Biodegradable plastics are one of the most innovative materials being developed in the packaging industry. How widespread biodegradable plastics will be used all depends on how strongly society embraces and believes in environmental preservation. There certainly are an abundant amount of materials and resources to create and fund more uses for biodegradable plastics. The advancement of biodegradable technology has skyrocketed in recent years and there are growing signs that the public shows a high amount of curiosity in the product. With the variety of biodegradable plastics available in the near future, there will be a place for them current age of plastics. Bioplastics are non-toxic, biocompatible, biodegradable that can be produced from renewable resources. These features make them highly competitive with polypropylene, the petrochemical-derived plastic. The aim of this work was isolation, identification of bacterial isolates and testing their for bioplastic production. Factors affecting bioplastic production by the active bacteria and some physical properties of bioplastic were studied.

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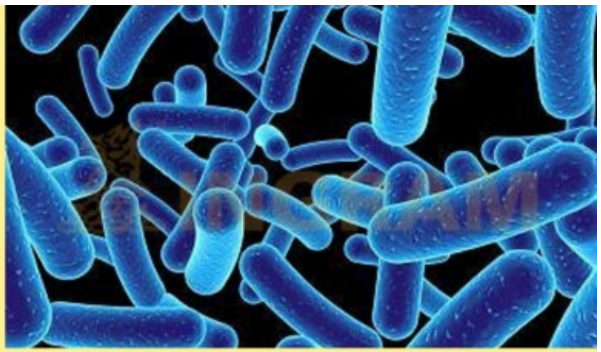
Production of biodegradable plastic



Sherif El-Kadi

Bioplastic production from inexpensive sources

Bacterial biosynthesis, Cultivation system, Production and Biodegradability

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INTRODUCTION

Past ages of human society have been called the Stone, Bronze, Copper, Iron, and Steel Ages, based on the material that was relied upon the most during that time. Today, the total volume of plastics produced worldwide has surpassed that of steel and continues to increase. Without a doubt, We have entered the Age of Plastics. Although plastics as we know them today are a relatively recent invention, they have become an important part of modern life and are here to stay **(Du and Yu 2002b)** .

The problem of environmental pollution caused by indiscriminate dumping of plastic wastes has assumed global proportions. These conventional plastics that are synthetically derived from petroleum are not readily biodegradable, It is considered as environmentally harmful wastes. In the search of environmentally friendly materials to substitute for conventional plastics, different biodegradable plastics have been developed either by incorporating natural polymers into conventional plastics formulations, by chemical synthesis, or by microbial

fermentations. However, physical limitations of these materials still exist **(Kahar *et al.*, 2004).**

Among the variety of biodegradable plastics a family of more than 40 poly-hydroxy alkanoates (PHAs) and their co-polymeric derivatives has emerged as very attractive materials due to their complete biodegradability. A number of bacteria accumulate these polymers or co-polymers as an intracellular carbon reserve when unfavorable environmental and nutritional conditions are encountered. Poly-b-hydroxybutyrate (PHB) is a microbial polyester produced by many bacteria and stored in cells in the form of granules. It is a candidate for the synthesis of environmentally benign, biodegradable plastics. Much efforts has been spent in optimizing the poly-β-hydroxybutyrate (PHB) production using pure substrates and pure cultures. But the product (PHB) cost is still around ten times higher than that of conventional plastics **(Wang & Lee, 1997).**

PHB can be completely degraded to water and carbon dioxide under aerobic conditions and to methane under anaerobic conditions by microorganisms in soil, sea, lake water and sewage **(Lee, 1996).** The main factor preventing the large-scale production and

commercialization of PHB is their high cost of production as compared with that of plastics based on petrochemicals. One of the major factors adding to the cost of PHB is the cost of substrates used for production. Therefore, less expensive substrates, improved cultivation strategies and easier downstream processing methods are required for reducing the cost . Thus, utilization of media containing cheaper carbon and nitrogen sources should be used to reduce the production costs of PHB **(Ahn *et al*., 2000)**.

PHB are non-toxic, biocompatible, biodegradable thermoplastics that can be produced from renewable resources. They have a high degree of polymerization, are highly crystalline and insoluble in water. These features make them highly competitive with polypropylene, the petrochemical-derived plastic **(Reddy *et al*., 2003).**

The aim of this work was isolation, identification of some local bacterial isolates and testing their for PHB production . Also, factors affecting PHB production by the active microorganism and some physical properties of PHB were studied .

REVIEW OF LITERATURE

What is plastic ?

A material can be called a plastic if it satisfies three conditions: it's main ingredient must be a polymer material, it must be fluid at some point during processing (usually processed using heat), and it must be solid in its final form. Plastics can be made up of many different kinds of polymer, and can be processed in many different ways, but as long as they satisfy these three conditions, they are bona fide plastics.

What is polymer ?

The main ingredient of any plastic is a polymer, a type of molecule that takes the form of a long chain . The word polymer comes from two Greek words, **poly** meaning many and **mer** meaning parts. So, as the name implies, polymers are made of many parts, called monomers or monomeric units, that are chained together. Polymers can come in different shapes. For example, microwaveable food containers and Dacron carpets are made of linear polymers. Soft and flexible shampoo bottle and milk jugs

are generally made using branched polymers. Car tires and bowling balls, on the other hand, are composed of cross-linked polymers. All of these polymer types are long and flexible molecules, so they can wind together and tangle like spaghetti on a plate. Some polymers are synthetically produced, such as nylon and polyester, while others can be found in nature : silk, hair, natural rubber, and even starch are examples of polymers . In principle, any of these polymers could be used to produce plastics; in practice, however, over 90% of all plastics are made from just five polymers, all of which are synthetic. The initial unprocessed mass of polymer, called resin, is processed into different shapes using a variety of methods, including: extrusion, injection molding, compression molding, transfer molding, and casting. Different processing techniques result in the wide variety of forms that plastic can take: ranging from thin films and elastic sheets, to resilient panels and hard, solid three-dimensional shapes **(El-Kady, 2008)**.

The plastics today :

Plastics were first invented in 1860. Plastics are made from natural resources, *e. g*., crude oil, natural gas and coal. Plastics are utilized in almost every

manufacturing industry ranging from automobiles to medicine. Plastic is not biodegradable and will persist in the environment for hundreds of years. Plastic is also lightweight and moisture resistant which means it can float easily in air and water, often traveling long distances. Plastics are very much advantageous because as synthetic polymers, their structure can be chemically manipulated to have a wide range of strengths and shapes. They have molecular weights ranging from 50,000 to 1,000,000 Dalton . The per capita consumption of plastics in the USA is 80, 60 Kg in the European countries and 2 Kg in India (**Reddy *et al*., 2003).**

Polyethylenes, polyvinyl and polystyrene are largely used in the manufacture of plastic. More than 200 million tones of plastic is produced every year (2005). And 300 million tones in (2010). Non-degradable plastics accumulate at the rate of 25 million tons per year. Plastics have been an environmental trepidation because of the lack of degradation. Plastics make up about 20% by volume waste per year. There are over 21,000 plastics facilities in the US, and the employment rate has increased by an average of three percent over the past two and a half decades. Since plastics are vital to people's everyday lives,

production of biodegradable plastics to make plastics more compatible with the environment is necessary**.**

Plastics are used because they are easy to make, cheap and they can last for a long time. Unfortunately theses useful qualities can make plastic a huge pollution problem, because plastic does not decompose, and requires high-energy ultraviolet light to break down, the amount of plastic waste in oceans is steadily increasing **(Sheu *et al*., 2000;Gouda *et al*., 2001 and Reddy *et al*., 2003).**

The problem of environmental pollution caused by indiscriminate dumping of plastic waste has assumed global proportions. These conventional plastics that are synthetically derived from petroleum are not readily biodegradable **(Kumar *et al*., 2004).** Harmful chemicals like hydrogen chloride and hydrogen cyanide are released during incineration. Recycling also presents some major disadvantages, as it is difficult sorting the wide variety of plastics and there are also changes in the plastics material such that its further application range is limited. Replacement of non-biodegradable by degradable plastics of major interest both to decision-makers and the plastic industry. Making eco-friendly products such as bioplastics

is one such reality that can help us overcome (**Reddy *et al*., 2003).**

The chemicals and processes used to create certain plastics can be highly toxic, affecting not only our groundwater and the air we breathe, but also leaching (slowly dissolving or migrating) into packaged foods, bottled water, and even our skin upon handling them. Several harmful synthetic organic compounds were presented in plastics such as, polystyrene, styrene butadiene copolymers, acrylonitrile, polychlorinated biphenyls, acetyl biburyl citrate, styrene oxide, styrene phenyl benzene, di(2-ethylhexyl) adipate, diisodecyl phthalate, diisononyl phthalate, (5)di-(2-ethylhexyl)phthalate, bisphenol A & Bisphenol B, diallyl phthalate, dioctyl phthalate and butyl benzyl phthalate **(Colon *et al*., 2000; Schecter, 2001; Warner *et al*., 2002 and El-Kady, 2008) .**

The major environmental source of dioxins is incineration because of some countries burn their plastic waste. Dioxins are a group of synthetic organic chemicals that contain 210 structurally related individual chlorinated dibenzo-p-dioxins (CDDs) and chlorinated dibenzofurans (CDFs). The term “dioxins” will refer to the aggregate of all CDDs and CDFs. Not all dioxins have the same toxicity

or ability to cause illness and adverse health effects. Dioxin exposure is associated with a wide array of adverse health effects in experimental animals, including death. Experimental animal studies have shown toxic effects to the liver, gastrointestinal system, blood, skin, endocrine system, immune system, nervous system, and reproductive system **(Colon *et al*., 2000; Schecter, 2001 and Warner *et al*., 2002).**

The environment-friendly materials :

There are at least three factors that affect how environment-friendly a material is:

1- **renewability**: how quickly are the ingredients that go into making the plastic created in the environment? (A material that is made from soybeans, for example, is more environmentally friendly than one made from wood, because nature can produce soybeans faster than it can produce trees.

2- **degradability:** how quickly can the plastic be re-integrated into the environment after it is no longer being used?

3- **production:** how much pollution or waste is created during the process of actually making the plastic?

Traditional plastics fail on all three of these points .

To minimize plastic pollution, some countries burn their plastic waste to generate steam, hot water or electricity, but there are some dangerous gases released. Plastic incineration generate toxic emissions such as carbon dioxide and methane. These greenhouse gases contribute to worldwide climate change. **(Khodair, 2003)**.

The three types of biodegradable plastics introduced are photodegradable, semi-biodegradable, and completely biodegradable. Photodegradable plastics have light sensitive groups incorporated directly into the backbone of the polymer as additives. Extensive ultraviolet radiation (several weeks to months) can disintegrate their polymeric structure rendering them open to further bacterial degradation. However, landfills lack sunlight and thus they remain non-degraded. Semi-biodegradable plastics are the starch-linked plastics where starch is incorporated to hold together short fragments of polyethylene. The idea behind starch-linked plastics is that once discarded into landfills, bacteria in the soil will attack the starch and release polymer fragments that can be degraded by other bacteria. Bacteria indeed attack the starch but are turned off by the polyethylene fragments, which thereby remain non-degradable. The third type of biodegradable plastics rather

new and promising because of its actual utilization by bacteria to form a biopolymer. Included are PHA, polylactides (PLA), aliphatic polyesters, polysaccharides, copolymers and/or blends of the above (**Reddy *et al*., 2003).**

Among the candidates for biodegradable plastic, PHAs have been drawing much attention because of their similar properties to conventional plastics and complete biodegradability **(Hong *et al*., 2003)**. PHB is degraded by specific hydrolyzing enzymes, extracellular and intracellular PHB depolymerases (EC 3.1.1.75). Extracellular depolymerases are able to degrade partially crystallized (denatured) PHB, whereas intracellular depolymerases act on amorphous (native) PHB **(Hisano *et al*., 2006).**

The additives used in manufacturing plastics :

During this process they are also often combined with plasticizers and other additives, such as coloring, to increase their strength or flexibility, or to improve their appearance. The pure polymer resin by itself may not always have the properties needed in the final product: it may be strong but too brittle, flexible but too elastic, or flexible and elastic but just plain ugly. Just like the polymer

material itself, additives come in different varieties: some can be found in the environment, while others are manufactured. The amounts and types of additives used in manufacturing plastics are another factor that influence how environmentally-friendly they are.

The restrictive legislation on the use of plastics :

Societal concern over the environment is already being reflected in governmental restrictive legislation on the use of plastics, particularly aimed at plastic packaging. Legislation has begun at the local, state, federal, and international levels, and legislation will undoubtedly increase in the future. New legislation will likely contain restrictions aimed at materials that are neither recyclable nor biodegradable. Labeling legislation may lead to an "ecolabel," based on a product's raw material usage, energy consumption, emissions from manufacture and use, and waste disposal impact. Most of all, what is needed is a paradigm shift. We have grown accustomed to having a wide variety of useful plastic materials that are attractive, long lasting, and inexpensive.

On the other hand, we are coming to realize, in retrospect, that we may have had too much of a good thing, and have given too little thought about the effect their

continually increasing use has on the future. Ignoring nature's way of building strong materials, we have, for many applications, over-engineered our plastics for stability, with little consideration of their recyclability or ultimate fate, and ended up transforming irreplaceable resources into mountains of waste .

Plastics pollution :

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The world consumption of plastic and bioplastic :

For the next 10 years the market for plastics is expected to continue the rapid growth it experienced in the last half of the last century. World per capita consumption of plastics is expected to increase from the current level of 24.5 kg to 37 kg by 2010 led by the US, Western Europe and Japan, but South-east and East Asia and India are expected to emerge as growth regions to account for 40 %

of world consumption of plastics by 2010. World consumption is expected to increase form the current 180 million tonnes to 258 million tonnes in 2010 . The world market for biopolymers could be between 4 million and 12.5 million tonnes by 2010. This implies, somewhat conservatively, that bio-plastics could capture between 1.5 and 4.8% of the total plastics market.

The history of bioplastics :

Significant commercialization of bioplastics only began in the middle of the nineteenth century. The American inventor, **John Wesley Hyatt**, **Jr**., was looking for a substitute for ivory in the manufacture of billiard balls, and in **1869** patented a cellulose derivative for coating non-ivory billiard balls. That attempt, however, was affected by the coating's flammability; balls were occasionally ignited when lit cigars accidentally came into contact with them. Hyatt continued working on the project and soon developed celluloid, the first widely used plastic, now most widely known for its use in photographic and movie film. The history of plastics changed dramatically in the early 1900s, as petroleum emerged as a source of fuel and of chemicals. The early bioplastics were simply displaced by plastics made from synthetic polymers. World

War II brought on a large increase in plastics production, a growth which continues to this day. In the 1920s Henry Ford experimented with using soybeans in the manufacture of automobiles. Ford was partly motivated by a desire to find non-food applications for agricultural surpluses, which existed then as they do now. Soy plastics were used for an increasing number of automobile parts, like steering wheels, interior trim, and dashboard panels. Finally Ford gave the go-ahead to produce a complete prototype "plastic car." Ford, a master at generating publicity, exhibited the prototype with great fanfare in 1941, but by the end of the year was no longer publicizing the "plastic car," probably for a variety of reasons. World War II played a role: armament work took precedent over almost everything else, and steel shortages limited all non-defense production. Today plastic automobile parts are common, but the use of plastics made from renewable raw materials got side-tracked. One well established bioplastic that has survived the growth of the synthetic plastics industry is cellophane, a sheet material derived from cellulose. Although production peaked in the 1960s it is still used in packaging for candy, cigarettes, and other articles. Demand for materials like plastics is continually growing and will not be abated .

Bioplastics sector registers continuous growth: As estimated by IBAW, pan-European consumption of bioplastics in 2003 was at 40,000 tons. This indicates that consumption has doubled from 2001. Compostable wastebags and starchbased loose fill had the biggest share of overall consumption in Europe. For the first time biopackaging made a substantial contribution to market growth. Especially in Great Britain, Italy and the Netherlands the market development was dynamic. In fall 2004 a big German retailer tested the filling and marketing of organic carrots in starch based packaging. For this purpose ca. 120.000 carrot bags of natura Packaging GmbH were filled with carrots by the company Theis. IBAW expects that in 2005 the first compostable packaging will be launched in Germany.

The Discovery of PHB :

The first PHA, poly(3-hydroxybutyrate) (PHB) was discovered in *Bacillus megaterium* by the French scientist Lemoigne in 1926 **(Lemoigne, 1926)** . He reported this bacterium to accumulate intracellularly a homopolymer that consisted of 3-hydroxybutyric acids that were linked through ester bonds between homopolymer that consisted

of 3-hydroxybutyric the 3-hydroxyl group and the carboxylic group of the next monomer.

The ability of bacteria to produce storage polyesters with compositions other than PHB was not realized until **1974** when **Wallen and Rohwedder** at the USDA Northern Regional Research Laboratory reported that a polyester isolated from activated sludge contained both HB and HV units, but they were not able to identify the microbial species in sludge which produced the polyester **(Wallen and Rohwedder, 1974)** .

PHA has been identified in more than 20 bacterial genera, such as *Alcaligenes* **(Khanna & Srivastava, 2007)**, *Azotobacter* **(Pozo *et al*., 2002)**, *Azospirillum* **(Sun *et al*., 2002)**, *Rhodospirillum* **(Berleman & Bauer 2004)**, *Herbaspirillum* **(Catalan *et al*., 2007)**, *Bacillus* **(Law *et al*., 2003)**, *Pseudomonas* **(Jiang *et al*., 2008)**, *Rhizobium* (**Encarnacion *et al*., 2002** and **Todd *et al*., 2002**), *Azorhizobium*, *Streptomyces* **(Ramachander & Rawal 2005)**, *Methylobacterium*, and *Nostoc* **(Sharma *et al*., 2007)**. In addition to bacteria, recombinant yeasts were used in PHA biosynthesis such as *Saccharomyces* **(Carlson & Srienc, 2006)**, *Candida* **(Kim *et al*., 1999)** and *Variovorax* **(Maskow *et al*., 2004).**

Bioplastics will find their place in the current age of plastics :

For bioplastics to become practical, they must have properties that allow them to compete with the current plastics on the market: bioplastics must be able to be strong, resiliant, flexible, elastic, and above all, durable. It is the very durability of traditional plastics that has helped them in the marketplace, and has been a major goal of plastics research throughout the years. However, it is exactly this durability that now has people increasingly worried. Now that we wrap our sandwiches in bags that will still be around when the sandwich, and even the person who ate it, are long gone, many people are wondering: have we gone too far? Current research on bioplastics is focusing on how to use nature's polymers to make plastics that are programmed-degradable: in other words, how to make products that allow you to control when and how it degrades, while insuring that the product remains strong while it is still in use .

S. Berkesch in Michigan State University reported in March 2005 "Biodegradable Polymers: A Rebirth of Plastic". The future of biodegradable plastics shows great potential. Many countries around the world have already

begun to integrate these materials into their markets. The Australian Government has paid $1 million dollars to research and develop starch-based plastics. Japan has created a biodegradable plastic that is made of vegetable oil and has the same strength as traditional plastics. The mayor of Lombardy, Italy recently announced that merchants must make biodegradable bags available to all of their customers. In America, McDonald's is now working on making biodegradable containers to use for their fast food ("Plastics", 1998). Other companies such as Bayer, DuPont, and Dow Cargill are also showing interest in biodegradable packaging. According to Dr. Mohanty, "demands for biodegradables are forecast to grow nearly 16% per annum." This increasing interest will allow the technology needed to produce biodegradable plastics became more affordable and the falling production costs will eventually lead to an increase in producers ("Plastics", 1998). America and Japan show the greatest potentials for the biodegradable markets. The estimated amount of biodegradable plastics produced per year is about 30,000-40,000 tons over the next five years.

Biodegradable plastics are one of the most innovative materials being developed in the packaging

industry. Companies cannot work fast enough to produce this highly valuable technology. How widespread biodegradable plastics will be used all depends on how strongly society embraces and believes in environmental preservation. There certainly are an abundant amount of materials and resources to create and fund more uses for biodegradable plastics. The advancement of biodegradable technology has skyrocketed in recent years and there are growing signs that the public shows a high amount of curiosity in the product. With the variety of biodegradable plastics available in the near future, there will be a place for them current age of plastics.

What is Biopol :

In **1982**, Imperial Chemical Industries Ltd. (ICI) in England announced a product development program on a new type of thermoplastic polyester which was totally biodegradable and could be melt processed into a wide variety of consumer products including plastics, films, and fibers (**Anderson and Edwin, 1999).** The polymer was to be manufactured by a large-scale fermentation process not unlike the brewing of beer but which, in this case, involved the production of the polymer inside the cells of bacteria grown in high densities and containing as much as 90% of

their dry weight as polymer. The bacterium capable of performing this feat was *Alcaligenes eutrophus*, and the commercial polyester product, tradenamed "Biopol", was a copolyester containing randomly arranged units of [*R*]-3-hydroxybutyrate, HB, and [*R*]-3-hydroxyvalerate, HV **(Lenz and Marchessault, 2005) .**

As mentioned at the start of this review, bacterial polyesters became an article of commerce when ICI began their production of "Biopol" in 1982, but "Biopol" was not PHB. PHB has a high melting point (180˚C) and forms highly crystalline solids which crystallize slowly and form large spherulitic structures that impart poor mechanical properties in molded plastics and films, although, addition of nucleating agents and suitable posttreatment after extrusion or casting can lead to much improved properties. Because of its high melting point, PHB is also susceptible to thermal degradation during melt processing by ester pyrolysis of the aliphatic secondary esters of the repeating units. These deficiencies were partly eliminated when it was found that, when *A. eutrophus* is grown on a mixture of glucose and propionic acid, the storage polyester formed is a random copolyester of HB and HV units which has a lower melting point. As a result, the copolymers have better

processing characteristics and considerably improved mechanical properties for use as plastics. Nevertheless, like PHB, the copolymer is fully biodegradable in a wide variety of natural environments as well as in waste disposal facilities, especially in municipal compost sites **(Lenz and Marchessault, 2005) .**

Despite the 75 years, on and off, of research on PHAs and 20 years of intense industrial interest, PHAs still appear to be far removed from large scale production. At this writing, two development programs on these biopolymers are receiving attention, namely (1) a joint program by the Proctor & Gamble Co. and Kaneka Corp. on a family of short and medium chain copolymers, especially on poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate), and (2) a program at Metabolix Inc. on PHAs for medical applications. The lack of commercialization of the initially promising bacterial poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymers has been generally attributed to the high investment for the fermentation and product recovery processes on a large scale and to the cost of the substrates. To reduce the latter limitation, alternative substrates are receiving much attention, including starch and vegetable oils, but no major

breakthroughs in this area have been announced. Nevertheless, in the long run, it is possible that advances in our understanding and control of the genetic pathways involved in the biosynthesis of PHAs in microorganisms and plants could make the industrial scale production of these biopolymers competitive with oil-based synthetic polymers **(Lenz and Marchessault, 2005).**

PHB biosynthesis pathway :

Bacteria able to synthesize PHA can be divided into two groups **(Lee 2004).** The first group, accumulating PHA during the stationary phase, requires limitation of N, P, Mg and oxygen, for example, and an excess of the carbon sources. The most important micro-organism for industrial PHA production, *Ralstonia eutropha*. *Alcaligenes eutrophus* (Known as *Ralstonia eutropha* **(Khanna and Srivastsva, 2005)**; *Wautersia eutropha* **(Khanna and Srivastava, 2006)** and *Cupriavidus necator* **(Oliveira *et al*., 2007).** *Pseudomonas oleovorans* belongs to the first group also.

The second group, accumulating PHA during the growth phase, includes *Alcaligenes latus*, a mutant strain of *A. vinelandii*, *A. beijerinckii* or recombinant strains of *Escherichia coli* bearing the PHA operon of *R. eutropha*,

and *B. mycoides*, belongs to the second group because PHB accumulates during growth. PHA accumulates in cells under the condition of limited nutrients in the presence of excess carbon sources. When the supply of the limited nutrient is restored, the PHA can be degraded by intracellular depolymerases and subsequently metabolized as carbon and energy source **(Du *et al*., 2001a and Borah *et al*., 2002**).

The biosynthetic pathway of PHB consists of three enzymatic reactions catalyzed by three different enzymes. The first reaction consists of the condensation of two acetyl coenzyme A (acetyl-CoA) (EC 2.3.1.9) molecules into acetoacetyl-CoA by β-ketoacylCoA thiolase (encoded by phbA). The second reaction is the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl-CoA dehydrogenase (encoded by phbB). Lastly, the (R)-3-hydroxybutyryl-CoA monomers are polymerized into PHB by P(3HB) polymerase, (encoded by phbC) (**Reddy *et al*., 2003).**

The metabolic pathway for the PHAs synthesis in *A. eutrophus* is as following: acetly coenzyme A (acetly-CoA) is converted to P(3HP) in tree enzymatic steps. A biosynthetic β-ketothiolase catalyzes the formation of

carbon-carbon of two acetly-CoA moieties. An NADPH-dependent acetoacetyl-CoA formed in the first reaction to 3hydroxybutyryl-moiety to an existing polymer molecule by an ester bond (**Ribera *et al*., 2001; Carlson *et al*., 2002; Encarnacion *et al*., 2002; Hong *et al*., 2003 and Khodair, 2003**).

PHA accumulates in the cells as discrete granules, the number per cell and size of which can vary among the different species, some 8 to 13 granules per cell having the diameter of 0.2 to 0.5 μm were observed in *A. eutrophus*. These granules appear as refract inclusions under electron or phase-contrast microscopic observation (**Aslim *et al*., 2002**).

The groups of PHAs :

PHAs can be divided into two broad groups based on the number of carbon atoms in the monomer units; the short chain length polyhydroxyalkanoates PHAs (SCL), which consist of C3-C5 atoms, and medium chain length polyhydroxyalkanoates PHAs (MCL) consisting of C6-C14 atoms. This grouping is due to the substrate specificity of the PHA synthesis that only accept 3-hydroxyalkanoates (3HAs) of a certain range of carbon length. The PHA synthetases of *A. eutrophus* can only polymerize 3HAs(SCL) while that of *Pseudomonas oleovorans* only

polymerize 3HAs (MCL)**.** For PHAs (SCL), the monomer units are oxidized at positions other than the third carbons while for PHAs(MCL), all the monomers units are oxidized at the third position except in few cases. A lot of PHAs (MCL) containing various functional groups such as olefins, branched alkyls, halogens, aromatic and cyano have been reported. This flexibility of PHA biosynthesis makes it possible to design and produce related biopolymers having useful physical properties ranging from stiff and brittle plastic to rubbery polymers **(Ojumu *et al*., 2004) .**

The molecular weight of the polymers :

The molecular weight of the polymers are in the range at 2×10^5 to 3×10^6 daltons, based on the type of microorganism and growth condition. PHAs are accumulated in the cells as discrete granules, the size and number per cell varies depending on the different species. About 8 to 13 granules per cell having diameter range of 0.2 to 0.5μm were observed in *Alcaligenes eutrophus* **(Byron, 1994).**

The granules appear as highly refractive inclusion under electron microscopic observation. The Microorganisms accumulating PHA are easily identified by

staining with Sudan black or Nile blue **(Ojumu *et al*., 2004)** .

The physical properties of PHAs and polypropylene :

PHAs are a versatile class of biopolymers into which more than 150 different monomers containing different groups have been incorporated thus far. These functionalities, which are always located in the polyster side chains, ecompass olefinic, ester, cyano, amino, nitroso, phenyl, nitrophenoxy, chloro, and carboxylic functions among others **(Roo *et al*., 2002)**. A number of MCL-PHAs containing various functional groups such as olefins, branched alkyls, halogene, phenyl, and cyano were identified by **(Khodair, 2003)**. This flexibility of the PHA biosynthesis allows the design of novel functional polymers having potentially properties. **Lotto *et al*. (2004)** repoeted that poly(ε-caprolactone) (PCL), a linaear polyester with high crystallinity, a low melting point, and good biocompatibility, can be molded by injection and extrusion or blended with other polymers .

The occurrence of thioether groups in the side chains provides new possibilities for chemical modifications, *e. g.*, oxidation of the sulfur atom with iodide to form sulfonated side chains. After such modification, cross-linking of the

PHA molecules will be possible. The derivatives may exhibit quite different properties after chemical modification and may thus allow special and novel technical applications, *e. g*., in medicine and or pharmacy. They might be used, for example, as a skin substitute because these new polymers may have antifungal and antibacterial properties. Little is known about the physical properties of these polymers. The newly isolated polymers generally appear light yellow and translucent . PHA with thioether linkages in the side chain are very glutinous and exhibit a weak **(Ewering *et al*., 2002)** .

The homopolymer PHB is a stiff and relatively brittle thermoplastic. Most studies of the physical properties of bacteria PHAs have been with PHB and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (HB*co*-HV). Polyhydroxybutyrate is 100% stereospecific with the asymmetric carbon atoms having D(-) configuration **(Steinbüchel, 1991)** thus, highly crystalline. It melting point (175˚C) is just slightly lower than it degrading temperature (185˚C), this makes it processing by injection molding difficult.

PHB has several useful properties such as moisture resistance, water insolubility and optical purity, this differentiate PHB from other currently available

biodegradable plastics which are either water soluble or moisture sensitive. PHB also shows good oxygen impermeability **(Lindsay, 1992 and Ojumu *et al*., 2004) .**

Bioplastic producing organisms :

PHAs are widely studied as potential alternatives to some petroleum-based thermoplastics and have been isolated from more than 240 strains of bacteria and have been found in more than 100 different structural variations **(Carlson & Srienc, 2006)**.

***Alcaligenes eutrophus* the bioplastic maker :**

Over 250 different bacteria, including gram-negative and gram-positive species, have been reported to accumulate various PHAs. Several bacteria synthesize and accumulate PHA as carbon and energy storage materials or as sink for redundant reducing power under the condition of limiting nutrients in the presence of excess carbon source **(Steinbüchel, 1991; Byron, 1994; Yu, 2001; Du and Yu, 2002b and El-Kady, 2008).**

The bacterium capable of performing this feat was *Alcaligenes eutrophus*, since renamed *Ralstonia eutropha* (more recently changed again to *Wautersia eutropha*) and the commercial polyester product, tradenamed "Biopol", was a copolyester containing randomly arranged units of

[*R*]-3-hydroxybutyrate, HB, and [*R*]-3-hydroxyvalerate, HV **(Lenz and Maechessault. 2005).**

1- Alcaligenes eutrophus is the most widely studied bacterium due to its ability to accumulate large amount of PHB from simple carbon sources **(Khanna & Srivastsva, 2005)**. *R. eutropha* was the production organism of choice for Imperial Chemical Industries (ICI) (a British chemical company in the United Kingdom) in the development of commercial production facilities for P(3HB-3HV)**.** This microorganism grows well in minimal medium at 30°C on a multitude of carbon sources but not on glucose. A glucose-utilizing mutant was therefore selected and used to produce P(3HB-3HV) at a scale of 300 tons per year**.** Chemic Linz GmbH, Linz, Austria, produced PHB from sucrose at up to 1,000 kg per week by using *Alcaligenes latus. A. latus* is substantially different from *R. eutropha* and produces PHB during exponential growth, whereas *R. eutropha* does not start PHA formation until stationary phase **(Lee *et al*., 2005)**.

Several author studied the PHB production by *Alcaligenes* such as **Jung & Lee, 2000; Sheu *et al*., 2000; Du *et al*., 2001a; Eversloh *et al*., 2001; Ewering *et al*.,**

2002; Khodair, 2003; Shang *et al*., 2003; Kahar *et al*., 2004; Patwardhan & Srivastava, 2004; Khanna & Srivastava, 2005; El-Kady, 2008 and **El-Sawah, *et al*., 2008.**

2- *Azotobacter* was the first organism to be grown by ICI for PHB synthesis. But now *A. eutrophus* was employed for this process, and the product was marketed under the trade name Biopol **(Byrom, 1992)**. *A. vinelandii* is a mutant strain that synthesizes PHB during growth**.** PHA production is particularly characteristic of Azotobacteraceae. Accumulation of PHAs by strains of different species of *Azotobacter* has been widely reported (**Toledo *et al*., 2002**).

Nutrient regulated hyper accumulation of PHB and PHA copolymers has been reported in *A.* v*inelandii*, which produced PHAs during growth on a variety of unrefined sugar sources including molasses, cane molasses and corn syrup **(Pozo *et al*., 2002).**

Many author studied the PHB production by *Azotobacter* sp. such as **Castaneda *et al*., 2000; Kim, 2000; Page *et al*., 2001; Pettinari *et al*., 2001; Pozo *et al*., 2002; Toledo *et al*., 2002**; **El-Kady, 2008** and **El-Sawah, *et al*., 2008.**

3- PHAs with various monomer structures can be synthesized by pseudomonads, such as *P. putida* , *P. oleovorans*, *P. aeruginosa*, *P. mendocina*, *P. stutzeri* and *P. nitroreducens* **(Qiang *et al*., 2001; Ribera *et al*., 2001 El-Kady, 2008** and **El-Sawah, *et al*., 2008).**

Jiang *et al*., 2008 reported that, *P. fluorescens* able to produce high amounts of PHB (up to 70% of dry cell weight) from a cheap sugarcane liquor was used instead of expensive yeast extract which could not be used for large scale cultivation. Use of inexpensive substrates such as sugarcane liquor could contribute to reducing the PHB production cost. Many author studied the PHB production by *Pseudomonas* such as **Ewering *et al*., 2002; Roo *et al*., 2002 and Sheu & Lee, 2004.**

4- Sun *et al*., 2002 reported that, some species, such as *Azospirillum brasilense* and *A. lipoferum*, can accumulate high levels of PHB under unbalanced nutrient conditions. *Azospirillum* can accumulate even more PHB up to 40% of the cell dry.

Also, **Sun *et al*., 2002; El-Kady, 2008** and **El-Sawah, *et al*., 2008.**reported that, in most PHB-producing bacteria, production of only a little PHB is observed during the active growth phase of cells such as *Azospirillum*.

Therefore, a long time is needed for bacteria to reach a high density non-PHB cell biomass before accumulation of large amounts of PHB can occur. Some bacteria, such as *Azotobacter vinelandii*, *Alcaligenes latus* and *Pseudomonas putida*, are able to accumulate a large amount of PHB during exponential growth .

5- Aslim *et al*., 2002 reported that, PHB was found in *B. subtilis*, *B. megaterium*, *B. firmus*, *B. sphaericus*, *B. theringiensis* and *B. pumilus*. The highest value of PHB in *B. megaterium* was 0.27 g/L and the cell dry weight was 1.04 g/L in *B. subtilis*, and the lowest value of PHB was 0.04 g/L in *B. theringiensis* and the cell dry weight was 1.04 g/L in *B. firmus* . Also, **Labuzek & Radecka 2001,** studied the PHB production by *B. cereus* in shake-flask experiments using 10 g/l glucose and they found that, *B. cereus* is able to produce PHB as a carbon storage material up to 25 % of dry biomass . And **He *et al*., 2002** reported that, The PHB contents in fermentation broth of *Bacilus thuringiensis* were determined and the weight of PHB was about 30% of the dried weight of the cells . In addition to that, **Kato *et al*., 992, Gouda *et al*., 2001; Borah *et al*., 2002 He *et al*., 2002a; Law *et al*., 2003 El-Kady, 2008** and **El-Sawah,**

***et al*., 2008** also studied the PHB production by *Bacillus* sp.

6- Synthesis and accumulation of PHB in *Rhizobium leguminosarum*, and *R. meliloti* were studied by **Tombolini & Nuti, 1989** . PHB is accumulated up to 55% of the cell dry weight of *R. leguminosarum*. *R. meliloti* accumulates 50% of its biomass (5.5 g/L dry weight) *R. meliloti* is able to synthesize the copolymer poly(β-hydroxybutyrate-co-β-hydroxyvalerate) Glucose and sucrose were used as carbon source . **Encarnacion *et al*., 2002** reported that, *Rhizobium etli* accumulates PHB both in symbiosis and in free life. **Tavernier *et al*., 1997; Encarnacion *et al*., 2002; Todd *et al*., 2002 El-Kady, 2008** and **El-Sawah, *et al*., 2008** also studied the PHB production by *Rhizobium* sp.

7- Among the actinomycetes, the occurrence of PHA has been reported from *Streptomyces* sp. and *Nocardia* sp **(Ramachander & Rawal 2005)** .

Verma *et al*., 2002 studied the production of PHB by different strains of *Streptomyces* (*S. albus*, *S. venezuelae*, *S. lividans*, *S. olivaceus*, *S. fradiae*, *S. rosa*, *S. parvus*, *S. kanamyceticus* and *S. coelicolor*) and found that all the tested isolates produced PHB and wide variation in the PHB

content . The maximum amount varying between 1.5 and 11.8% dry cell weight . Maximal PHB production was by *S. coelicolor* (11.8% dry cell weight) . Also, **Ramachander & Rawal 2005** studied the PHB production *Streptomyces aureofaciens* and they used 10 g/l glucose as carbon source.

Among many different microorganisms that are known to synthesize PHAs, only few bacteria have been employed for the production of bioplastic . Each bacterium requires different conditions for growth and PHA production . **(Kim, 2000; Khodair, 2003; Reddy *et al*., 2003 El-Kady, 2008** and **El-Sawah, *et al*., 2008).**

8- PHA has been identified in more than 20 bacterial genera, including *Azorhizobium*, *Herbaspirillum* **(Catalan *et al*., 2007),** *Caulobacter* **(Qi & Rrhm, 2001**), *Methylobacterium* **(Yellore *et al*., 1999 and FitzGerald & Lidstrom, 2003**), *Legionella* **(James *et al*., 1999**), *Rhodospirillum* **(Berleman & Bauer 2004**), *Halomonas* **(Quillaguaman *et al*., 2006);** *Haloferax* **(Chen *et al*., 2006**), *Rhodobacter* **(Eroglu *et al*., 2004**), *Lactobacillus* **(Ganduri *et al*., 2005**), *Synechocystis* **(Wu *et al*., 2002**), *Nostoc* **(Sharma & Mallic, 2005** and **Sharma *et al*., 2007**) and *Aeromonas* **(Kahar *et al*., 2004**)

9- Archaea are a group of ancient organisms that live in adverse environmental conditions such as high salinity, high temperature, and high or low pH conditions . This group of organisms is known as ‘‘extreme halophiles.’’ Some members of the family Halobactericeae have been shown to accumulate large amounts of the intracellular reserve polymer (PHB). Strain 56 was recently isolated from a soil sample collected from the surface of hyper-saline soil close to Aswan city - Egypt . The growth was conducted at 40°C for 8 days at 200 rpm **.** Sodium acetate and butyric acid were used as carbon source . PHB accumulation contributing to about 50% of cellular dry weight **(Hezayen *et al*., 2002)**

Natural PHA-producing bacteria have a long generation time and relatively low optimal growth temperature . These are often hard to lyse and contain pathways for PHA degradation . Bacteria such as *E. coli* are incapable of synthesizing or degrading PHA; however *E. coli* grows fast, even at high temperature and is easy to lyse . Fast growth will enable it to accumulate a large amount of polymer . The easy lysis of the cells saves the cost of the purification of the PHA granules . Metabolic engineering is being intensely explored to introduce new

metabolic pathways to broaden the utilizable substrate range, to enhance PHA synthesis and to produce novel PHA. Recombinant *E. coli* strains harboring the *Alcaligenes eutrophus* PHA biosynthesis genes in a stable high-copy-number plasmid have been developed and used for high PHA productivity . Since *E. coli* can utilize various carbon sources, including glucose, sucrose, lactose and xylose, a further cost reduction in PHA is possible by using cheap substrates such as molasses, whey and hemicellulose hydrolysate (**Reddy *et al*., 2003 and Ganduri *et al*., 2005).** Several author studied the PHB production by *Echerichia* such as **Wang & Lee, 1997; Choi & Lee, 1999; Ahn *et al*., 2000; Yu *et al*., 2002; Hong *et al*., 2003 and Lu *et al*., 2003.**

PHA from yeasts :

In addition to bacteria, recombineant yeasts were used in PHA biosynthesis such as *Saccharomyces* **(Carlson *et al*., 2002)**,*Candida* **(Kim *et al*., 2002)** and *Variovorax* **(Maskow *et al*., 2004)**. *C. rugosa* were developed for production of D-β-hydroxyisobutyric acid (D-HIBA) from isobutyric acid **(Kim *et al*., 1999)**.

The synthesis of PHA from potentially toxic substrates appears to be difficult, because bacteria synthesize

and accumulate it under growth-limiting conditions only in the presence of excess carbon source. But *V. paradoxus* can use toxic substrates such as phenol or sodium benzoate as a carbon source for PHB production, and it was (8-12% of cell mass) **(Maskow *et al*., 2004).**

The bioplastic production:

PHA are synthesized and intracellularly accumulated in most bacteria a under unfavourable growth condition such as limitation of nitrogen, phosphorus, oxygen or magnesium in the presence of excess supply of carbon source **(Du *et al*., 2001b; Du and Yu, 2002a and Lee, 1996)** .

Strategies are still being developed to simulate conditions for efficient production of PHAs. **(Yu, 2001; Du *et al*., 2001b and Du and Yu, 2002b)** . Some bacteria such as *A. eutrophus*, *A. latus* and mutant strain of *Azotobacter vinelandii* are known to accumulate PHA during growth in he absence of nutrient limitation.

Several factors need to be considered in the selection of microorganism for the industrial production of PHA such as the ability of the cell to utilize an inexpensive carbon source, growth rate, polymer synthesis rate and the maximum extent of polymer accumulation of a particular

cell based on the substrate. Some workers have derived equation that predicts the PHA yield on several carbon source which could be used for the preliminary calculation of PHA yields **(Yu and Wang, 2001)** .In order to reduce the overall cost, it is important to produce PHA with high productivity and high yield. Several methods such as Fed-batch and continuous cultivations have been carried out to improve productivity **(Du and Yu, 2002a; Du and Yu, 2002b; Du *et al* 2001b and Yu and Wang, 2001)** .

Only three prominent PHAs [PHB, poly (3-hydroxybutyrate-co-3-hydroxyvalerate) and poly (3-hydroxyhexanoate-co-3-hydroxyoctanoate)] have been produced to a relatively high concentration with high productivity. Recently, workers have been exploring cultivation strategies involving inexpensive, renewable carbon substrates in order to reduce production cost and obtain high productivity **(Yu and Wang, 2001)** .

Fungi were capable of PHB degradation :

Fungi isolated from a variety of environmental samples were capable of PHB degradation. This is consistent with their role in nature as organic polymer degraders. Samples yielding the greatest proportion of fungal isolates of different morphologies were compost,

garden soil, and hay. These samples were likely to contain the greatest bacterial diversity, and thus more likely to contain PHB and more likely to support the existence of PHB-degrading fungi. Another, less expected source of PHB-degrading fungi was lichen. In retrospect, it should not be surprising that lichen would be a source of PHBdegrading organisms. Nutritional condition in lichens are high in carbon (through photosynthesis) and low in other nutrients, conditions that promote bacterial synthesis of PHB. It would follow that lichen-associated organisms would include PHBdegraders. It would be interesting to identify the specific lichen-associated bacteria involved in PHB synthesis as a first step in following perturbations in carbon flow in lichens, an organisms negatively impacted by air pollution **(Lee *et al*., 2005)** .

The end products of PHA degradation in aerobic environments are carbon dioxide and water, while methane is also produced in anaerobic conditions. The effect of different environments on the degradation rate of PHB and P(HB-HV) has been studied by several workers. Degradation occurs most rapidly in anaerobic sewage and slowest in seawater. P(HBHV) completely degraded after

6, 75 and 350 weeks in anaerobic sewage, soil and sea water, respectively **(Ojumu *et al*., 2004)** .

Commercial production of PHAs :

PHA is now being produced industrially by several companies over the world for use as natural, biodegradable, and biocompatible thermoplastics with a variety of potential applications. PHB is the most well known member of the family of PHAs . PHB has been shown to be completely biodegraded by bacteria into water and carbon dioxide (and methane under anaerobic conditions) in natural environments, including water, soil, and compost **(Lee et al., 2005).**

Commercial production of PHAs is proven, but only as expensive specialty plastics . Important contributors to cost of production are the fermentation substrate and downstream processing . Inexpensive and scaleable PHA recovery schemes need to be developed to achieve low-cost production that is competitive with traditional thermoplastics **(Tamer & Young 1998).**

Much research has been focused on the efficient production of PHA using inexpensive carbon sources, because its production cost is still high in comparison with those of chemically synthesized plastics. Using sugars as

inexpensive carbon sources, PHB production with high productivity has been archived in an optimized fermentation process. In spite of such many efforts, PHB production from sugars has an unsolvable disadvantage on a low PHA yield from carbon substrate. The yield of PHB production from glucose is ranging within 0.30–0.40 g PHB/g glucose used . In face to the further reduction on the production cost, it is necessary to alter the carbon source from sugar to other inexpensive one, which allows bacteria to produce PHA with a high yield. Plant oils are desirable feedstocks for PHA production because they are also inexpensive carbon sources. In contrast to the other carbon sources, the theoretical yield coefficients of PHA production from plant oils are as high as over 1.0 g PHA/g plant oils used, since they compose a much higher number of carbon atoms per weight **(Kahar *et al*., 2004)** .

Chemie Linz GmbH, Linz, Austria, produced PHB from sucrose at up to 1,000 kg per week by using *Alcaligenes latus. A. latus* is substantially different from *R. eutropha* and produces PHB during exponential growth, whereas *R. eutropha* does not start PHA formation until stationary phase (**Hrabak, 1992**).

PHB synthesis in *A. latus* is a shorter culture period . Moreover, a one-stage fermentation is also more amenable to continuous culture. Whereas the fermentation methodology and the choice of microorganism can reduce costs, low-cost product recovery remains essential **(Tamer *et al*., 1998)**

A number of applications have now been commercialized for Biopol. These include bottles for cosmetics and other personal care products in Germany, Japan and the USA **(Byrom, 1992).**

A PHA copolymer (poly-3-hydroxybutyrate-co-hydroxyvalerate) was commercially produced by Imperial Chemical Industries (ICI) a British chemical company in the United Kingdom. ICI is now part of AkzoNobel, one of the world's leading industrial companies . *Azotobacter* was the first organism to be grown by ICI for PHB synthesis. But now *A. eutrophus* was employed for this process, and the product was marketed under the trade name Biopol. *A. eutrophus* has high polymer content, good molecular mass and the PHA can be relatively easily extracted. **(Byrom, 1992 and Lee *et al*., 2005**). Zeneca, a former subsidiary of ICI, inherited the production process which was later acquired by Monsanto. A different product, the PHB

homopolymer, was produced by the Austrian company Chemie Linz GmbH via continuous culture of *A. latus* on sucrose **(Tamer *et al*., 1998).**

PHB provides products and services to companies throughout the world including Biotechnolgische Forschungs gesellschaft mbH (Austria), Metabolix Inc. (USA), Monsanto (USA), ZENECA Seeds (UK), Bio Ventures Alberta Inc. (Canada), Warner's Lambert (USA), Fertec (Ferruzi e Technologia) (Italy), Biotec (Melitta) Emmerich (Germany), BASF Ludwigshafen (Germany), Bayer/Wolf Walsrode Leverkusen (Germany), Novamont Novara (Italy), Polyferm Inc. (Canada), Biocorp (USA), Asahi Chemicals and Institute of Physical and Chemical Research (Japan). Markets currently serviced include; appliance 24% , telecommunications 13% , electrical 5% , automotive 18% , transportation 12% , medical 3%, industrial 6% , instrumentation 7% , defense and aerospace industries 10% **(Reddy *et al*., 2003)** With over 650 dedicated employees and over 750,000 of production floor space, manufacturing capabilities consist of die casting, machining, plastic and rubber molding, tool & die as well as product assembly. America and Japan show the greatest potentials for the biodegradable markets . The estimated

amount of biodegradable plastics produced per year is about 30,000 - 40,000 tons over the next five years **((Lenz and Marchessault, 2005).**

The production cost of plastic and bioplastic :

The production cost of bacterial lyproduced PHA was between $3 and $5 per kg, while that of propylene was less than $1 per kg. There is therefore a need to decrease the production cost and also increase productivity.

The primary applications PHB in some markets :

PHAs are natural thermoplastic polyesters, and hence the majority of their applications are as replacements for petrochemical polymers currently in use for packaging and coating applications . The extensive range of physical properties of the PHA family of polymers and the broadened performance obtainable by compounding and blending provide a correspondingly broad range of potential end-use applications, as described in numerous patents (**Madison and Huisman, 1999).**

PHA are generally biodegradable, with good biocompatibility, making them attractive as tissue engineering biomaterials. So far, only several PHA including poly 3-hydroxybutyrate (PHB), copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV), poly 4-

hydroxybutyrate (P4HB), copolymers of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx) and poly 3-hydroxyoctanoate (PHO) are available in sufficient quantity for application research **(Chen & Wu, 2005)**.

The applications of biodegradable polymers have been focused on three major areas: medical, agricultural, and consumer goods packaging. Some of these have resulted in commercial products . Because of their specialized nature and greater unit value, medical device applications have developed faster than the other two. Over the past years, PHA and its composites are used to develop devices including sutures, suture fasteners, meniscus repair devices, rivets, tacks, staples, screws (including interference screws), bone plates and bone plating systems, surgical mesh, repair patches, slings, cardiovascular patches, orthopedic pins (including bone filling augmentation material), adhesion barriers, stents, guided tissue repair/regeneration devices, articular cartilage repair devices, nerve guides, tendon repair devices, atrial septal defect repair devices, pericardial patches, bulking and filling agents, vein valves, bone marrow scaffolds, meniscus regeneration devices, ligament and tendon grafts, ocular cell implants, spinal fusion cages, skin substitutes,

dural substitutes, bone graft substitutes, bone dowels, wound dressings, electronic applications and hemostats. The changing PHA compositions also allow favorable mechanical properties, biocompatibility, and degradation times within desirable time frames under specific physiological conditions **(Chen & Wu, 2005).**

In addition to its range of material properties and resulting applications, PHAs promise to be a new source of small molecules . PHA can be hydrolyzed chemically, and the monomers can be converted to commercially attractive molecules such as are β-hydroxy acids, 2-alkenoic acids, β-hydroxyalkanols, β-acyllactones, β-amino acids, and β-hydroxyacid esters (**Williams and Peoples, 1996**) . The last class of chemicals is currently receiving attention because of potential applications as biodegradable solvents **(Madison and Huisman, 1999).**

The role of genetic Engineering in the bioplastic production :

In 1988, a group of scientists at James Madison University cloned the entire set of genes in *R. eutropha* for the three enzymes involved in the synthesis of PHB from acetyl CoA. The three genes are clustered in one operon, and they were able to introduce this operon into *E. coli.*

The genetically engineered *E. coli* containing the operon can express all three enzymes and can synthesize PHB in large quantities from a wide range of organic compounds. Some recombinant strains of *E. coli* can also produce the HB/HV copolymer, or alternatively as reported by them at the Massachusetts Institute of Technology in 1994, strains containing only the synthase gene can express this protein in sufficiently large quantities for isolation and purification **(Jung *et al*., 2004 and Lenz and Marchessault, 2005).**

The transgenic plants :

A small oil seed plant (transgenic plant) was engineered to harbour the *A. eutrophus* PHA biosynthesis genes, this was found to accumulate PHB granules of 0.2-0.5 μm diameter in the nucleus, vacuole and cytoplasm. The accumulation was 100 μg/g fresh weight **(Lee, 1996 and Poirier *et al*., 1995)**.

Nature Biotechnology opened new prospects for the large-scale production of a very good quality PHA from the leaves of transgenic *Arabidopsis* as well as form the seeds of transgenic oilseed-rape. The US researchers had transferred four bacterial genes to the plants, that could be expressed in two distinct metabolic pathways. However, both transgenic *Arabidopsis* and oilseed-rape produced

only 2.5% of their biomass as plastic, which implied that more genetic transformations would be necessary to obtain commercially-exploitable results. The extraction process was also an important element of a profitable production of plastics by transgenic crops.

The green plastics :

Commercial ventures are aiming to translate these new technologies into useful products, to show that environment friendly plastics - Green Plastics - is not a contradiction in terms . plastics that are biodegradable and are made mostly or entirely from renewable resources. Green plastics are the focus of an emerging industry focused on making convenient living consistent with environmental stability. One reason to make a shift toward the use of green plastics is the availability of raw materials. Green plastics can be made using polymers that come from agricultural and marine feedstocks .

In 1992, French researchers were able to transfer two genes of *R. eutropha* into *Arabidopsis*, which could produce 0.1% of its biomass as PHA. In 1994, this proportion was increased up to 14% after the transfer of a third gene and a better expression of alien genes. This yield was considered satisfactory, but the polymer had poor

physical properties. In 1998, small quantities of a good quality PHA, called MCL-PHA, could be derived from a transgenic plant; this plastic resembled elastomeres, rubber or glue . In 1997, these genes were transferred into cotton and tobacco in 2002.

MATERIALS AND METHODS

Microorganisms :

Some local isolates were isolated from different soil samples taken from different regions in Damitta and Dakahlia Governorates, Egypt. The soil samples were collected from rhizosphere of different plants (*Zea mayz*, *Vicia faba* and *Trifolium alexandrinum*) from 0-15 cm layer.

Rhizobium spp. :

Seven isolates of *Rhizobium* sp. were obtained from Microbiology Department, Fac. of Agric., Mansoura Univ. 4 strains belong to *Rhizobium leguminosarum*, 2 strains belong to *R. meliloti* and one strain belong to *R. japonicam*

Isolation and purification of the other organisms :

Ten gram of each soil sample was suspended in 90 ml of sterile tap water and shaked vigorously, serially diluted in sterile tap water and the dilutions from 10^{-1} to 10^{-6} were plated on specific media.

Azotobacter spp. :

Azotobacter sp. was isolated from Most Probable Number tubes (MPN tubes) by successive streaking onto plates containing modified Ashby's agar medium **(Abd-El-**

Malek and Ishac, 1968) and incubated at 30°C for 15 days. Single colonies of different morphologies developed on the N-deficient medium were picked-up. Purified isolates were kept on modified Ashby's agar medium.

***Azospirillum* spp. :**

Tubes containing 5 ml N-deficient semi-solid medium **(Döbereiner, 1988)** were inoculated with 1 ml aliquots of prepared serial dilutions of rhizospheric soil. Incubation took place at 30°C for 7 days, and positive tubes were recognized by the formation of subsurface fine white pellicles. Microscopic examination of these preparations confirmed the predominance of typical azospirlla cells with characteristic spiral movement. Purification was carried out using the streaking technique on malate agar **(Döbereiner, 1988)**. Colonies were further purified and finally kept in N-deficient semi-solid malate medium.

***Alcaligens* spp. :**

The dilutions from 10^{-1} to 10^{-6} were plated on **Kim *et al.* (1995)** medium and incubated at 30°C for 3 days. Single colonies of different morphologies developed on **(Kim *et al.*, 1995)** medium were picked-up.

***Bacillus* spp. :**

The dilutions were pasteurized at 80°C for 15-20 min in water bath, then the dilution from 10^{-1} to 10^{-6} was plated on nutrient agar medium and incubated at 30°C for 3 days. Colonies of various morphologies were further purified and finally kept in nutrient agar medium **(Aslim *et al*., 2002)**.

***Pseudomonas* spp. :**

The dilutions from 10^{-1} to 10^{-6} was plated on **(Atlas, 2000)** medium and incubated at 30°C for 3 days. Single colonies of different morphologies developed on **(Atlas, 2000)** medium were picked-up.

***Streptomyces* spp. :**

The dilutions from 10^{-1} to 10^{-6} was plated on yeast extract malt extract medium (YEME medium) **(Verma *et al*., 2002)** and incubated at 30°C for 3 days. Single colonies of different morphologies developed on (YEME medium) were picked-up.

Methods of identification of isolates:

The following microbiological methods were carried out identify the obtained isolates according to **Holt *et al*. (1994)**.

The media were used for bioplastic production :

Pozo *et al*. (2002) medium:

This medium was used for production of PHB by *Azotobacter* strains. The composition of the mineral salts solution was: 0.64 g K_2HPO_4; 0.16 g KH_2PO_4; 0.2 g NaCl; 0.2 g $MgSO_4.7H_2O$; 0.05 g $CaSO_4.2H_2O$; 0.01 g $Na_2MoO_4.2H_2O$; 0.02 g Ferric citrate; 1000 ml distilled water and the pH was 7.2. This medium was supplemented with 0.12% ammonium acetate and 1% fructose as carbon source. Carbon and nitrogen sources were sterilized separately and added to the sterilized medium.

Minimal medium (Vanstockem *et al*., 1987):

This medium was used for PHB production by *Azospirillum* isolates. The composition of minimal medium was as follows: 3 K_2HPO_4; 1 NaH_2PO_4; 0.3 $MgSO_4.7H_20$; 0.15 KCl; 0.01 $CaCl_2.2H_20$; 0.0025 $FeSO_4.7H_20$; 0.005 biotin; 1 ml of trace metal solution; 1000 ml distilled water and the pH was 6.8. The trace metal solution consisted of (g/l) 20 $FeSO_4.7H_2O$; 0.3 H_3BO_3; 0.2 $CoCl_2.6H_2O$; 0.03 $ZnSO_4.7H_2O$; 0.03 $MnCl_2.4H_2O$; 0.03 $(NH_4)_6Mo_7O_{24}.4H_2O$; 0.03 $NiSO_4.7H_2O$ and 0.01 $CuSO_4.5H_2O$. Minimal medium was supplemented with 10 g/l of sodium malate as a carbon

source and 2 g/l of NH_4Cl as a nitrogen source **(Sun *et al.*, 2002).**

Wang and Lee (1997) medium :

Two different media were used for PHB production by *Alcaligenes*. The first one was **Wang and Lee (1997)** medium and it consists of**:** 1.0 g $(NH_4)_2SO_4$; 0.6 g KH_2PO_4; 3.6 g $Na_2HPO_4.12H_2O$; 1.0 g $MgSO_4.7H_2O$; 0.1 g $CaCl_2$; 0.1 g citric acid; 3 ml trace metal solution; 1000 ml distilled water and the pH was 6.8. The trace metal solution consists of (g/l) 20 $FeSO_4.7H_2O$; 0.3 H_3BO_3; 0.2 $CoCl_2.6H_2O$; 0.03 $ZnSO_4.7H_2O$; 0.03 $MnCl_2.4H_2O$; 0.03 $(NH_4)_6Mo_7O_{24}.4H_2O$; 0.03 $NiSO_4.7H_2O$ and 0.01 $CuSO_4.5H_2O$. Sucrose was used as carbon source at a concentration of 30 g/l. Sucrose, $(NH_4)_2SO_4$ and salt solutions were sterilized separately and then aseptically reconstituted at room temperature prior to inoculation.

Beaulieu *et al.* (1995) medium :

The second one was **Beaulieu *et al.* (1995)** medium and it consists of: 30 g of glucose, 0.79 g of $MgSO_4.7H_2O$, 2.98 g of Na_2HPO_4, 1.5 g of KH_2PO4, 15 mg of $FeSO_4.7H_2O$, and 24 ml of trace element solution. Each liter of trace element solution contain 20 mg of $CuSO_4.5H_2O$, 100 mg of $ZnSO_4.6H_2O$, 100 mg of $MnSO_4.4H_2O$, and 2.6 g of

$CaCl_2.2H_2O$. After the medium was sterilized, the nitrogen source (ammonium sulfate, 2.5 g/l) was added to the medium. The pH was adjusted to 7.0.

Nutrient broth medium :

This medium was used for PHB production by *Bacillus* strains: 5 g peptone; 3 g beef extract; 1000 ml distilled water and the pH was 7.0. This medium supplemented with 2 % glucose as carbon source. Carbon source was sterilized separately and added to the sterilized medium **(Aslim *et al*., 2002)**.

Qiang *et al*. (2001) medium :

This medium was used for PHB production by *Pseudomonas* strains: The mineral salt medium consists of (g/l): 0.5 $(NH)_2SO_4$; 0.4 $MgSO_4.7H_2O$; 9.65 $Na_2HPO_4.12H_2O$; 2.65 KH_2PO_4 in distilled water. The pH was 7.0. In addition, 1 ml of microelement solution was added to the medium. The microelement solution contains (g): 20 $FeC1_3.6H2O$; 10 $CaCl_2.H_2O$; 0.03 $CuSO_4.5H_2O$; 0.05 $MnCl_2.4H_2O$ and 0.1 ZnSO4.7H2O in 1 L 0.5 N HC1. This medium supplemented with 10 g glucose in 100 ml distilled water as carbon source. Carbon source was sterilized separately, cooled to 25°C and added to the sterilized medium.

Tavernier *et al*. (1997) medium :

This medium was used for PHB production by *Rhizobium* strains. 1 g NH_4NO_3; 1 g K_2HPO_4; 0.2 g $MgSO_4.7H_2O$; 1000 ml distilled water and the pH was 7.2. The medium was supplemented with filter-sterilized biotin (10 mg liter and thiamine 500 mg liter). Stock solution of fructose was separately sterilized and added to a final concentration of 6 g liter.

Yeast extract malt extract medium (YEME medium):

This medium was used for PHB production by *Streptomyces* strains. (g/l) peptone, 10; yeast extract, 4; malt extract, 4 and the pH was 7.0. This medium was supplemented with 2% glucose as carbon source. Carbon source was sterilized separately and added to the sterilized medium **(Verma *et al*., 2002).**

Cultivation system :

The inocula were prepared in 250 ml conical flasks containing 20 ml of different media, inoculated with a loop of tested cultures and incubated in a rotary shaker at 200 rpm at 30°C for 48 h. Then the inocula were transferred into 250 ml conical flasks containing 50 ml of the production medium and incubated in a rotary shaker at 200 rpm at 30°C for 48 h. **(Khanna and Srivastava, 2006)**.

Biomass determination :

Cells from culture broth were centrifuged at (5000×*g*, 10 min), washed twice with sterile distilled water and recentrifuged, dried for 24 h at 100°C and used for total cell dry weight determination **(Khanna and Srivastava, 2005)**.

Poly-β-hydroxybutyrate determination :

For the quantitative estimation of PHB, cells from culture broth were collected by centrifugation (5000×*g*, 10 min). 10 ml of hot chloroform was added in cells at 70°C for 10 min and incubated at 30°C for 24 h. The resulting solution was collected, allow to the chloroform to evaporate, dried at 100°C for 24 h and the non PHB cell dry weight was determined **(Pozo *et al*., 2002).**

Determination of total carbohydrate

Total carbohydrates were determined as glucose according to the method of **Dubois *et al*., (1956)** as follows:

Reagents: - 5% (w/v) solution of phenol in water.

- Concentrated sulfuric acid (sp. gr. 1.84).

Procedure: Into thick walled tubes, 1 ml of sample was piptted containing the equivalent of 20-100 µg glucose.

A reagent blank containing 1 ml of water and a set of glucose standards (20-100 μg glucose, in a volume of 1 ml) were prepared at the same time. To all tubes 1 ml of 5% phenol solution was added. Then 5 ml of concentrated sulfuric acid were added, directing the stream of acid on the surface of liquid with shaking. The tubes were allowed to stand in water bath at 25°C. for 10 to 20 min. before reading at 490 μm.

Infra-red (IR) spectroscopic analysis of PHB :

The polymer was confirmed by infra-red (IR) (MATTISON 5000 FTIR spectrometer) in Mansoura University, Faculity of Science, Chemistry Department, Spectral Analysis Unit. The IR spectra were taken with a Fourier transform spectrometer IFS 28 (Bruker). The samples were dissolved in chloroform and deposited as a film on a sodium chloride disk. IR spectra of the polymer was measured at a 2 cm^{-1} spectral resolution by using a MATTSON 5000 FTIR spectrometer equipped. An excitation wavelength at 3900 nm was provided and the laser power at the sample position was typically 500 nm . The spectra was obtained with a spectral resolution of 4 cm^{-1}, and 1024 (**Eversloh *et al*., 2001).**

RESULTS AND DISCUSSION

Part 1 : Characterization and identification of isolates

Since species of *Azotobacter*, *Azospirillum*, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Streptomyces*, and *Alcaligenes* are the most active producers for polyhydroxybutyrate (PHB) and alginate **(Reddy *et al*., 2003)**, different bacterial isolates of these genera were isolated on specific culture media form the rhizosphere of several plants. The isolates were purified, identified and investigated for their potentialities in PHB and alginate formation. The most active species in PHB and alginate formation were selected for use in subsequent experiments.

***Azotobacter* spp.:**

Identification testes of the isolates showed that the cells are oval, negative to Gram-stain, motile, capsulated, formed dark brown pigments in old cultures, catalase positive, not hydrolyzing gelatin, starch or casein, indole negative, M.R. positive, V.P. test positive, acid produced from glucose, fructose, galactose, arabinose, maltose, sucrose, xylose, mannitol, sorbitol and ribose. Acid not produced from lactose. From these characteristics, the

isolates were identified and designated as *A. chroococcum* **(Holt *et al*., 1994).**

***Azospirillum* spp.:**

Identification testes of the isolates showed that the cells were vibroid, negative to Gram stain, motile, colonies are round, white, slightly viscid, convex and translucent, catalase positive, M.R., V.P. and indole tests negative, not hydrolysing starch, gelatin and casein, acid produced from glucose, fructose, galactose, arabinose, xylose, mannitol, sorbitol and ribose and acid not produced from maltose, sucrose and lactose. From these characteristics, the isolates were identified and designated as *A. lipoferum* **(Holt *et al*., 1994).**

***Alcaligenes* spp.:**

Identification of the isolates showed that the cells were rods and cocobacilli, negative to Gram stain, motile, capsulated, catalase positive, indole negative, M.R. negative, V.P. negative, citrate negative, not hydrolyzing gelatin, starch or casein, acid not produced from glucose, Lactose, mannitol, maltose, sucrose, xylose, positive anaerobic growth with nitrate and negative anaerobic growth with nitrite therefore, the isolates were placed under the species of *A. eutrophus*. The last three isolates had

negative anaerobic growth with nitrate and positive anaerobic growth with nitrite. Therefore, these three isolates were placed under the species of *A. latus* **(Holt *et al*., 1994).**

***Bacillus* spp.:**

Identification testes of the isolates showed that the cells were bacilli, positive to Gram stain, endospore forming, motile, catalase positive, M.R. positive, hydrolyzing starch and casein. Three isolates (No. 1, 2 and 3) produced acid from glucose, arabinose, xylose, manitol, not produced gas from glucose, oxidase positive, hydrolyzing gelatin, indole negative, V.P. test positive, and citrate utilization positive. From these results, these isolates were identified and designated as *B. subtilis*. Two isolates (No. 4 and 5) not produced acid from glucose, arabinose, xylose, manitol, not produced gas from glucose, oxidase positive, hydrolyzing gelatin, indole positive, V.P. test positive, and citrate utilization negative. From these tests, these isolates were identified and designated as *B. alvei*. Two isolates (No. 6 and 7) not produced acid from glucose, arabinose, xylose, manitol, not produced gas from glucose, oxidase positive, hydrolyzing gelatin, indole negative, V.P. test positive, and citrate utilization positive. These isolates were identified

and designated as *B. cereus*. Two isolates (No. 8 and 9) produced acid from glucose, arabinose, xylose, not acid produce from manitol, not produce gas from glucose, oxidase negative, not hydrolyzing gelatin, indole negative, V.P. test positive and citrate utilization positive. These isolates were identified and designated as *B. coagulans*. Five isolates (No. 10, 11, 12, 13 and 14) produced acid from glucose, manitol, xylose, arabinose, produced gas from glucose, oxidase positive, hydrolyzing gelatin, indole negative, V.P. test negative, and citrate utilization positive. These isolates were identified and designated as *B. megaterium*. Three isolates (No. 15, 16 and 17) produced acid from glucose, manitol, xylose, not acid produce from arabinose, not produced gas from glucose, oxidase positive, hydrolyzing gelatin, indole negative, V.P. test positive, and citrate utilization negative. From these testes, these isolates were identified and designated as *B. polymyxa*. Four (No. 18, 19, 20 and 21) isolates produced acid from glucose, not from manitol, xylose, arabinose, not produced gas from glucose, oxidase positive, hydrolyzing gelatin, indole negative, V.P. test positive, and citrate utilization positive. These isolates were identified and designated as *B. thuringiensis* **(Holt *et al*., 1994)**.

***Pseudomonas* spp.:**

Identification testes of the isolates showed that the cells were short rods, negative to Gram stain, non endospore forming, motile, catalase positive, oxidase positive, not hydrolyzing starch, indole negative, V.P. negative, citrate utilization negative, acid produced from fructose and not produced from lactose and maltose. Two isolates (No. 1 and 2) produced acid from glucose, not produced from sucrose, xylose, manitol, not hydrolyzing gelatin and M. R. positive. From these results, these isolates were identified and designated as *P. putida*. Two isolates (No. 3 and 4) produced acid from glucose, sucrose, xylose, manitol, hydrolyzing gelatin and M. R. positive. From these results, these isolates were identified and designated as *P. fluorescence*. Two isolates (No. 5 and 6) produced acid from glucose, manitol, not produce from sucrose, xylose, hydrolyzing gelatin and M. R. positive. From these characteristics, these isolates were identified and designated as *P. aeruginosa*. Two isolates (No. 7 and 8) produced acid from manitol, not produce from glucose, sucrose, xylose, not hydrolyzing gelatin and M. R. negative. Thus, the isolates Nos. 7 and 8 may be fall under the species *P. alcaligenes* **(Holt *et al*., 1994)**.

***Streptomyces* spp.:**

Identification testes of the isolates showed that the cells are filamentous, positive to Gram stain, non motile, catalase positive, hydrolyzing gelatin, starch and casein, indole negative, M.R. positive, V.P. negative, acid not produced from fructose, lactose, glucose, maltose, sucrose or xylose. From these testes, the isolates were identified and designated as *S. albus* **(Holt *et al*., 1994)**.

Part 2: Efficiency of the bacterial isolates for PHB production :

The ability for biopolymers production is widely distributed among microbial species. Large numbers of bacterial biopolymers are potentially available, but relatively few have been commercially developed. Several products from bacteria including PHB are accepted products of modern biotechnology. Several more may be developed in the next few years, especially as we look to renewable resources for alternatives to products of the chemical industry **(Sutherland, 2001).**

PHB production by *Azotobacter* species :

Data in Fig 1 show that all isolates of *Azotobacter* produced PHB. The cell dry weight (C. D. W.) ranged from 0.54 to 2.28 g/l. The PHB concentration ranged from 0.12 to 0.78 g/l. *A. chroococcum* No. 16 recorded the highest values of PHB and the cell dry weight. PHB concentration was 0.78 g/l. The values of PHB are higher than those of **Cho *et al*. (2001)** who investigated the production of PHB by *Azotobacter* and found that the PHB concentration was 0.69 g/l.

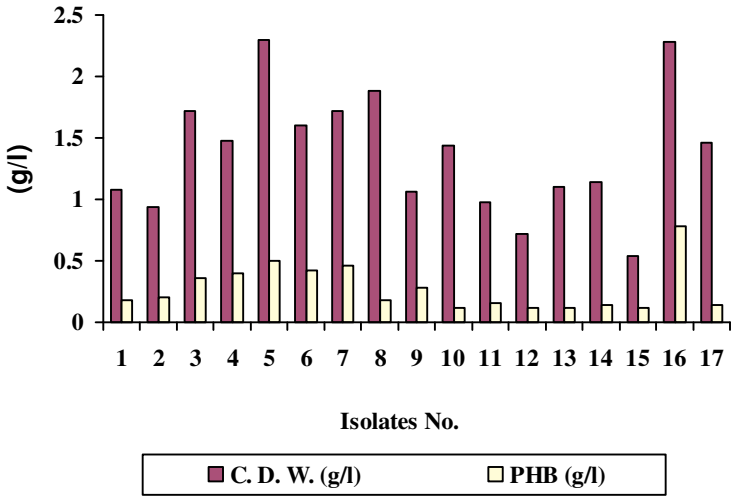


Fig. 1. PHB synthesis by *Azotobacter* isolates on Pozo *et al*., (2002) medium.

PHB production by *Azospirillum* species :

Figs. 2 show the synthesis of PHB by *Azospirillum* isolates. Cell dry weight of the used isolates varied from 0.30 to 4.20 g/l. The PHB concentration ranged from 0.12 to 0.58 g/l. *A. lipoferum* No. 5 has the highest values of PHB and the cell dry weight, PHB concentration was 1.44 g/l. Results are similar with those obtained by **Sun *et al*. (2002)** who studied the production of PHB by *A. brasilense* and *A. lipoferum*. They found that *Azospirillum* can accumulate even more PHB up to 40% of the dried cell.

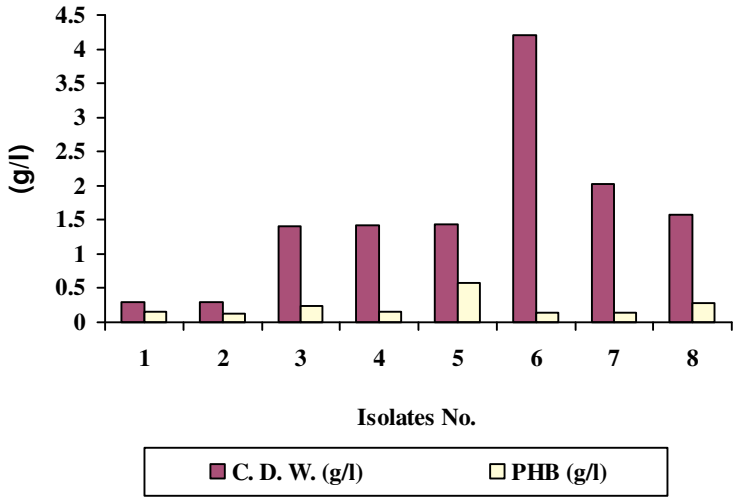


Fig. 2. PHB production by some *Azospirillum* isolates on Vanstokem *et al*., (1987) medium.

PHB production by *Alcaligenes* species :

Tow different media were used to produce PHB by *Alcaligenes* species. Figs 3 and b show that cell dry weight of *Alcaligenes* isolates varied from 0.40 to 7.30 g/l. The PHB production ranged from 0.02 to 0.52 g/l. *A. eutrophus* No. 21 on **Wang and Lee (1997)** medium recorded the highest amount of PHB (0.52 g/l). Several authors studied the PHB production from *Alcaligenes* isolates.

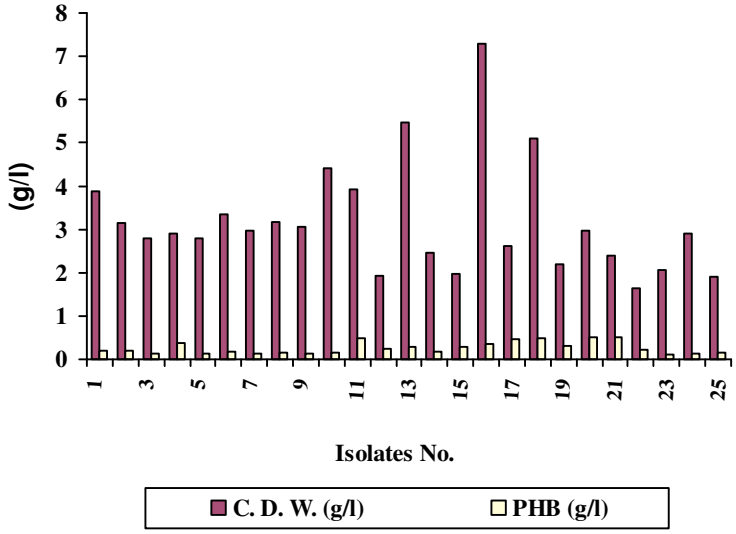


Fig.3. PHB production by some *Alcaligens* isolates on (Wang and Lee, 1997) medium.

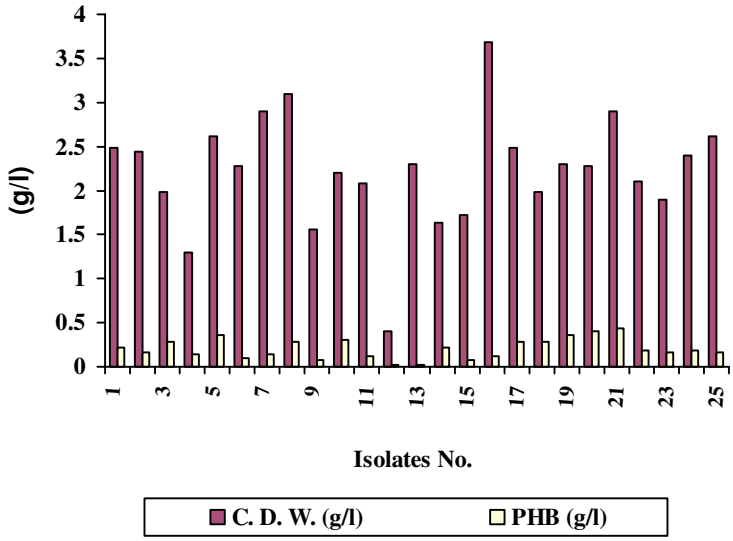


Fig. 4. PHB production by some *Alcaligens* isolates on (Beaulieu *et al*., 1995) medium.

Khanna and Srivastava (2005) found that, *A. eutrophus* exhibited a maximum biomass of 3.25 g/l with a PHB concentration of 1.4 g/l in 48 h. **Wang *et al.* (2007)** studied PHB production by *A. latus* and reported that, the cell growth was 1.10 g/l and the storage polymer accumulation was 0.248 g/l.

PHB production by *Bacillus* species :

Figs 5a and 5b show that all isolates of *Bacillus* produced PHB. Cell dry weight varied from 0.22 to 2.92 g/l. The PHB concentration ranged from 0.02 to 0.16 g/l. *B. megaterium* No. 5 showed the highest values of PHB and cell dry weight. PHB concentration was 0.16 g/l.

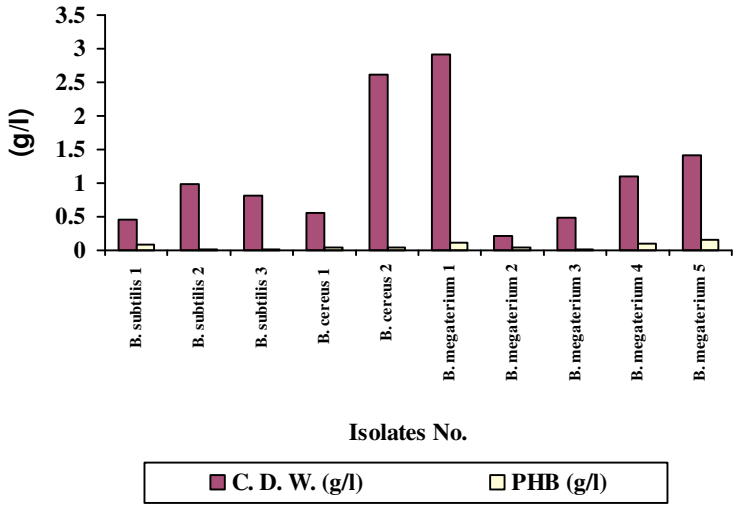


Fig. 5a. PHB production by some *Bacillus* isolates on Aslim (2002) medium.

Similar results were found by **Aslim *et al.* (2002)** who reported that PHB was common among *B. subtilis*, *B. megaterium*, *B. firmus*, *B. sphaericus*, *B. theringiensis* and *B. pumilus*. The highest value of PHB in *B. megaterium* was 0.27 g/l and the cell dry weight was 1.04 g/l in *B. subtilis*, and the lowest value of PHB was 0.04 g/l in *B. theringiensis* and the cell dry weight was 1.04 g/l in *B. firmus*.

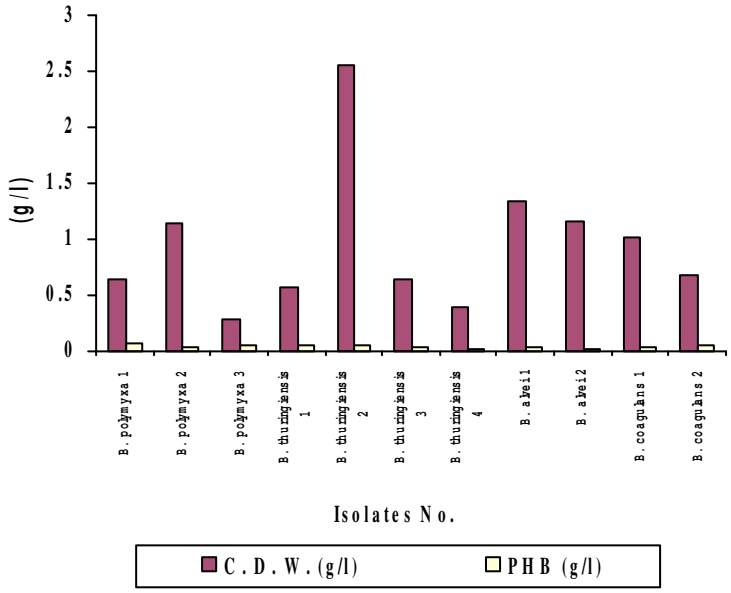


Fig. 5b. PHB production by some *Bacillus* isolates on Aslim (2002) medium.

There was no relationship between the PHB production and the cell dry weight. **Labuzek and Radecka**

(2001) studied the PHB production by *B. cereus* in shake-flask experiments using 10 g/l glucose and they found that *B. cereus* is able to produce PHB up to 25 % of dry biomass. **He *et al.* (2002)** reported that, The weight of PHB in *B. theringiensis* was about 30% of the dried weight of the cells.

PHB production by *Pseudomonas* species :

Fig 6 show that cell dry weight of *Pseudomonas* isolates varied from 0.32 to 2.92 g/l. The PHB concentration ranged from 0.10 to 0.30 g/l. PHB% ranged from 4.00 to 43.75%. *P. fluoresens* No.1 recorded the highest values of PHB (0.30 g/l), while the cell dry weight was 1.68 g/l, and the yield of PHB was 17.86 %.

Qiang *et al.* (2001) reported that, PHAs with various monomer structures can be synthesized by pseudomonads, such as *P. putida* , *P. oleovorans* , *P. aeruginosa* , *P. mendocina* , *P. stutzeri* and *P. nitroreducens*. **Ribera *et al.* (2001)** studied the production of PHB by *P. putida* in wastewater from olive oil mills and they found that biomass (g/l) was 4.2495 and PHB was 3.59 %.

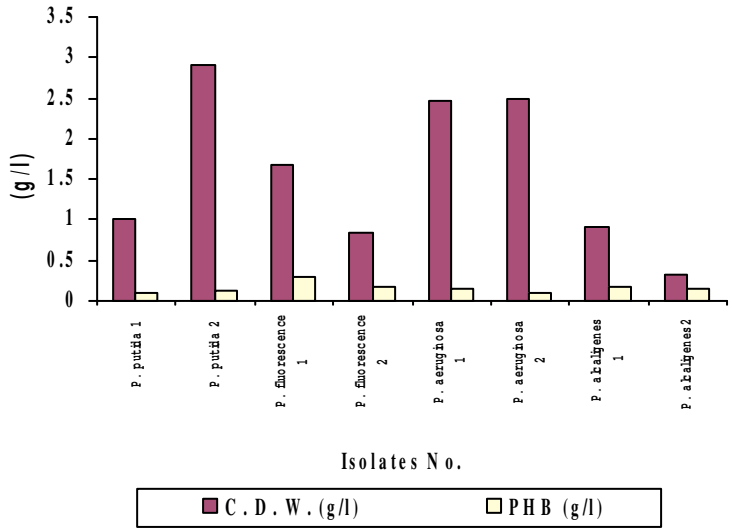


Fig. 6. PHB production by some *Pseudomonas* isolates on Qiang *et al*., (2001) medium.

PHB production by *Rhizobium* species :

Figs 23-24 show that cell dry weight of *Rhizobium* isolates varied from 0.38 to 2.44 g/l. The PHB concentration ranged from 0.06 to 0.36 g/l and PHB% ranged from 7.38 to 29.51 %. *R. leguminosarim* No. 3 produced the highest values of PHB concentration and PHB yield. Similar results were obtained by **Tavernier *et al*. (1997)** and **Encarnacion *et al*. (2002)**.

Tombolini and Nuti (1989) studied the synthesis and accumulation of PHB in *R. leguminosarum*, and *R. meliloti* and they found that, PHB is accumulated up to

55% of the cell dry weight of *R. leguminosarum* and *R. meliloti* accumulates 50% of its biomass.

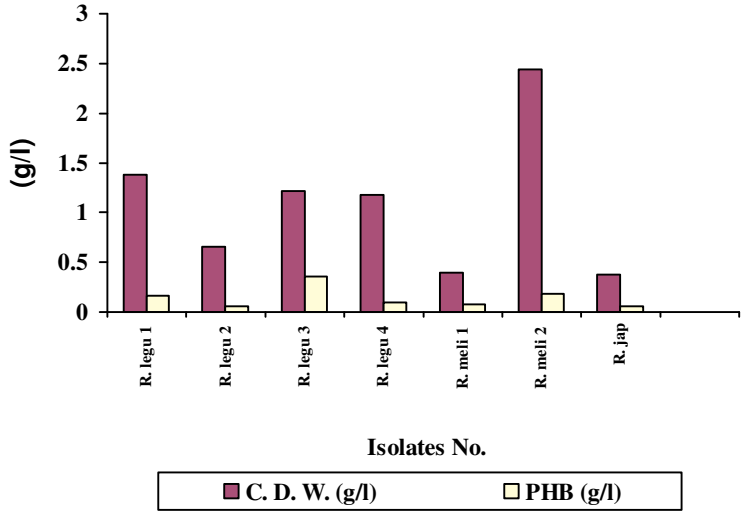


Fig.7. PHB production by some *Rhizobium* isolates on Travernier *et al*., (1997) medium.

PHB production by *Streptomyces* species :

Figs 25-26 show that cell dry weight of *Streptomyces* isolates varied from 0.90 to 3.04 g/l The PHB concentration ranged from 0.02 to 0.12 g/l. *Streptomyces albus* No. 10 seemed to be the highest producer of PHB. **Verma *et al.* (2002)** studied the production of PHB by different strains of *Streptomyces* (*S. albus*, *S. venezuelae*, *S. lividans*, *S. olivaceus*, *S. fradiae*, *S. rosa*, *S. parvus*, *S. kanamyceticus* and *S. coelicolor*) and they found that all the tested isolates

produced PHB and wide variation in the PHB content. The maximum amount of dry cell weight varying between 1.5 and 11.8%. Maximal PHB production was by *S. coelicolor*.

Ramachander and Rawal (2005) studied the PHB production by *S. aureofaciens* and they used 10 g/l glucose as carbon source, and they reported that, among the actinomycetes, the occurrence of PHA has been reported from *Streptomyces* sp. and *Nocardia* sp.

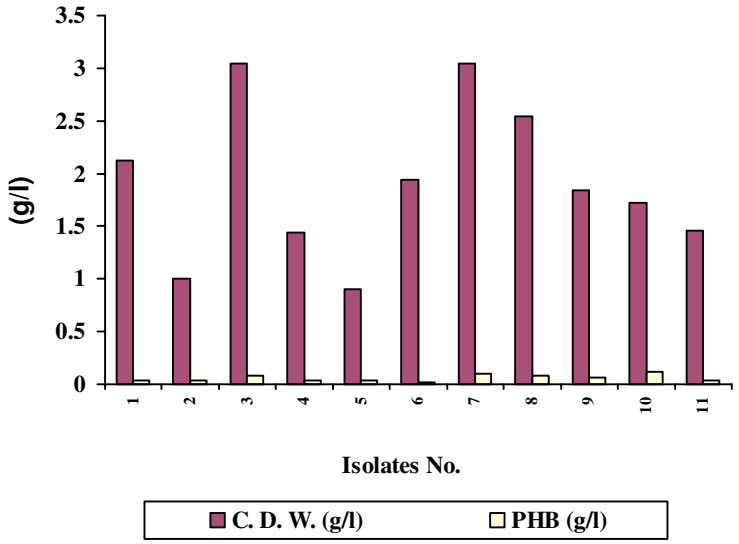


Fig. 8. PHB production by some *Streptomyces* isolates on Verma *et al*., (2002) medium.

It could observe from Figs. 1-26 that the best producer for PHB was *A. chroococcum* No. 16 on **Pozo *et al*. (2002)** medium. Thus *A. chroococcum* No. 16 was

employed for PHB production in the following experiments on **Pozo *et al*. (2002)** medium. Fig 27 summarized the best isolates for PHB production which obtained from the previously experiments.

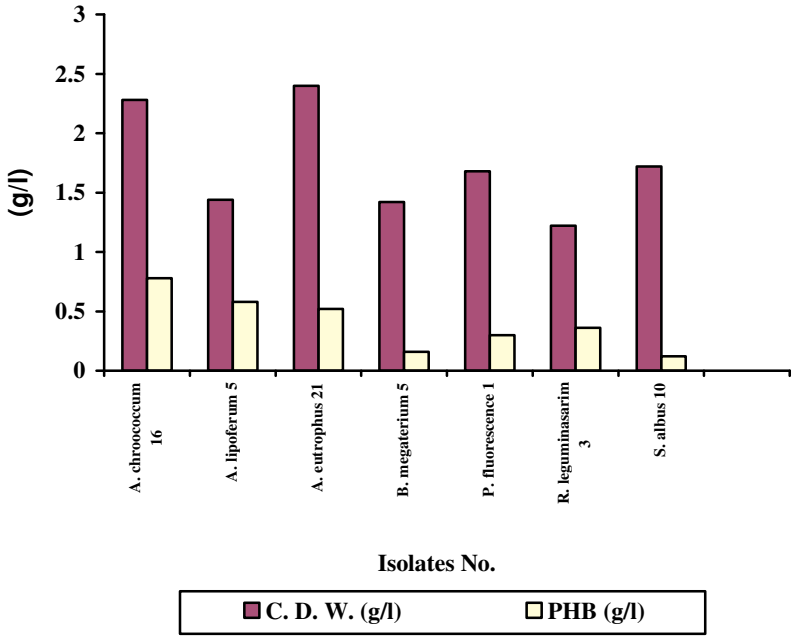


Fig. 9. The best producer isolates of PHB.

Part 3: Influence of environmental conditions on the accumulation of PHB in *Azotobacter chroococcum*:

A. chroococcum the free-living nitrogen-fixing bacterium, is well known for its intracellular accumulation of PHB. A. *chroococcum* accumulates PHB as an intracellular carbon reserve when unfavorable environmental and nutritional conditions are encountered. It is a candidate for the synthesis of environmentally benign, biodegradable plastics. Much effort has been spent in optimizing the PHB production using pure substrates and cultures **(Saha *et al*., 2007)**.

The purpose of this section is to investigate the optimal conditions for PHB production by *A. chroococcum* No. 16 using **Pozo *et al*. (2002)** medium. Thus, cultural conditions such as time course, initial pH, carbon sources, nitrogen sources and different concentrations of K_2HPO_4; KH_2PO_4; NaCl; $MgSO_4.7H_2O$; $CaSO_4.2H_2O$; $Na_2MoO_4.2H_2O$ and ferric citrate were investigated.

Effect of time course on PHB production :

The results of the effect of time course on PHB production are presented in Fig.10. The results showed that the concentration of PHB increased with the

increasing the fermentation time the maximum PHB concentration (0.85 g PHB/l) was obtained after 54 hours of fermentation then declined, at this time (54 h) the cell dry weight (C. D. W.) was 2.59 g/l and the consumed sugars (C. S.) was 5.38 g/l. The biomass dry weight followed a pattern similar to PHB production. The highest biomass dry weight (3.64 g dried biomass/l culture) was obtained after 72 hours.

Relationship between time and PHB production could be represented by the following equation :

$y = 0.3846Ln(x) - 0.0873.$

From this previous equation, it was easily seen that, the progress of fermentation time occur the rise of PHB production is noted. The determination factor (R^2) was 0.9237, it was high enough to ensure the mathematical model showing time via production.

These results are agreement with **Toledo *et al.* (1995)** who reported that the cultivation time was after 48 hours, and **Kim (2000)** who used a rotary shaker for 58 hours. Therefore, this time (54 hours) which proved to be the optimum, was used in the following experiments.

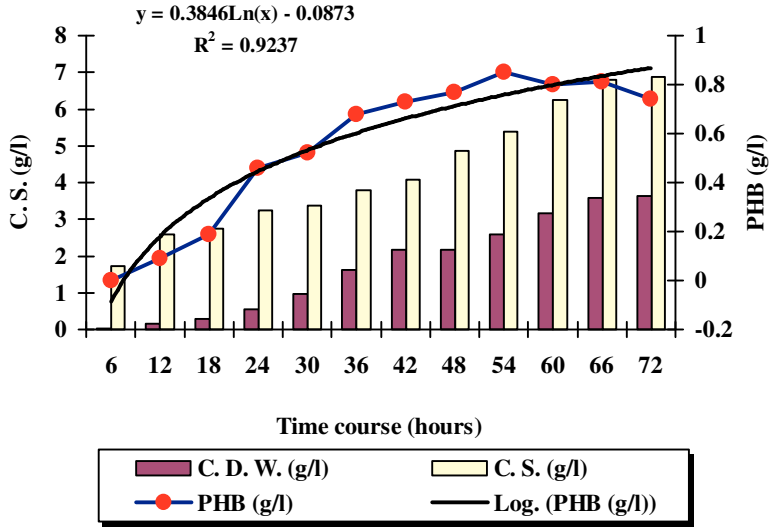


Fig.10. Effect of time course on PHB production by *A. chroococcum*.

Effect of the initial culture pH on PHB production :

Since the initial pH of the culture is an important factor that affect the PHB production and the cell growth of *A. chroococcum*, this experiment was undertaken to determine the optimum initial pH.

The PHB concentration increased with the increasing of the initial pH from 6.0 to 7.5 and then decreased (Fig. 11). The final pH was between 4.25 and 7.45. The maximum amount of PHB was at pH 7.5, in this point, the biomass was (2.88 g/l) and the concentration of consumed sugars was 5.51 g/l.

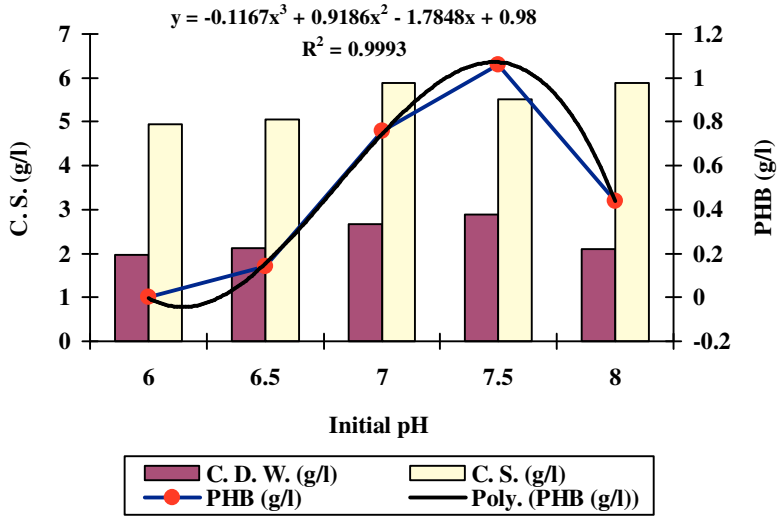


Fig 11. Effect of initial pH on PHB production by *A. chroococcum*.

Relationship between initial pH and PHB production could be represented by the following equation :

$$y = -0.1167x^3 + 0.9186x^2 - 1.7848x + 0.98.$$

From this previous equation, it was easily seen that, the increasing of initial pH increase the PHB production and then decline. The determination factor (R^2) was 0.9993, it was high enough to ensure the mathematical model showing time via production. These results are in line with **Toledo *et al.* (1995)** and **Pozo *et al.* (2002)** where the optimum pH value was 7.2.

Kim (2000) found that the optimum pH was 7.0. **Hezayen *et al.* (2002)** studied the effect of pH on the PHB

synthase with a pH range from 5 to 10 and they found that, the optimum pH of the PHB synthase was 7.5, and rather high activity was obtained for the pH range 7– 8.5. **Khanna and Srivastava (2005)** studied the initial pH and they found that the pH value measured at the end of the cultivation was found to be 5.26 as against an initial value of 7.0. On the other had, **Wang *et al.* (2007)** reported that, the pH was fluctuated between 6.50 and 7.00 and it had no obvious effect on the process. Therefore, this pH value (7.5), which proved to be the optimum, was used in the following experiments.

Effect of different carbon sources on PHB production :

The PHB production was carried out using glucose, sucrose, mannitol, galactose, fructose, ribose and mannose. These carbon sources were added to the mineral salts solution to study the effect of carbon sources. As shown in Fig.12a the highest amount of PHB was obtained with galactose being 1.46 g/l, biomass was 3.13 g/l and consumed sugars concentration was 5.99 g/l. The other tested carbon sources gave lower PHB concentration. The C.D.W. was between 2.72 g/l when fructose was used and 4.46 g/l when mannitol was used as a carbon source. The lowest value of PHB was 0.12 g/l when ribose was used as carbon source.

The maximum value of consumed sugars was 7.66 g/l and the lowest value was 3.63 g/l in the case of lactose.

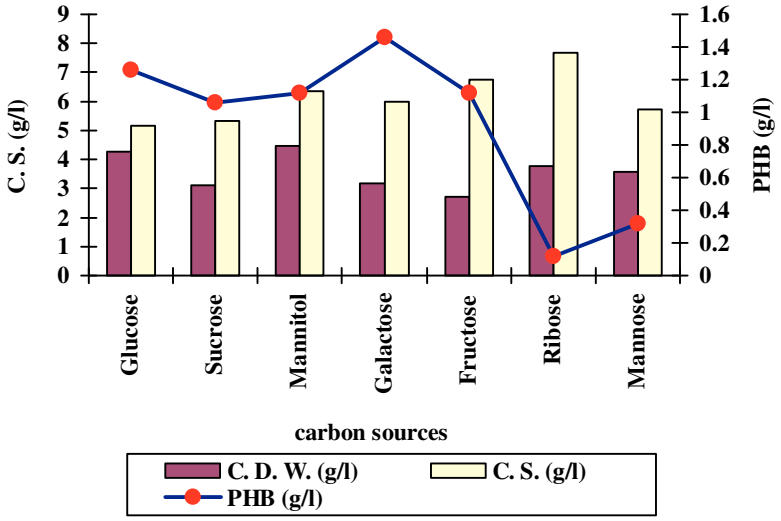


Fig.12a Effect of carbon sources on PHB production by *A. chroococcum*.

Bioplastic production from inexpensive sources (carbon sources):

Lactose, starch, cane molasses, beet molasses and glucose syrup were used as carbon inexpensive sources for PHB production (10 g/l from total sugar equivalent about 23.81 g/l from glucose syrup (42% total sugars); 19.23g/l from cane molasses (52% total sugars) and 17.86 g/l beet molasses (56% total sugars). These carbon sources were added to the mineral salts solution to study the effect of

carbon sources. As shown in Fig.12b the highest amount of PHB was obtained with glucose syrup being 1.76 g/l, biomass was 3.92 g/l and consumed sugars concentration was 5.61 g/l, The other tested carbon sources gave lower PHB concentration.

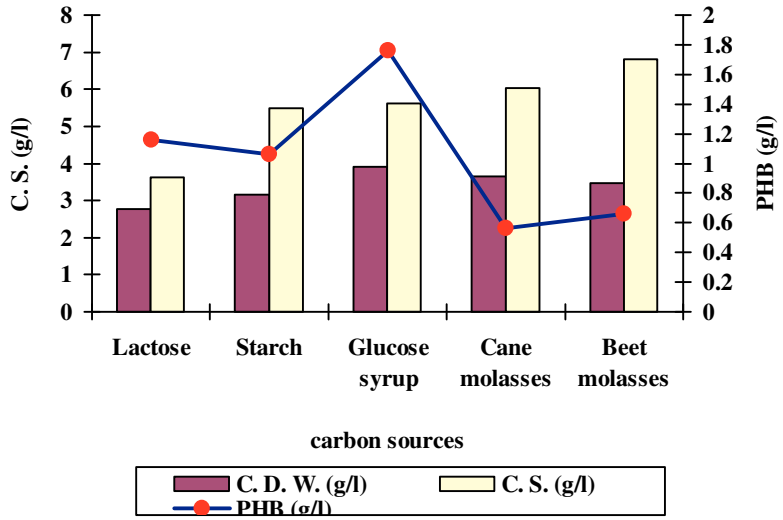


Fig.12b Effect of carbon sources on PHB production by *A. chroococcum*.

In this sense, the possibility that glucose syrup (a by-product produced during the manufacture of starch) could be utilized as an inexpensive substrate for the industrial application of this microorganism could be of considerable important. Therefore, glucose syrup was the best carbon source for PHB production, replaced for fructose of **Pozo *et al*. (2002)** medium in the following experiments.

Kim (2000) was used starch as carbon source for PHB production by *A. chroococcum*. The cell growth and PHB production by *A. vinelandii* in the 40 g/l glucose medium was as following, cells grew with the consumption of glucose after a 12 h lag period. Only a homopolymer, poly(hydroxybutyrate) (PHB) was produced, and the PHB content increased steadily with fermentation time. After 48 h of incubation, the dry cell mass and PHB mass were 10.2 g/l and 7.6 g/l, respectively **(Cho *et al*., 2001).**

Pozo *et al*. (2002) found that *A. chroococcum* formed homo- and copolymers of polyhydroxyalkanoates (PHAs) up to 80% of the cell dry weight after 48 h of incubation at 100 rev/min and 30°C. Production of PHAs by *A. chroococcum* specially on the cheap substrates is essential if bioplastics are to become competitive products. In an optimized production system, around 2.8 kg of sugar (as glucose equivalents) are needed to produce 1.0 kg of polymer.

Effect of glucose syrup (as total sugar) concentrations on PHB production :

The concentration of in the culture medium had been reveled to be important for PHB synthesis. To investigate the effect of sugar concentrations on PHB production, an

experiment was designed using different amounts of glucose syrup which the sugar content varied from 5 to 30 g/l. Every one was supplemented with the inorganic nutrients of the basal medium. As shown in Fig.13 carbon source consumption followed cell growth. The PHB level was increased with the increasing of glucose syrup from 5 to 12.5 g/l total sugars, and then declined. The C.D.W. was between 1.63 and 5.04 g/l. The lowest value of PHB was 0.13 g/l when total sugars was 5 g/l. The maximum value of consumed sugars was 9.72 g/l at this point the concentration of total sugars was 22.5 g/l. and the lowest value was 2.65 g/l in the case of 5 g/l of total sugars. These results are in harmony with **Khardenavis *et al*. (2007)** and **Koutinas *et al*. (2007)**. At 12.5 g/l sugar concentration PHB reached its maximum being 1.81 g/l and cell dry weight was 4.39 g/l, consumed sugars concentration was 7.99 g/l, Therefore, this concentration of total sugars (12.5 g/l) which proved to be the optimum, was used in the following experiments, (12.5 g/l from total sugar equivalent about 29.76 g/l from glucose syrup).

Relationship between glucose syrup (as total sugar) concentrations and PHB production could be represented by the following equation :

$y = 0.0397x^3 - 0.5723x^2 + 2.4894x - 1.48.$

From this previous equation, it was easily seen that, the increasing of total sugar concentrations increase the PHB production and then decline. The determination factor (R^2) was 0.9408, it was high enough to ensure the mathematical model showing time via production.

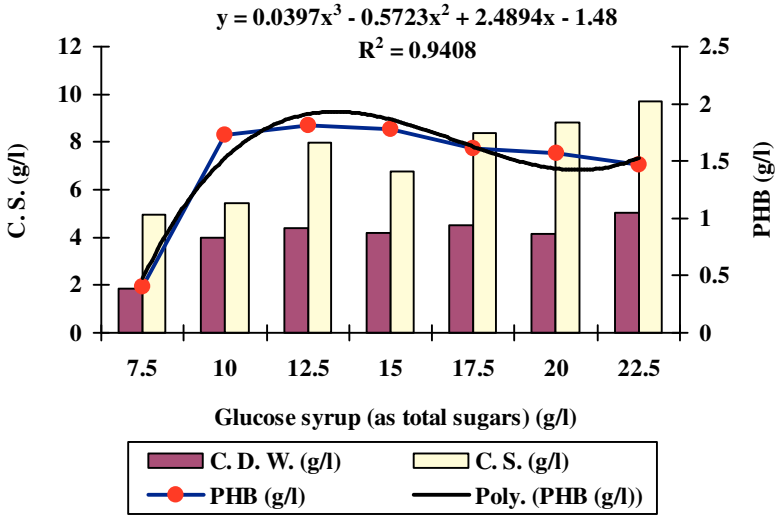


Fig13. Effect of glucose syrup (as total sugar) concentrations on PHB production by *A. chroococcum*.

Yellore *et al.* (1999) reported that, the use of cheap and easily available raw materials such as molasses can make the production of such polymer considerably more economical. **Cho *et al.* (2001)** studied the production of PHB by *Azotobacter* and they fount that the PHB

concentration was 0.69 g /l when the medium was supplemented with 20 gram glucose.

Pettinari, *et al*. (2001) reported that, when *Azotobacter* was grown in a medium containing glucose, PHB was only polymer detected.

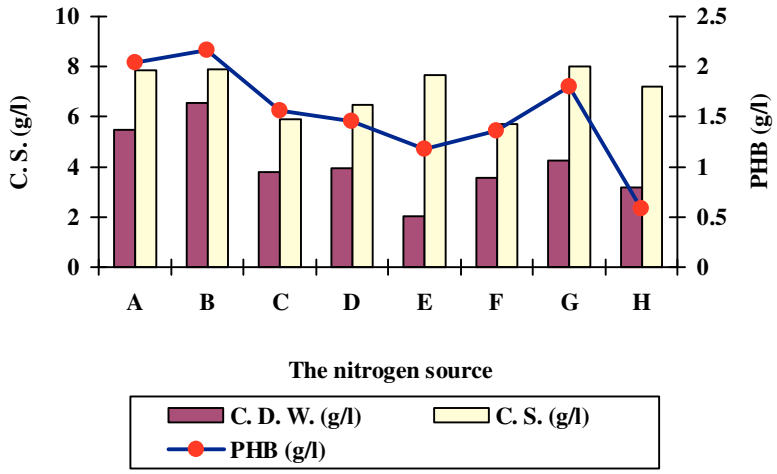
Effect of different nitrogen sources on PHB production:

Different nitrogen sources investigated were: $NH_4H_2PO_4$, NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$, ammonium oxalate, ammonium acetate, ammonium citrate, $NaNO_3$, urea, yeast extract, malt extract, beef extract, peptone and corn steep liquor. Because of *A. chroococcum* is a N_2 fixer, one treatment was used without nitrogen source Fig. 14a and 14b.

Tested nitrogen sources were added separately to the previous medium to give final nitrogen concentration of 0.2182 g N/l, (equivalent 1.2 g/l ammonium acetate). The highest amount of C. D. W. and PHB production were obtained with NH_4NO_3 as a nitrogen source (6.54 g/l and 2.16 g/l, respectively) followed by urea and $NH_4H_2PO_4$.

PHB production in the case of organic nitrogen sources (peptone, yeast extract, malt extract, beef extract

and CSL) was lowest than inorganic sources. The lowest value of C.D.W. was 2.02 g/l when $NaNO_3$ was used.

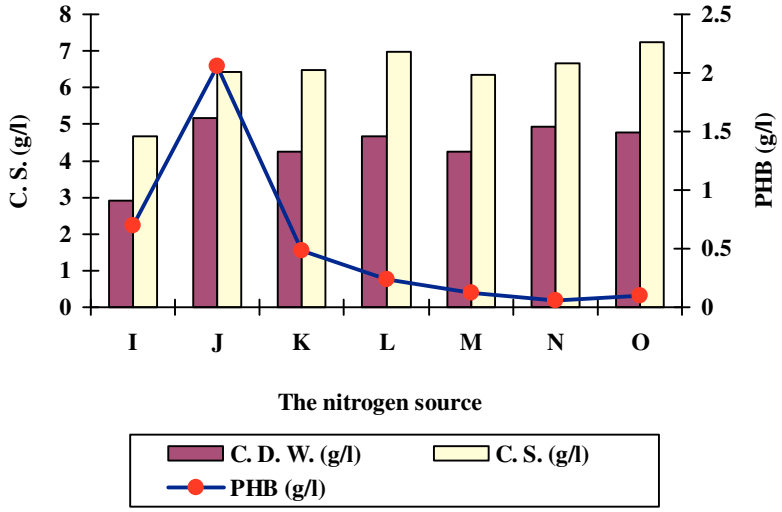


Where : A = $NH_4H_2PO_4$; B= NH_4NO_3; C= NH_4Cl; D=$(NH_4)_2SO_4$; E= $NaNO_3$; F= Ammonium oxalate; G= Ammonium acetate and H= Ammonium citrate;

Fig.14a Effect of nitrogen sources on PHB production by *A. chroococcum*.

The value of consumed sugars was 4.66 g/l when the medium was without nitrogen source at this point the concentration of PHB was 0.7 g/l and maximum value of consumed sugars was 8.01 g/l in the case of ammonium acetate. **Khodair (2003)** recommended to use ammonium sulfate as nitrogen source for highest polymer production. **Khanna and Srivastava (2005)** used relatively cheap corn steep liquor (CSL) in place of expensive yeast extract as a source of vitamins and minerals. The presence of

ammonium as a source of nitrogen is an important requirement during the growth phase in order to maximize the concentration of biomass responsible for accumulation of PHB.



Where : I= Without nitrogen; J= Urea; K= Yeast extract; L= Malt extract; Beef extract=M; Peptone=N and Corn steep liquor=O

Fig.14b Effect of nitrogen sources on PHB production by *A. chroococcum*.

Effect of NH_4NO_3 concentrations on PHB production :

The effect of ammonium nitrite on the cell growth and PHB production was investigated by increasing or reducing levels. The PHB concentration was increased with the increasing of NH_4NO_3 in the range from 0.00 to 1.25 g/l then declined (Fig.15).

The highest value of PHB concentration, 2.66 g/l, was obtained with 1.25 g NH_4NO_3/l. Also at 1.25 g/l NH_4NO_3, the dried biomass was 6.28 g/l and consumed sugars concentration was 8.29 g/l.

When NH_4NO_3 was 3.50 g/l, the C.D.W. and PHB reached at the lowest values (2.13 and 0.50 g/l, respectively). The maximum amount of C.D.W. was 6.76 g/l when NH_4NO_3 concentration was 1.00 g/l.

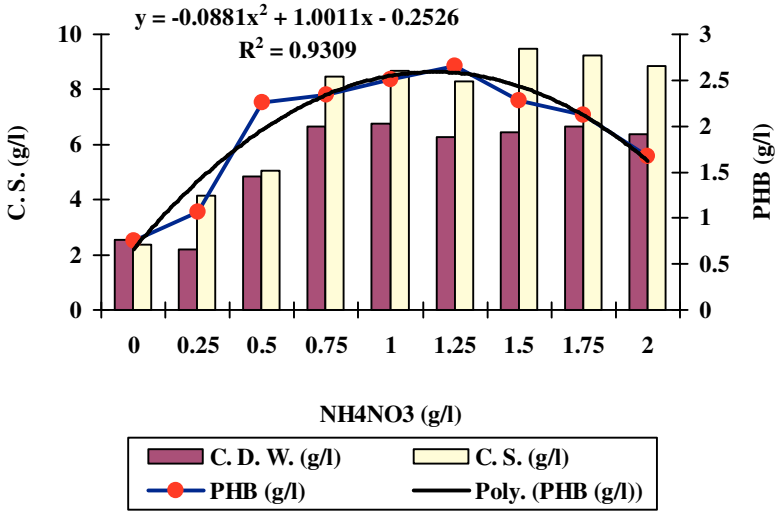


Fig. 15. Effect of NH_4NO_3 concentrations on PHB production by *A. chroococcum*.

The lowest value of consumed sugars was 2.36 g/l when the medium was without nitrogen source at this point the concentration of PHB was 0.75 g/l. The maximum value of consumed sugars was 9.46 g/l when the

concentration of NH_4NO_3 was 1.5 g/l. Therefore, this concentration of ammonium nitrate (1.25 g/l), which proved to be the optimum, was used in the following experiments.

Relationship between NH_4NO_3 concentrations and PHB production could be represented by the following equation :

$$y = -0.0881x^2 + 1.0011x - 0.2526.$$

From this previous equation, it was easily seen that, the increasing of NH_4NO_3 concentrations increase the PHB production and then decline. The determination factor (R^2) was 0.9309, it was high enough to ensure the mathematical model showing time via production.

Bioplastic production from inexpensive sources (nitrogen sources):

Different inexpensive nitrogen sources were used for bioplastic production such as : N2, NH4Cl, urea, malt extract, N2 was and corn steep liquor. Bioplastic production was 0.7, 1.57, 0.58, 0.24 and 0.1 g/l, respectively. But the highest amount bioplastic production was obtained with NH_4NO_3 as a nitrogen source.

Wang *et al.* (2007) reported that, the nitrogen deficient condition affected the normal growth and cytoplasmic synthesis of biomass. Therefore, nitrogen seems to be important for normal growth of biomass but unfavorable for polymer accumulation. On the other hand, the increased nitrogen caused an increased specific polymer yield from 0.047 to 0.422 g /l.

A. chroococcum grew in NH_4^+-medium supplemented with 1 % glucose under aerobic conditions, formed high amounts of PHB. It appears that under that culture condition *A. chroococcum* takes up carbon source and store then after conversion to PHB with higher than in nitrogen-fixing cultures. This interpretation could be supported by the fact that energy charge of the cells grown on NH_4^+-medium was lower than that of cells grown in N-free medium. Also, the ratios of ATP to ADP in cells grown in NH_4^+-medium were low when compared with cells grown in N-free medium **(Toledo *et al.*, 1995)**.

Effect of K_2HPO_4 concentrations on PHB production :

The effect of K_2HPO_4 concentrations on the cell growth and PHB production was investigated by increasing or reducing K_2HPO_4 levels (Fig. 16). The highest value of PHB concentration 3.04 g/l was obtained with 0.20 g/l

K_2HPO_4. Also at 0.20 g/l K_2HPO_4 biomass was 8.24 g/l, consumed sugars concentration was 9.10 g/l. Therefore, this concentration of K_2HPO_4 (0.20 g/l) which proved to be the optimum, was used in the following experiments.

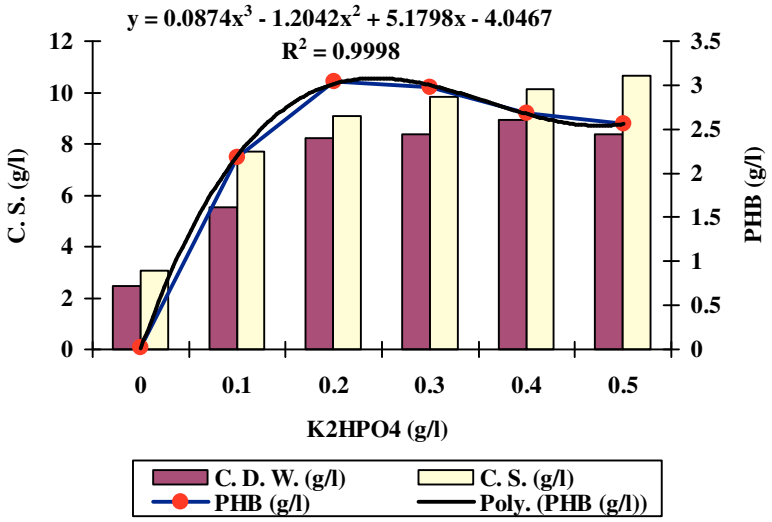


Fig.16. Effect of different concentrations of K_2HPO_4 on PHB production by *A. chroococcum*.

The lowest value of C.D.W. was 2.48 g/l without K_2HPO_4, at this point the PHB concentration was 0.02 g/l (the lowest value of PHB). The PHB% was between 0.81 and 39.35%. The lowest value of consumed sugars was 3.05 g/l without K_2HPO_4 and maximum value was 10.99 g/l when the concentration of K_2HPO_4 was 0.70 g/l.

Relationship between K_2HPO_4 concentrations and PHB production could be represented by the following equation :

$$y = 0.0874x^3 - 1.2042x^2 + 5.1798x - 4.0467.$$

From this previous equation, it was easily seen that, the increasing of K_2HPO_4 concentrations increase the PHB production and then decline. The determination factor (R^2) was 0.9998, it was high enough to ensure the mathematical model showing time via production.

Limitation of phosphate appeared to be a suitable stimulant for PHB accumulation. Where PHB accumulation was enhanced when growth is restricted due to unavailability of phosphorus **(Cho *et al*., 2001).** They studied the PHB production by *A. vinelandii* and they used 0.3 g/l of KH_2PO_4.

Pozo *et al*. (2002) studied the PHB production by *A. chroococcum* and they used 0.64 g/l of K_2HPO_4. **Khanna and Srivastava (2005)** used 1.5 g/l KH_2PO_4 and 4.0 g/l Na_2HPO_4 for PHB production. **Borah, *et al*. (2002)** found that the maximum of growth was at 2.0 g/l K_2HPO_4, whereas the maxima of PHB yield was obtained at 1.0 g/l K_2HPO_4. This concentration of phosphate is suboptimal for

cell growth but stimulated PHB accumulation. A further increase in K_2HPO_4 concentration suppressed the PHB yield and productivity.

Effect of KH_2PO_4 concentrations on PHB production :

The effect of KH_2PO_4 concentrations on the cell growth and PHB production were investigated by increasing or reducing KH_2PO_4 levels. The PHB concentration was increased with the increasing of KH_2PO_4 then declined (Fig. 17). The highest PHB concentration (3.12 g/l) was obtained with 0.15 g/l KH_2PO_4. Also at 0.15 g/l KH_2PO_4 biomass was 8.54 g/l and consumed sugars concentration was 10.73 g/l.

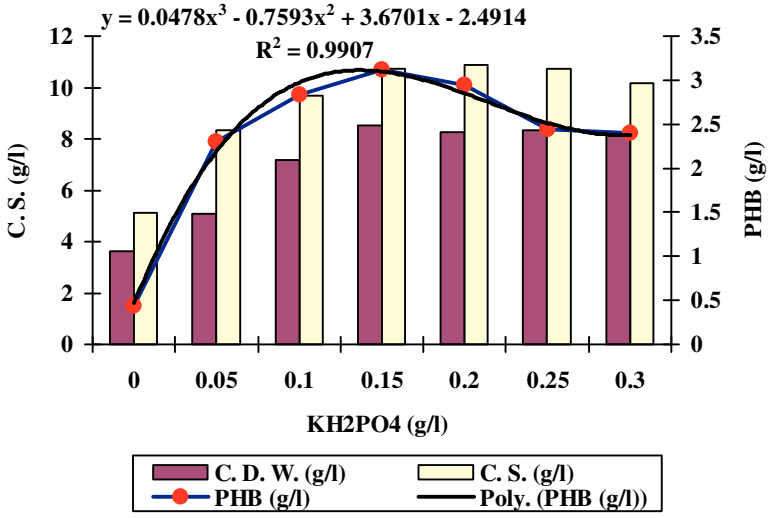


Fig. 17. Effect of different concentrations of KH_2PO_4 on PHB production by *A. chroococcum*.

Therefore, this concentration of KH_2PO_4 (0.15 g/l) which proved to be the optimum, was used in the following experiments. When the medium was without KH_2PO_4 the C.D.W., PHB and consumed sugars were at the lowest values (3.62 g/l, 0.44 g/l and 5.11 g/l, respectively).

Relationship between KH_2PO_4 concentrations and PHB production could be represented by the following equation :

$$y = 0.0478x^3 - 0.7593x^2 + 3.6701x - 2.4914.$$

From this previous equation, it was easily seen that, the increasing of KH_2PO_4 concentrations increase the PHB production and then decline. The determination factor (R^2) was 0.9907, it was high enough to ensure the mathematical model showing time via production.

Pozo *et al.* (2002) studied the PHB production by *A. chroococcum* and they used 0.16 g/l of KH_2PO_4. **Cho *et al.* (2001)** found that the maximum PHA production was obtained at 165 : 1 of C:P ratio, and the dry cell mass and PHA content were 8.0 g/l and 72%, respectively. At phosphorus concentrations higher than the optimum value, the cell mass and PHA production decreased. In particular, a large amount of phosphorus inhibited PHA formation.

Effect of NaCl concentrations on PHB production :

The effect of NaCl on the cell growth and PHB production was investigated by increasing or reducing NaCl levels. The PHB concentration was increased with the increasing of NaCl then declined (Fig. 18).

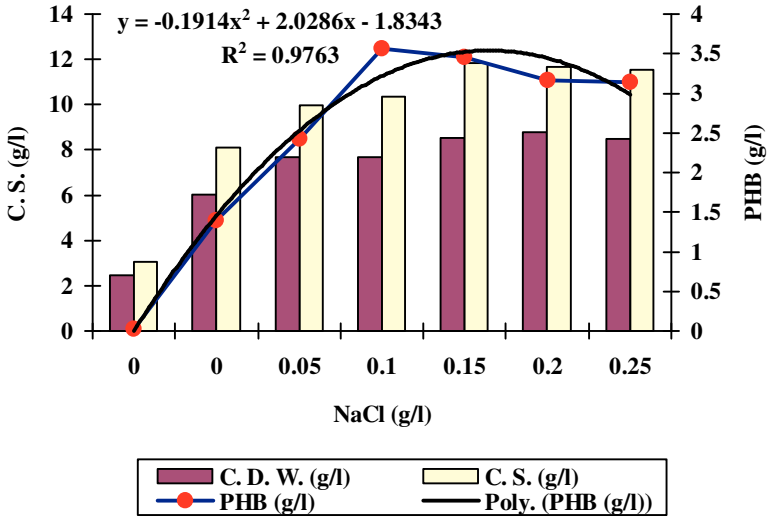


Fig.18. Effect of different concentrations of NaCl on PHB production by *A. chroococcum*.

The highest value of PHB concentration 3.56 g/l was obtained with 0.10 g/l NaCl. Also at 0.10 g NaCl/l biomass was 7.66 g/L, consumed total sugars concentration was 10.36 g/l. Therefore, this concentration of NaCl (0.10 g/l) which proved to be the optimum, was used in the following experiments. When the medium was without NaCl the

C.D.W., PHB and consumed sugars were at the lowest values (6.02 g/l, 1.40 g/l and 8.12 g/l), respectively.

Relationship between NaCl concentrations and PHB production could be represented by the following equation :

$y = -0.1914x^2 + 2.0286x - 1.8343.$

From this previous equation, it was easily seen that, the increasing of NaCl concentrations increase the PHB production and then decline. The determination factor (R^2) was 0.9763, it was high enough to ensure the mathematical model showing time via production.

Hezayen *et al.* (2002) studied the effect of NaCl on the PHB synthase and they found that, in the absence of salt the PHB synthase still exhibited about 40% of the maximum activity . **Pozo *et al.* (2002)** studied the PHB production by *A. chroococcum* and they used 0.20 g/l of NaCl.

Effect of $MgSO_4.7H_2O$ concentrations on PHB production :

The effect of $MgSO_4.7H_2O$ on the cell growth and PHB production were investigated by increasing or reducing $MgSO_4.7H_2O$ levels. The PHB concentration increased with the increasing of $MgSO_4.7H_2O$ in the range

from 0.00 to 0.25 g/l then declined (Fig. 19). The highest value of PHB concentration (3.74 g/l) was obtained with 0.25 g $MgSO_4.7H_2O$/l. Also at 0.25 g $MgSO_4.7H_2O$/l biomass was 7.22 g/l and consumed sugars concentration was 10.32 g/l.

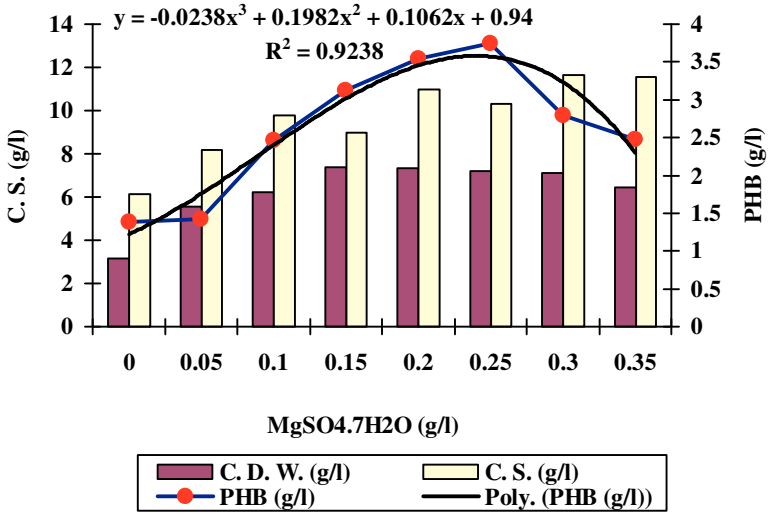


Fig. 19. Effect of different concentrations of $MgSO_4.7H_2O$ on PHB production by *A. chroococcum*

Relationship between $MgSO_4.7H_2O$ concentrations and PHB production could be represented by the following equation :

$$y = -0.0238x^3 + 0.1982x^2 + 0.1062x + 0.94.$$

From this previous equation, it was easily seen that, the increasing of $MgSO_4.7H_2O$ concentrations increase the PHB production and then decline. The determination factor

(R^2) was 0.9238, it was high enough to ensure the mathematical model showing time via production.

Cho *et al*. (2001) studied the PHB production by *A. vinelandii* and they used 0.3 g/l of $MgSO_4.7H_2O$. **Pozo *et al*. (2002)** studied the PHB production by *A. chroococcum* and they used 0.20 g/l of $MgSO_4.7H_2O$.

Interestingly, the addition of Mg^{+2} ions, which have been found to serve as cofactors for PHA synthases, showed the strongest effect. However, high enzyme activity without Mg^{+2} ions was observed, when other ions were present, suggesting that Mg^{+2} ions do not serve as cofactors **(Hezayen *et al*., 2002).**

Khanna and Srivastava (2005) used 0.51 g/l $MgSO_4.7H_2O$ and they reported that the trace metal solution had a positive effect on residual biomass production presumably because they are needed for the growth of the cells. Therefore, this concentration of $MgSO_4.7H_2O$ (0.25 g/l) which proved to be the optimum, was used in the following experiments.

Effect of $CaSO_4.2H_2O$ concentrations on PHB production:

The effect of $CaSO_4.2H_2O$ on the cell growth and PHB production were investigated by increasing or reducing $CaSO_4.2H_2O$ levels. The PHB concentration was increased with the increasing of $CaSO_4.2H_2O$ in the range from 0.00 to 0.08 g/l then declined (Fig. 20).

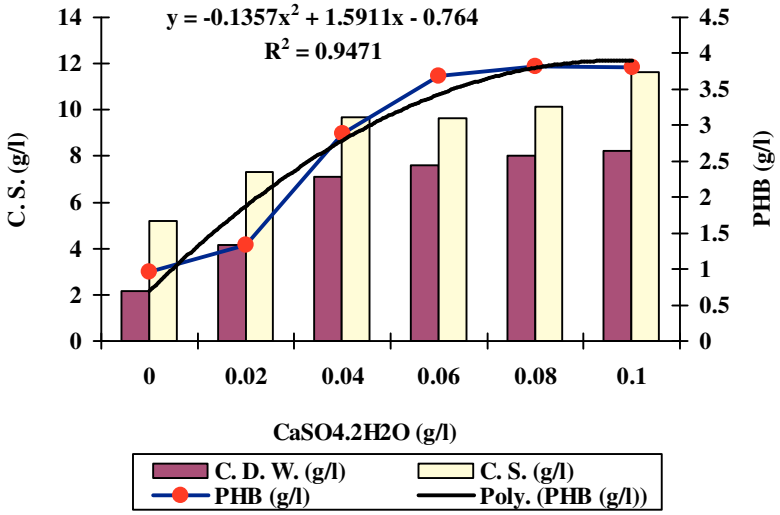


Fig. 20. The effect of different concentrations of $CaSO_4.2H_2O$ on PHB production by *A. chroococcum*.

Also, increasing the concentration of $CaSO_4.2H_2O$ increasing the cell growth from 2.16 g/l to 8.46 g/l and then declined. The highest value of PHB concentration 3.82 g/l was obtained with 0.08 g/l $CaSO_4.2H_2O$. Also at 0.08 g/l

$CaSO_4.2H_2O$ biomass was 8.00 g/l and consumed total sugars concentration was 10.12 g/l.

Relationship between $CaSO_4.2H_2O$ concentrations and PHB production could be represented by the following equation :

$$y = -0.1357x^2 + 1.5911x - 0.764.$$

From this previous equation, it was easily seen that, the increasing of $CaSO_4.2H_2O$ concentrations increase the PHB production and then decline. The determination factor (R^2) was 0.9471, it was high enough to ensure the mathematical model showing time via production.

Our resules are in agree with those obtained by **Cho *et al.* (2001)**, they studied the PHB production by *A. vinelandii*. They used 0.015 g/l of $CaSO_4.2H_2O$. **Pozo *et al.* (2002)** studied the PHB production by *A. chroococcum* and they used 0.05 g/l of $CaSO_4.2H_2O$. **Khanna and Srivastava (2005)** used 0.02 g/l $CaCl_2$.

Therefore, this concentration of $CaSO_4.2H_2O$ (0.08 g/l) which proved to be the optimum, was used in the following experiments.

Effect of $Na_2MoO_4.2H_2O$ concentrations on PHB production:

The effect of $Na_2MoO_4.2H_2O$ on PHB production as well as cell growth was investigated by increasing or reducing $Na_2MoO_4.2H_2O$ levels. The PHB concentration increased with increasing of $Na_2MoO_4.2H_2O$ concentrations in the range from 0.00 to 0.010 g/l then declined (Fig. 21). The highest value of PHB concentration (3.80 g/l) was obtained with 0.01 g/l $Na_2MoO_4.2H_2O$.

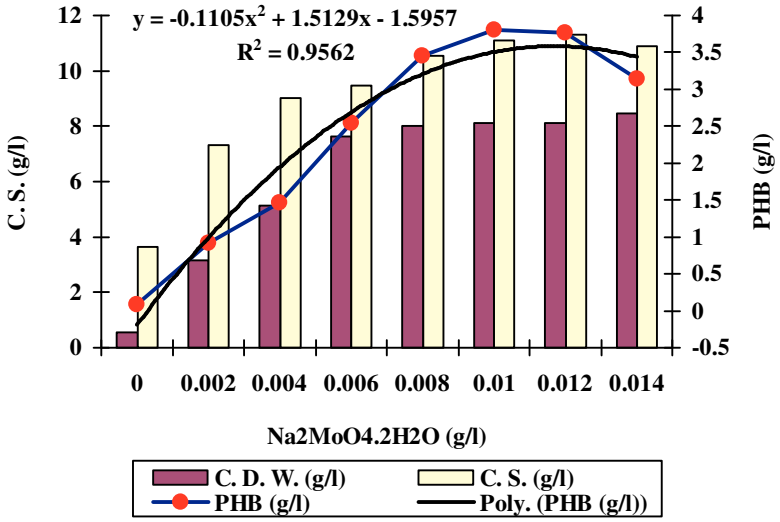


Fig. 21. Effect of different concentrations of $NaMoO_4.2H_2O$ on PHB production by *A. chroococcum*.

Also at 0.01 g/l of $Na_2MoO_4.2H_2O$ biomass was 8.12 g/l and consumed sugars was 11.11 g/l. When the

medium was without $Na_2MoO_4.2H_2O$ the C.D.W., PHB and consumed sugars was at the lowest values.

Relationship between $Na_2MoO_4.2H_2O$ concentrations and PHB production could be represented by the following equation :

$$y = -0.1105x^2 + 1.5129x - 1.5957.$$

From this previous equation, it was easily seen that, the increasing of $Na_2MoO_4.2H_2O$ concentrations increase the PHB production and then decline. The determination factor (R^2) was 0.9562, it was high enough to ensure the mathematical model showing time via production.

Cho *et al*. (2001) studied the PHB production by *A. vinelandii* and they used 0.00036 g/l of $Na_2MoO_4.2H_2O$. **Pozo *et al*. (2002)** investigated the PHB production by *A. chroococcum* and they used 0.01 g/l of $Na_2MoO_4.2H_2O$.

Therefore, this concentration of $Na_2MoO_4.2H_2O$ (0.01 g/l) which proved to be the optimum, was used in the following experiments.

Effect of ferric citrate concentrations on PHB production

The effect of ferric citrate on the cell growth and PHB production was investigated by increasing or reducing

ferric citrate levels. The PHB concentration was increased with the increasing of ferric citrate in the range from 0.00 to 0.020 g/l then declined (Fig. 22). The highest value of PHB concentration 3.82 g/l was obtained with 0.02 g/l ferric citrate. Also at 0.02 g/l ferric citrate biomass was 8.14 g/l and consumed sugars concentration was 10.23 g/l.. When the medium was without ferric citrate the C.D.W., PHB and consumed sugars were at the lowest values.

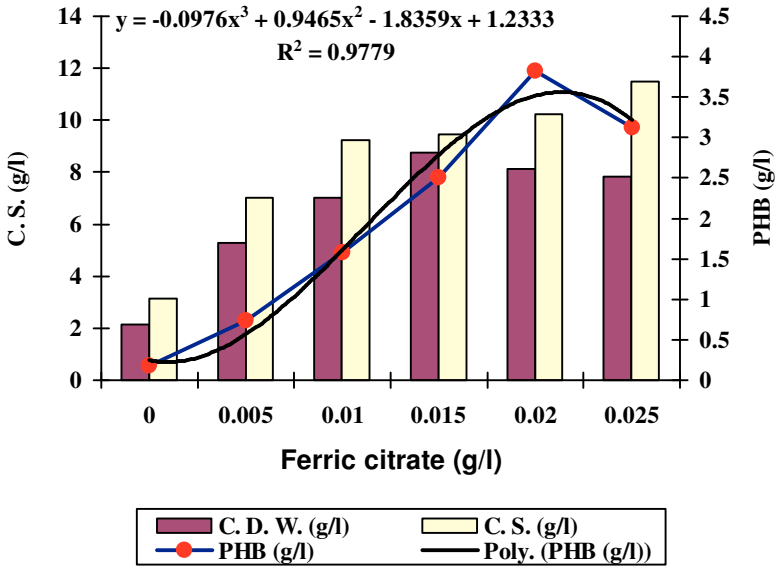


Fig. 22. Effect of different concentrations of ferric citrate on PHB production by *A. chroococcum*.

Relationship between ferric citrate concentrations and PHB production could be represented by the following equation :

$y = -0.0976x^3 + 0.9465x^2 - 1.8359x + 1.2333.$

From this previous equation, it was easily seen that, the increasing of ferric citrate concentrations increase the PHB production and then decline. The determination factor (R^2) was 0.9779, it was high enough to ensure the mathematical model showing time via production.

Cho *et al*. (2001) studied the PHB production by *A. vinelandii* and they used 0.01029 g/l of ferric citrate and 0.0075 g/l of $FeSO_4.7H_2O$. **Pozo *et al*. (2002)** studied the PHB production by *A. chroococcum* and they used 0.02 g/l of ferric citrate .

The spectroscopic analysis of PHB :

Within the cell, PHB exists in a fluid, amorphous state. However, after extraction from the cell with organic solvents, PHB becomes highly crystalline and in this state is a stiff but brittle material. Because of its brittleness, PHB is not very stress resistant. Also, the relatively high melting temperature of PHB (around 170°C) is close to the temperature where this polymer decomposes thermally **(Novikova *et al*., 2008)**.

The polymer formation was confirmed by several characterization methods such as, elemental analysis, X-ray diffraction, infra-red and NMR spectroscopic analysis.

Thus, in this section IR analysis was used to confirm the polymer formation.

By means of genetic and metabolic engineering and by the control of the cultivation conditions in combination with the feeding of suitable precursor substrates, more than 100 different hydroxyalkanoate (HA) monomers have been found as constituents of biosynthetic PHAs and many different types of copolymers have been synthesized, *e.g.*, poly(3HB-*co*-4-hydroxyvalerate (4HV)), poly(3HB-*co*-3-hydroxyhexanoate (3HHx))**,** and poly(3HB-*co*-4-hydroxybutyrate (4HB)**.** A range of additional favorable material properties have been revealed with these newly found PHA copolymers. To date, these copolyesters are mainly produced in pure culture, commonly involving complex genome mutation and expensive sterilized feedstock **(Dai *et al*., 2008)**.

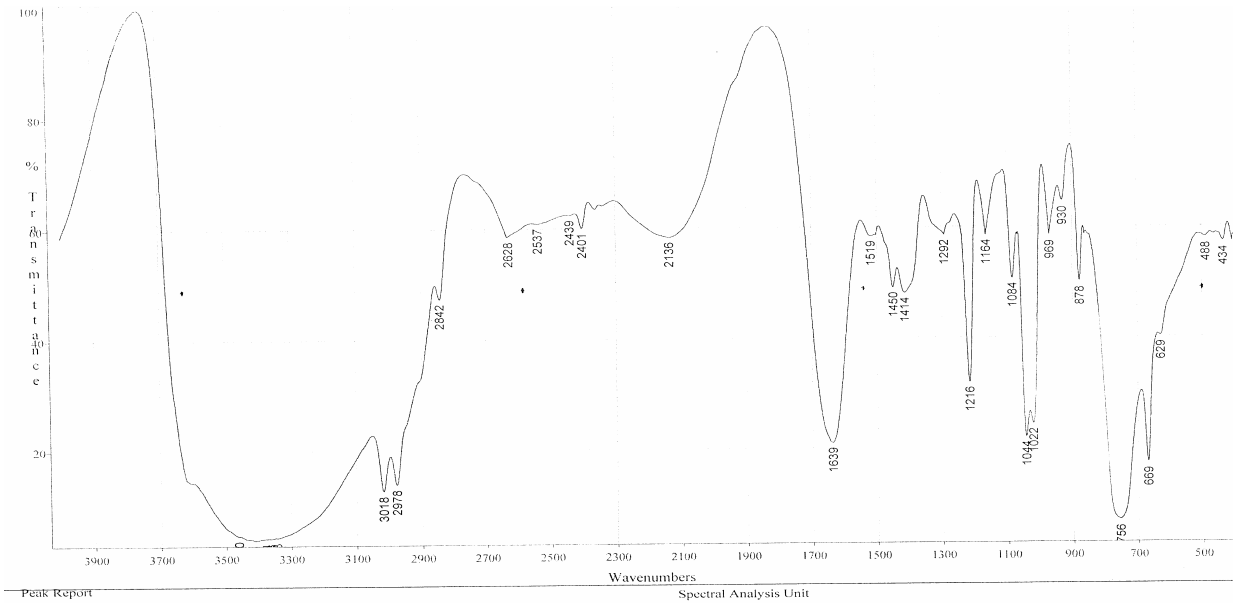
As showen in Fig. (23), analysis of the spectrum of PHB was observed at 3400, 1639, 3018, 2978, 2842, 1216, 1044, 1164, 434 and 669-765δ, respsctively, for (-OH) broad peak, (C=C) double bond, (≡C-H) acetylenic bond, (-CH_3) methyl group, (-S-H) thiol weak adsorption, (≡C-O-C≡) ether, (-O-C-O), (C-C=O), sulfoxide and haloalkanes. The chains have the C=O and CH_3 groups in the

amorphous regions is responsible for the good mechanical properties of the PHB films and fibers. The present of ether-linkage give the polymer elasticity and flexibility **(Murakami *et al*., 2007).**

Similar results were obtained by **Saha *et al*. (2007)** who studied the composition of PHB polymer produced by *A. chroococcum* when grown in glucose containing medium.

Murakami *et al*. (2007) studied the composition of PHB polymer using IR spectra and they found that spectrum of the structure shows four characteristic bands at 951, 902, 878, and 843 $cm^{-}1$.

Chen and Wu (2005) studied the properties of PHB and they found that the major peaks were due to the resonance absorption of methyl (CH_3), methylene (CH_2) and methine (CH) groups. Since PHB is made up solely of 3-hydroxybutyrate monomers, it possesses comparatively high melting and glass transition temperatures, as well as being both stiffer and more brittle than synthetic plastics, such as polypropylene. The incorporation of 3-hydroxyvalerate monomer units into PHB polymer chains addresses some of these issues.



Peak Report
File: e:\irdata~1\tmp915.ras
Title: Dr Sherf El-Kady
Filter: Five Point Center of Gravity

cm-1	%T	cm-1	%T	cm-1	%T	cm-1	%T
434	57.31	488	58.05	629	40.23	669	17.29
756	6.96	878	50.18	930	64.57	969	58.48
1022	24.22	1044	21.89	1084	50.70	1164	58.48
1216	31.79	1292	58.48	1414	48.11	1450	49.07
1519	58.32	1639	20.91	2136	58.32	2401	60.03
2439	62.34	2537	60.86	2628	58.48	2842	47.36

Spectral Analysis Unit
Chemistry Department
Faculty of Science
Mansoura University

MATTSON 5000 FTIR Spectrometer
9/3/2008

Fig. 23. IR spectrum of PHB producing by *Azotobacter chroococcum*

With increasing concentrations of 3-hydroxyvalerate units from 0 to 25%, the flexibility and toughness are greatly improved, while a decrease in high melting creates a more suitable temperature range within which the polymer can be processed without being degraded. The glass transition temperatures also decreases, allowing the use of these materials at low temperatures without embitterment **(Dai *et al*., 2008).** four characteristic bands at 966, 935, 908, and 858 $cm^{-}1$. The calculated spectrum of the structure is characterized by an intense band at 815 $cm^{-}1$ with a shoulder at 797 $cm^{-}1$, due to coupling mode of C-O stretching, O- C=O stretching, and C-O-C deformation and that of O-C stretching, C-C-O stretching, and C-O-C deformation, respectively, while the calculated

Final conclusion :

Maximum PHB obtained was 3.82 g/L. The selected strain was *A. chroococcum* No.16. The medium employed for PHB production contained glucose syrup : 29.76 g/L, ammonium nitrate : 1.25 g/L, 0.2 g K_2HPO_4; 0.15 g KH_2PO_4; 0.2 g NaCl; 0.25 g $MgSO_4.7H_2O$; 0.08 g $CaSO_4.2H_2O$; 0.01 g $NaMoO_4.2H_2O$; 0.02 g ferric citrate and pH was 7.4 at 30°C for 54 hours using shaking culture at 200 rpm. The IR analysis confirmed the polymer formation and it had a good mechanical properties.

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