polymer inclusions were visible in microphotographs.

### 3.11.4. Calibration curve of Nile red with E. coli

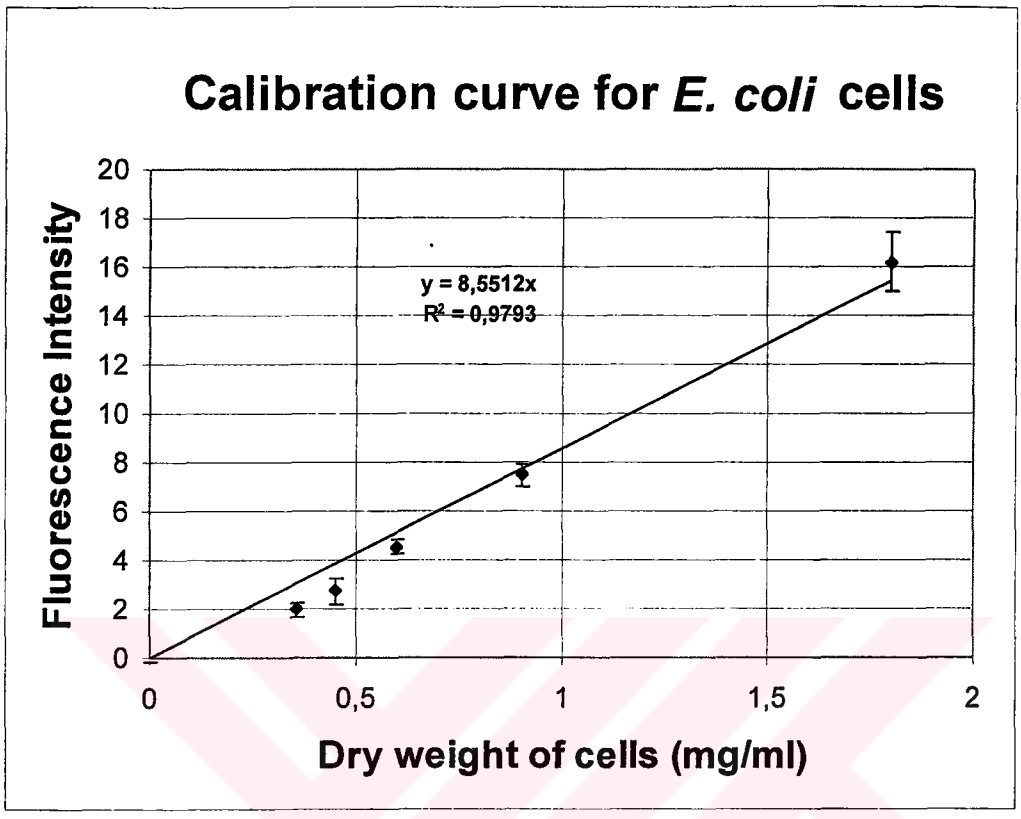



**Figure 26:** Calibration curve of *E. coli* cells after staining (control)

Calibration curve of Nile red stained *E. coli* cells is only sketched to serve as a control in the experiments since the organism is known as a non-producer of PHA's.

As seen, it was quite clear that the fluorescence measured might be due to the cell membrane lipids and other lipoid structures present in *E. coli* cells. As we compare the fluorescence intensity arising from 0.65 mg/ml cells as we do in the

others, we observe that it corresponds to a fluorescence about 4 units which is 4 times lower than *P. aeruginosa*, 25 times lower than *A. latus* and 82.5 fold lower than *R. eutropha* fluorescence in the same concentrations. Standard deviations of several studies concerning *E. coli* is given as error bars in the graph.

### 3.12. Calibration curves with Nile red in acetone:

#### 3.12.1 Calibration curve of *R. eutropha* with Nile red in acetone:

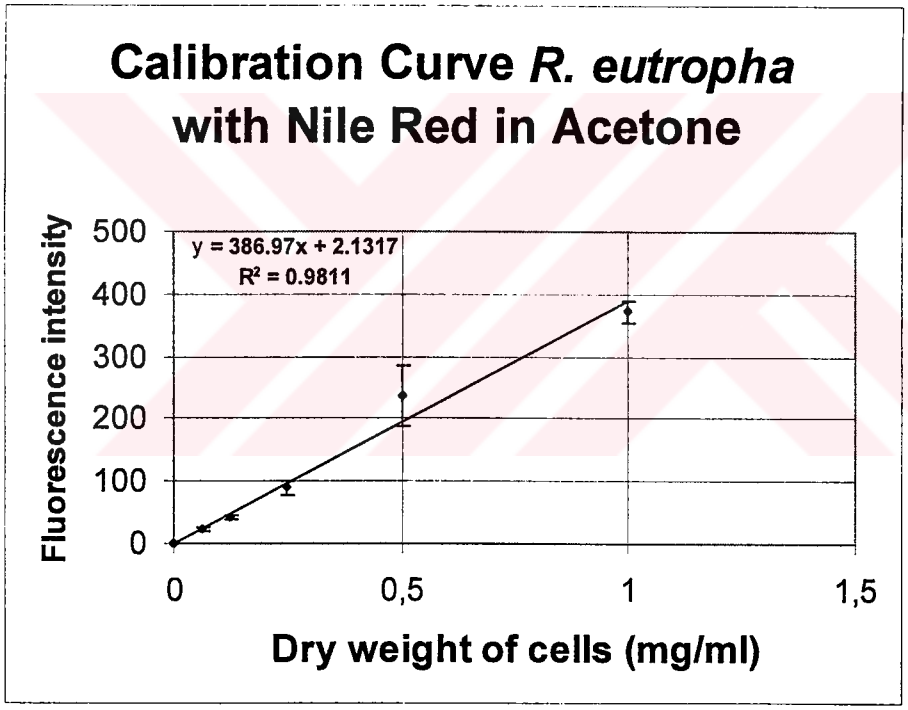



**Figure 27:** Calibration curve of *R. eutropha* cells after staining with Nile red in acetone.

For the observation of Nile red activity on the *R. eutropha* cells, a calibration curve was constructed , but this time using acetone as the solvent.. The scanned cell concentration ranged between 0 to 1 mg/ml. The error bars were used to show the standard deviations in the experiment.

Eventhough Nile red shows a higher fluorescence in acetone than in ethanol, 0.65 mg/ml of cells were coarsly corresponds to 250 units of fluorescence, which is 350 when ethanol is used as a solvent. This may have a few reasons;

First of all acetone is a much more effective solvent against lipid structures. This may cause cell disruption during the measurements which can effect the results. Secondly due to acetone's lower hydrophilicity than ethanol and better solubility of Nile red in acetone, this may cause a lower binding of the ionized Nile red molecules to the granular surfaces. Also the excitation and emission wavelengths of Nile red in acetone is different from the ones in ethanol, which is a counter effect for one to one correspondence of these two solvents.

Lets think when cells of *R. eutropha* were dissolved in an solution of Nile red in acetone. It was clear that the stain diffuses through cell membrane and attaches itself to the surface of PHA granules, both using its affinity and the activity of acetone during this process. But what happens when we remove the

excess stain and wash the cells with distilled water. The excess unbound stain is removed as acetone is diffused in water, and partial diffusion is also possible with the dye molecules absorbed on the surface of granules. But we know that Nile red quickly loses in fluorescence in water so this diffusion can hardly effect the fluorescence intensity.

### 3.12.2. Calibration curve of *P. Aeruginosa* with Acetone:

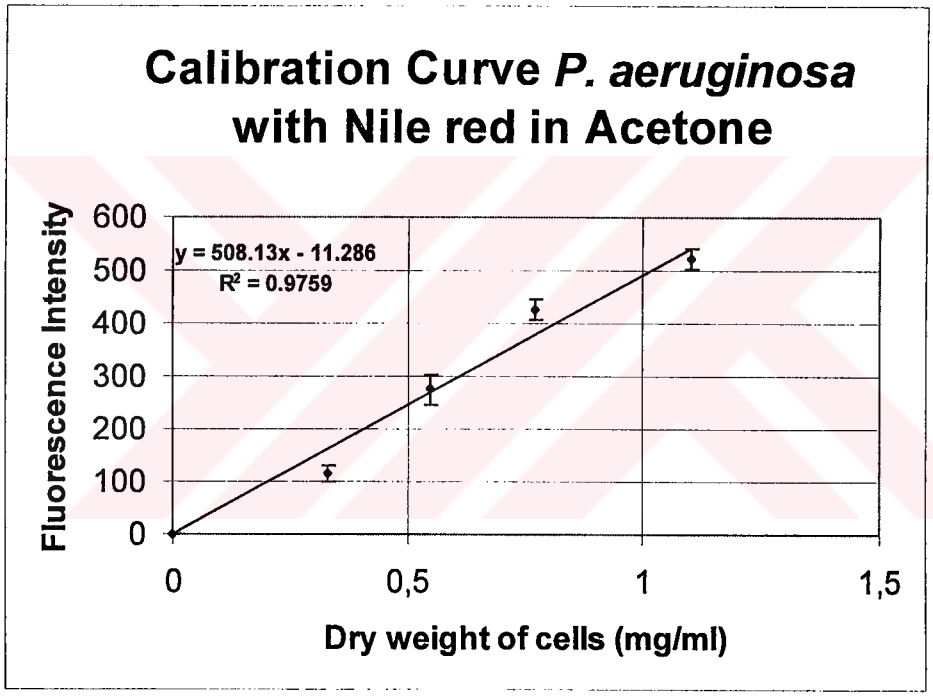



**Figure 28:** Calibration curve of *P. aeruginosa* cells after staining with Nile red in acetone

Interestingly, when Nile red in acetone was used in *P. aeruginosa* cells, there was a drastic increase in fluorescence. To understand this more clearly we should compare the values of 0.65 mg/ml of *P. aeruginosa* cells stained with Nile red in ethanol and in acetone. The former gives a fluorescence intensity around 15 units but in the latter it abruptly increases to an approximate value of 300 units. (20 fold increase) The reason can be several, but it was obvious in this study that the fluorescence was higher when Nile red was dissolved in acetone, eventhough this readings were done with an emulsion of cells in water, and acetone was used only in staining step of the cells. It is likely that the affinity of mcl PHA's towards Nile red dissolved in acetone was higher.

### 3.13. Use of colony staining for an attempt in isolation of new producers

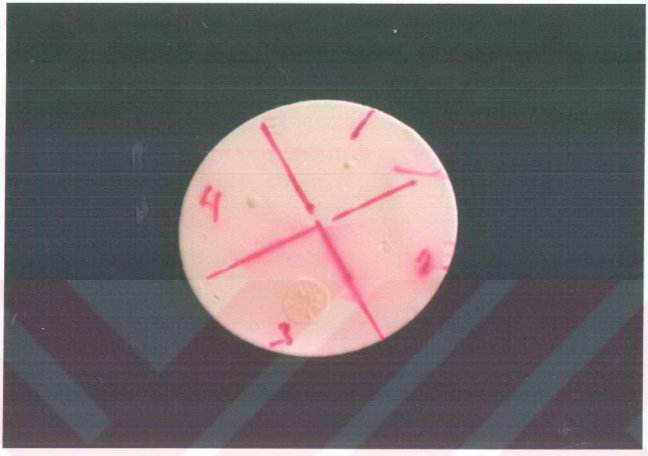



**Figure 29:** Cellulose nitrate membrane used for an attempt to isolate new producers 1-*A. latus* colony 2-*E.coli* colony 3-New strain A 4-New strain B Grown on minimal sucrose solid culture, before staining with Nile red, Actual dimensions



T.C. YÜKSEKÖĞRETİM KURULU
DOKÜMANTASYON MERKEZİ

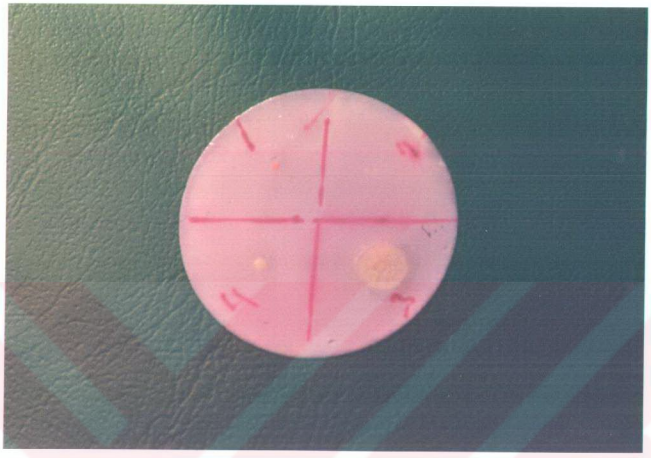


**Figure 30:**Cellulose nitrate membrane used for an attempt to isolate new producers 1-*A. latus* colony 2-*E.coli* colony 3-New strain A 4-New strain B Grown on minimal sucrose solid culture, after staining with Nile red, actual dimensions

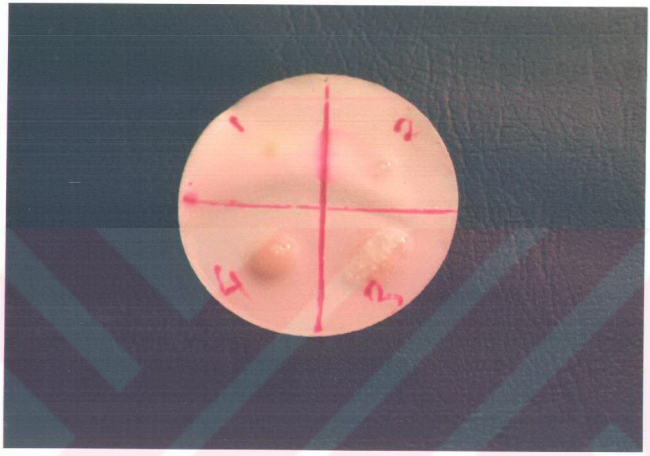


**Figure 31:** Polycarbonate membranes filters used for an attempt to isolate new producers 1-*R. eutropha* 2- *E.coli* V517 3- New strain A 4- New strain B Grown on nutrient rich medium and then transferred to solid production medium lacking nitrogen; before staining with Nile red; actual dimensions

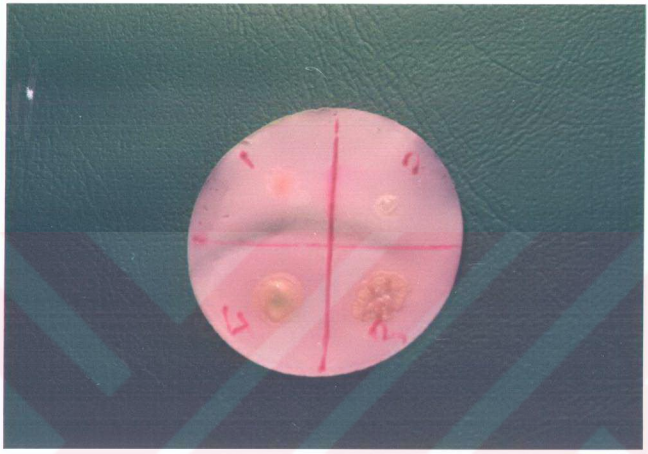


**Figure 32:** Polycarbonate membranes filters used for an attempt to isolate new producers 1-*R. eutropha* 2- *E.coli* V517 3- New strain A 4- New strain B Grown on nutrient rich medium and then transferred to solid production medium lacking nitrogen, after staining with Nile red, actual dimensions

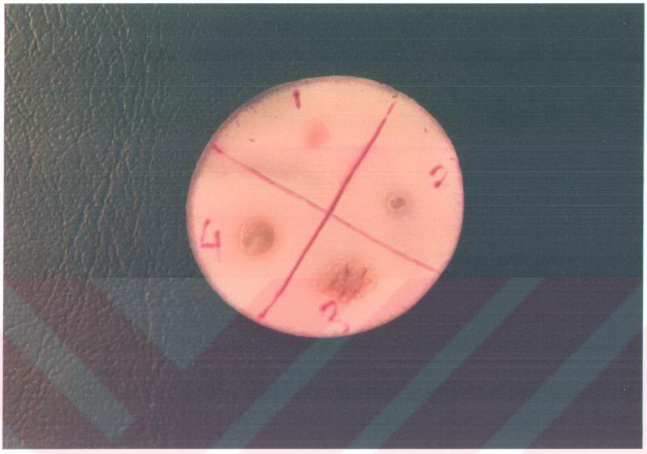


**Figure 33:** Polycarbonate membranes filters used for an attempt to isolate new producers 1-*R. eutropha* 2- *E.coli* V517 3- New strain A 4- New strain B Grown on nutrient rich medium and then transferred to solid production medium lacking nitrogen, after decolorization , actual dimensions

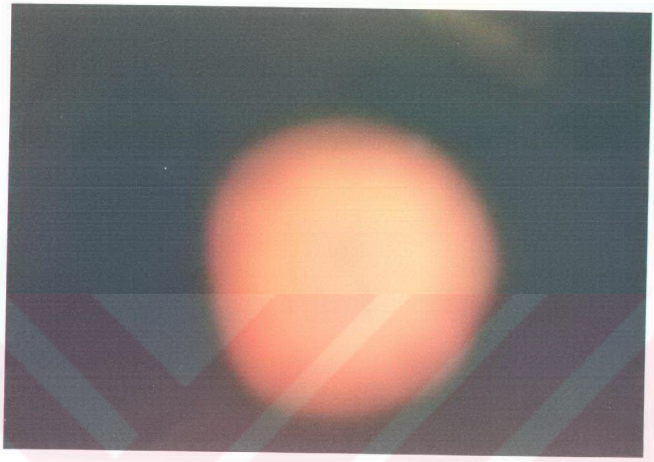


**Figure 34:** Photograph of a *R. eutropha* colony under UV light (40X)

### 3.14. Monitoring of Metabolism and polymer production.

#### 3.14.1. Monitoring of metabolism in *R. eutropha*:

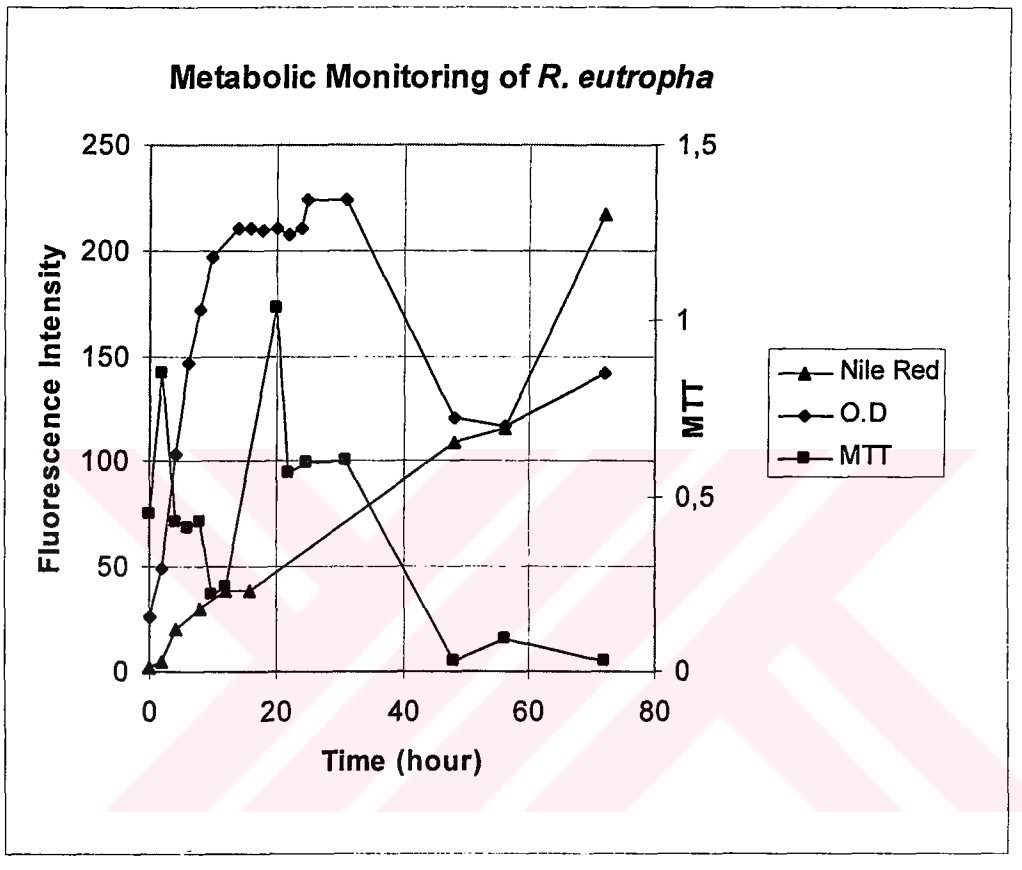



**Figure 35:** Metabolic monitoring of *R. eutropha*

Metabolic monitoring experiments were done in a single batch, simultaneously measuring three variables of $OD_{550}$, Nile Red fluorescence as a measure of polymer content and MTT reduction for monitoring of oxidative metabolism. The aim is to see the relationship between polymer production

(functioning as a pool for the reducing equivalents within the cells) and the oxidative metabolic activity of the cell. The latter can be measured due to its direct relationship with the MTT reduction. The relation, however, is also variable within the lag and log periods of growth, where cell mass in the unit volume increases with time.

This increase was obvious when we focused on the OD and Nile Red readings in the log phase of the growth. Here the OD and Nile red showed a one to one direct relationship, showing increase in the same structure between the 0 to 19 hours. This increase was the result of the fluorescence coming from the cell membrane rather than polymer deposition, as deduced from the shape of curves. During this period fluctuations in Nile red fluorescence were observed as the high metabolic rate of the cells during the logarithmic period was expected..

When the cells entered the stationary phase, the metabolic activity was stabilized on a level which was an indicator of stabilisation of the oxidative metabolism. However, Nile red fluorescence was showing a gradual increase, with the increased amount of polymer deposition that accelerated in this stage. This time, the fluorescence increase was solely depended on polymer deposition rather than fluorescence arising from cell membranes. This was further varified as no

more increase in $OD_{550}$ was observed, indicating no further increase in unit cell mass.

In the late stationary and death phase, the oxidative metabolism was at its lowest value as expected, and the rapid increase in Nile red fluorescence was observed, indicating high levels of polymer deposition.

### 3.14.2. Monitoring of Metabolism in *P. aeruginosa* :

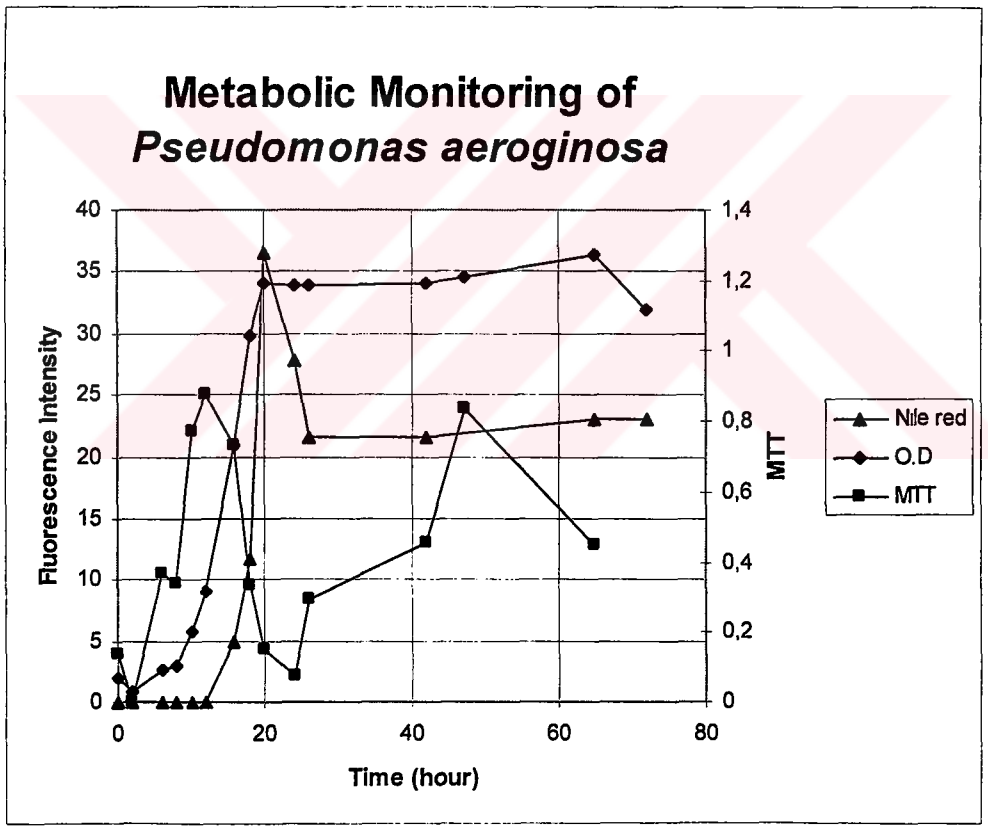



**Figure 36:** Metabolic monitoring of *P. aeruginosa*

*P. aeruginosa* seems to have a mixed metabolic activity. The fluorescence of *P. aeruginosa* cells measured after staining with Nile red was considerably lower than the other two producer microorganisms. This might be the basis of explanation for the fluorecence increase in the lag and log phases. This increase is both a sresult of cell membrane lipid amount increase originating from cell mass increase and less effectively the amount of polymer deposition. Metabolic activity measured with MTT is fluctuating, either because the local changes in the activities of the cells in log phase or due to some experimental errors.

This metabolic activity shows a rapid decrease, unlike to its expected behaviour with the enterance of the culture to the stationary phase. Another unexpected thing is the decrease of the Nile red fluorescence in the stationary phase. This cannot be explained in the decrease of cell mass as OD 550 is still stationary, without any decrease. Possible explanation is the decrease of polymer amount within the cells, may be due to depolymerisation. I should also note here the increase of MTT activity, hence the metabolic activity, when polymer amount is decreasing. This can be another evidence of the depolymerisation of the polymers.

The stationary phase continues until the 65 $^{th}$ hour where no unexpected changes is observed in absorbance and Nile red fluorescence. The fluctuations seen in MTT reduction in these stages needs more variables to be better understood as an explanation only depending on this variable is not very satisfactory.

# CHAPTER 4
# CONCLUSION

Nile red was used to monitor the polymer amount within producer microorganisms *R. eutropha* , *A. latus* (scl PHA producers), *P. aeruginosa* (mcl PHA producer) and *E. coli* (non-producer control).

- *R. eutropha* is a larger microorganism with respect to others, it has a faster metabolic rate which can be seen in growth curve and later during the metabolic monitoring.
- The polymers produced by *A. latus* and *R. eutropha* can easily be extracted by using the proper techniques that we optimise.
- Nile red is a proper fluorescent probe due to its desirable properties that we observe. It is non-toxic to the cells , can easily penetrate through cell membrane and well adhered to the polymer surface, and strongly fluoresce when bound. Therefore it is simple , easy to use and convenient.
- Solubility of Nile red is better in acetone and chroroform that in ethanol, so it can be more feasible to use acetone as a solvent as chloroform is immiscible with water, which makes it unsuitable for studying living samples.
- Nile red is both suitable for scl and mcl PHA's as our studies with *P. aeruginosa* demonstrate.However, further studies were needed for optimising this method to mcl PHA producers.
- Low amount of background fluorescence is possible due to binding of Nile red to the cell membrane lipids as can be observed with *E. coli*. However this is not a disadvantage for our method of quantification.
- Polymer quantification with Nile red fluorescence is in agreement with polymer extraction and gravimetric detection of polymer content.

- Colony screening can be feasibly used for isolation of new polymer producer strains from the nature, as our studies show and a few recently published studies were mentioning.
- Polymer produced by microorganisms seem to act as a sink for reducing equivalents , when MTT reduction is correlated with polymer production which is followed by us with the help of Nile red fluorescence during the metabolic monitoring studies.
- It will be better if we can use *pha*$^-$ mutants of producer microorganisms rather than a microorganism belonging to another genus as a control.
- As a conslusion, Nile red fluorescence is a measure for polymer amount within cells when appropriate calibration curves were used, and it is a strong indicator when cells were examined under fluorescent microscope.

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# APPENDICES:

# APPENDIX A

# CHEMICALS AND SUPPLIERS

| Chemical | Supplier |
|---|---|
| $Na_2HPO_4.7H_2O$ | Merck |
| $MgSO_4.7H_2O$ | Merck |
| $KH_2PO_4$ | Merck |
| KOH | Sigma |
| NaOH | Sigma |
| $(NH_4)_2SO_4$ | Carlo Erba |
| $H_3BO_3$ | Sigma |
| $CoCl_2.6H_20$ | Sigma |
| $ZnSO_4.7H_2O$ | Sigma |
| $MnCl_2.4H_2O$ | Merck |
| $NaMoO_4.2H_2O$ | Merck |
| $NiSO_4.7H_2O$ | Sigma |
| $CuSO_4.5H_2O$ | Merck |
| $Fe(III)NH_3$.citrate | Merck |
| $CaCl_2.2H_2O$ | Merck |
| $NiCl_2.6H_2O$ | Sigma |
| NaCl | Carlo Erba |
| Bacto Agar | Difco |
| Yeast extract | Oxoid, ADSA Micro |
| Meat extract | ADSA Micro |
| Nutrient broth | Difco |
| Bacteriological Peptone | Difco |
| Bacteriological Agar | Difco |
| Nutrient agar | Difco |
| D(+) Glucose | Sigma |
| D(-) Fructose | Sigma |
| Sucrose (Saccharose) | Difco |

| | |
|---|---|
| Methylene Blue | BDH Chemicals |
| Safranin | Fisher Scientific |
| Sudan Black B | Sigma |
| Nile Red | Sigma |
| Xylene | Merck |
| Acetone | Kimetsan |
| Methanol | Merck |
| Ethanol | Tekel |
| Chloroform | Meck |
| HCL | Merck |
| $H_2SO_4$ | Merck |
| Isopropyl alcohol | Atabay Kimya |
| Iodine | BDH Chemicals |
| Crystal violet | Sigma |
| Parafilm | American National Can |
| Gelatin | Merck |
| Lithmus milk | Difco |
| Sodium hypochloride | Birka Kimya |

# APPENDIX B

# MEDIA COMPOSITIONS AND PREPERATION

**Minimal Sucrose medium:**

**I-Minimal Base solution :**

| | |
|---|---|
| $KH_2PO_4$ | 1.5 g/l |
| $MgSO_4.7H_2O$ | 0.2 g/l |
| $(NH_4)_2SO_4$ | 2.0 g/l |
| $Na_2HPO_4.7H_2O$ | 6.7 g/l |

These chemicals were weighed and dissolved in distilled water in the given order. Before addition of the following chemical, total solution of the former was assured. Finally pH was adjusted to 7.2 with 1M KOH. Sterilization was done with an autoclave at 121°C at 1 atm pressure for 15 minutes.

**II-Iron-Calcium cocktail :**

| | |
|---|---|
| $Fe(III)NH_3$-citrate | 0.05 g/l |
| $CaCl_2.2H_2O$ | 0.01 g/l |

Chemicals were weighed and dissolved in distilled water. pH was adjusted to 5.5 and it was filter sterilised. This solution waskept as stock in a dark bottle at 4°C.

**III-Trace element solution :**

| | |
|---|---|
| $H_3BO_3$ | 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 |
| $NaMoO_4.2H_2O$ | 0.03 g/l |
| $NiSO_4.7H_2O$ | 0.028 g/l |
| $CuSO_4.5H_2O$ | 0.01 g/l |

All the chemicals were dissolved in distilled water in the following order. The solution's pH was adjusted to 5.5 and it was filter sterilised.It was kept cold and dark.

**IV- Carbon source :**

| | |
|---|---|
| Sucrose | 0.5 g/ml |

It was dissolved in distilled water and filter sterilised. Stock was kept cold at 4°C.

For the preperation of minimal sucrose solution , to the minimal base solution
1 ml/l iron-calcium cocktail, 1ml/l trace element solution and 20g/l sucrose solution was added asceptically. If minimal sucrose agar was desired, 15 g/l bacteriological agar was added before autoclaving and poured to petri plates before solidification after sterilization.

***R. eutropha* Inoculation medium :**

| | |
|---|---|
| Bacteriological peptone | 10 g/l |
| Yeast extract | 10 g/l |
| Meat extract | 10 g/l |
| Fructose | 20 g/l |

All microbiological ingredients were dissolved in distilled water and their pH wasadjusted to 7.2. Only fructose was prepared as a stock solution with concentration 0.5 g/ml filter sterilized and kept cold. After sterilization of microbiological media , fructose was added aseptically in the given concentration.

**Growth medium :**

| | |
|---|---|
| Bacteriological peptone | 10g/l |
| Yeast extract | 10 g/l |
| Meat extract | 5 g/l |
| $(NH_4)_2SO_4$ | 5 g/l |
| Fructose | 20g/l |

All microbiological ingredients were dissolved in distilled water and their pH wasadjusted to 7.2. The solution was steam sterilised thereafter. As stated before. Fructose was added aseptically after sterilization from the stock solution in the given concentration.

**Production medium (nitrogen free) :**

| | |
|---|---|
| $KH_2SO_4$ | 2.65 g/l |

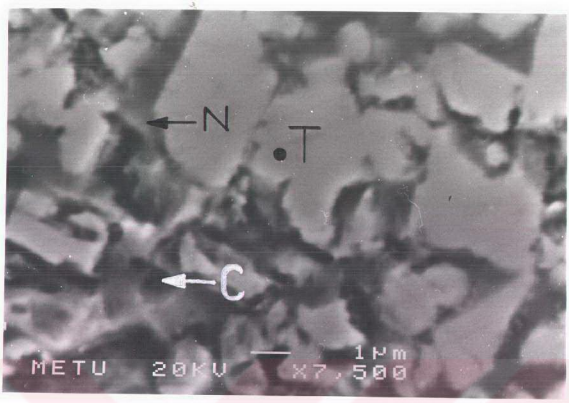



**Figure 41** Microstructure of WC-10%Co alloy sintered at 1420°C for 1 hr ( In the figure, T represent the recrystallized WC grain, N refers to the binder and C illustrates the closed porosity)

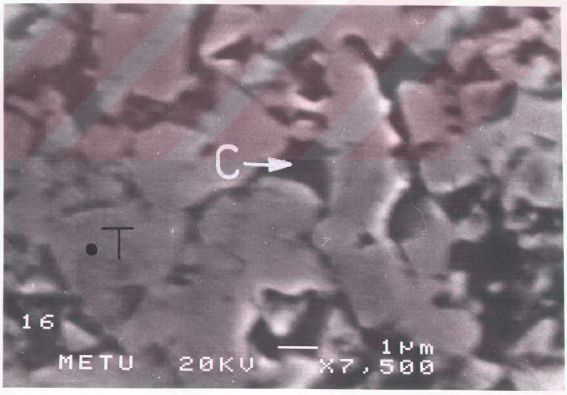



**Figure 42** Microstructure of WC-10%(Fe/Ni : 6/4) alloy sintered at 1420°C for 1 hr (In the figure, T represent the recrystallized WC grain while C refers to the closed porosity)



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