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Biopolymers by *Azotobacter vinelandii*

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1. Introduction

Alginate is a polysaccharide typically extracted from cell walls of brown algae (*Phaeophyta*), where is as a structural component of cell walls and intracellular spaces. The Giant kelp *Macrocystis pyrifera*, which grows abundantly on the shores of North America and South America, New Zealand, Australia and Africa, is the main world alginate source supply. There are also used varieties of *Laminaria, Ecklonia* and *Aschophyllum nodosum*. These different brown algae species produce alginates with different proportions of polyguluronic acid structure, resulting in different properties and functions of these compounds (Glicksman, 1987; Garcia-Cruz et al., 2008). The alginic acid structure consists of acid β-D-mannuronic (M) linear chains connected by links type (1 → 4) and its epimer of acid, α-L-guluronic (G) in various proportions. These residues are arranged in mannuronic (M) or guluronic (G) blocks, connected so that the sequence of these residues in the molecule is cycled. The molecule of this polymer is composed of homo-blocks, M and G, and block heteropolymer MG (Smidsrød, 1970). (Fig. 1).

Currently, production is concentrated mainly in the brown algae cultivation, however several bacteria belonging to the *Pseudomonas* and *Azotobacter* genus produce alginate and monomer blocks structure is similar in alginate produced by marine algae and synthesized by *A. vinelandii*. In contrast, the alginate produced by *Pseudomonas* does not have a block G. Because the property of alginic acid being insoluble in water at room temperature, the sodium, calcium and potassium salts of the acid, soluble in water, are preferred to be employed in the food industry. The compound most widely used is sodium alginate, which becomes insoluble by the divalent cations addition, usually calcium, resulting in gels or films, and the gel strength depends on the divalent cation nature. The alginate gels are capable of forming micro-beds and incorporate enzymes or whole living cells and this application has generated much interest in the food industry, biotechnology and biomedical sector (Smidsrød & Skjåk-bræk, 1990; Sabra et al., 2001). In the food industry, alginates are widely used as additives with the ability to increase viscosity, stabilize, emulsify and gelling aqueous solutions. The main application is in the production of ice cream, which is used to prevent crystallization and shrinkage, resulting in a homogeneous product. Another application is in salad dressings, where the sodium alginate or ester of propylene glycol alginate (PGA) are used as stabilizers to prevent phase separation, with the same purpose as it is applied to stabilize the mayonnaise emulsion phase water/oil. Because of its low calorie (1.4 Kcal / g), these polymers can be used as "body agents" in the formulation of low calorie products such as mayonnaise and pasta. The bacterium *Azotobacter vinelandii* as well as

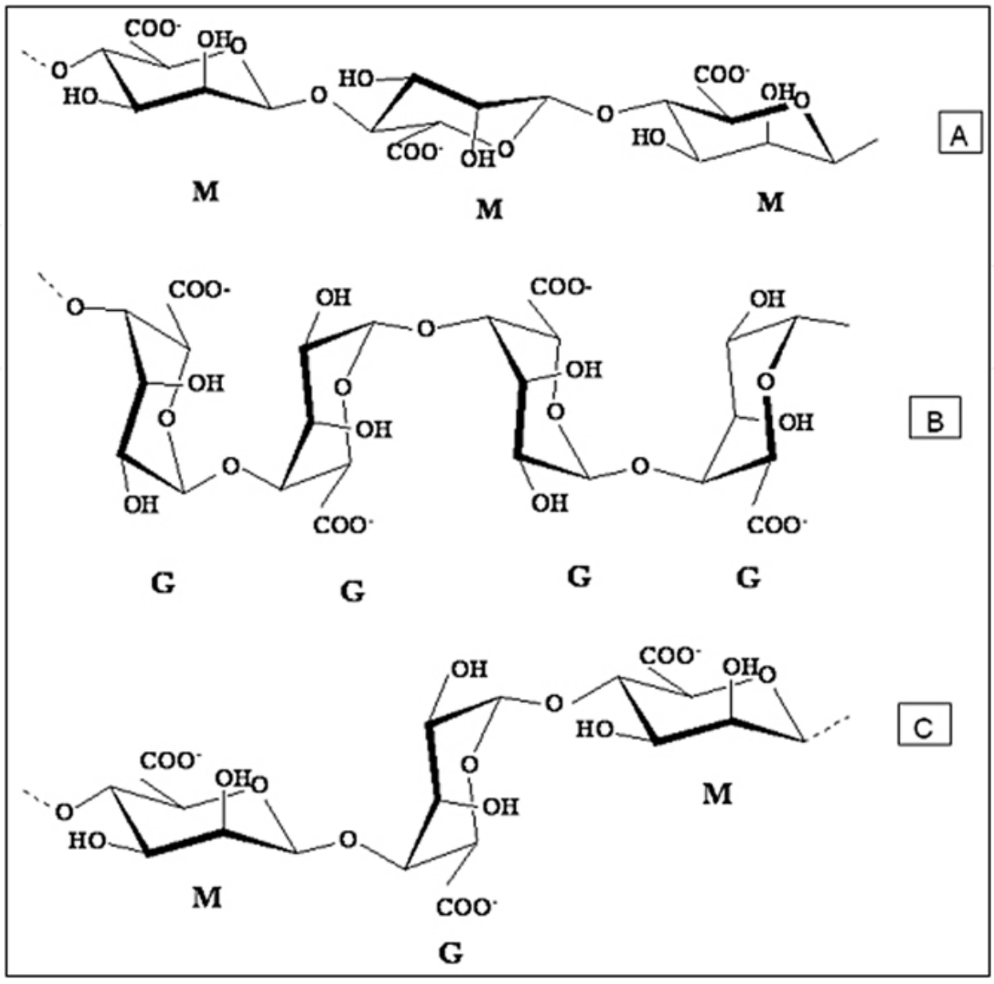


Fig. 1. Block structure homopolymeric M- and G-, and MG- block heteropolymeric, which constitute the molecule of alginate. At the top of the figure (A) has a sequence MM-; the center (B) and a GG- sequence at the bottom of the figure (C) a sequence MGM-. Adapted from Smidsrød (1970).

producing the alginate has another important feature: by limiting nutrients such as phosphorus, oxygen and the presence of an excess carbon source, produces polyhydroxyalkanoate (PHAs), intracellular polymers belonging to the polyesters family. The PHAs can be synthesized by many bacteria in bioreactors from sugars under stress. These polymers can represent up to 80% of total dry mass of the cell and are 100% biodegradable and biocompatible with the animal tissue. Conventional plastics of petrochemical origin, take decades to decompose in nature and also produce toxins during the degradation process. Therefore, there is a special interest in the plastics production from materials that can be easily eliminated from our environment (Suriyamongkol et al., 2007; Franchetti & Marconato, 2006). The PHAs are also known as bioplastics, they have thermoplastic properties and performance characteristics similar to those of conventional plastics, however, bioplastics are easily degraded by the microorganisms action in the environment. PHAs examples: polyhydroxybutyrate (PHB), poly-β-hydroxyvalerate (PHV) and polyhydroxybutyrate-co-valerate (PHB-V) (Franchetti & Marconato, 2006). (Fig. 2).

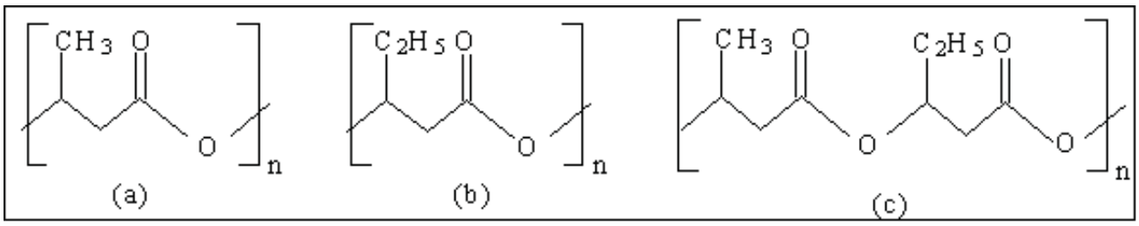


Fig. 2. Chemical structure of polyhydroxyalkanoates (PHAs): a) polyhydroxybutyrate, b) polyhydroxyvalerate, and c) polyhydroxybutyrate-co-valerate. Adapted from Franchetti & Marconato (2006).

Some possible PHAs applications include: biodegradable carriers that demonstrate the ability to deliver drugs for a given time within the individual's body, surgical needles, suture materials, bone tissue replacement, etc. The advantage of using biodegradable plastics is that it does not require surgical removal. Many microorganisms are producers of these PHAs and the *Azotobacter vinelandii* is a bacterium that can accumulate large PHB amounts with the advantage of use during its growth a wide variety of not necessarily refined sugars, like those found in cane sugar molasses, beet molasses and corn syrup.

Given the global alginate and polyhydroxybutyrate importance, this work shows the study of the both compounds production by the bacterium *Azotobacter vinelandii* in submerged fermentation using different parameters (pH, incubation temperature, incubation time, salt concentrations and also different molasses cane sugar concentrations as carbon source) and the production evaluation by methodology response surface used as a statistical tool.

2. Bacterium *Azotobacter vinelandii*

The *Azotobacteriaceae* family comprises of the *Azotobacter* genus, which are Gram-negative eubacteria that possess a cell wall complex consists of an outer membrane and an inner peptidoglycan layer containing muramic acid and murein. These bacteria reproduce by binary fission, live in soil and in fresh waters, and large ovoid cells have diameters of 1.5 to 2.0 micrometers. It can also exhibit characteristics such as the pleomorphism, varying their morphology from rod-shaped to cocci-shaped cells (Allman et al., 1990; García et al., 2002).

Bacteria of the *Azotobacter* genus are chemo-organotrophs, use sugars, alcohols and inorganic salts to grow. When free, setting an average of 10 mg of nitrogen per gram of carbohydrate (glucose) consumed and this activity requires molybdenum which can be partially replaced by vanadium. The optimum pH for growth when they fix nitrogen is 7.0 to 7.5. The *Azotobacter vinelandii* is polyploid, whose reproduction depends on the culture medium and cultivation conditions used, as well as the growth phase in which the organism (Allman et al., 1990). The biological functions that are attributed to the PHB present in *A. vinelandii* are different, being the main material to constitute a reserve of carbon and energy for the bacterial cell, which can be used during periods of nutrient limitation in the middle. These polymers store large amounts of carbon that remain condensed in the form of insoluble intracellular granules without affecting the osmotic pressure inside the cell (Page & Knosp, 1989).

Another function that is attributed to PHB in *A. vinelandii* is related to biological nitrogen fixation, specifically with the nitrogenase protection, ensuring the respiratory function of the bacterium in the absence of an exogenous carbon source by depriving the cell of a carbon and energy source rapidly oxidized. Thus, it allows maintaining an adequate respiratory

avoiding decrease the concentration of oxygen, thus contributing to the protection of this enzyme (Page et al., 1992; Almeida et al., 2004).
The *Azotobacter vinelandii* advantage is the PHB production during its growth through the use of a wide variety of refined sugars do not necessarily like those found in molasses cane sugar, beet sugar and corn syrup, for example. Another advantage is the easy attainment of this bacterium, since *A. vinelandii* is found in soils and in freshwater (Page et al., 1992).

3. Alginate

3.1 Alginate from seaweed

Brown algae (*Phaeophyceae*) containing alginate are in a diverse plants family which grow in rocky beaches or areas of the ocean with clear, rocky bottom. Some species are found at high tide, others occur in a belt along the beach, the depth shallower than 38 meters (the limit for sunlight penetration). Only a few brown algae species are used as commercial alginates source. The Giant kelp *Macrocystis pyrifera*, which grows abundantly on the shores of North America and South America, New Zealand, Australia and Africa, is the main alginate source supply of world. Are also used *Laminaria, Ecklonia* and *Aschophyllum nodosum* varieties which are also taken by some producers. These different brown algae species produce alginates with different ratios of polyguluronic acid structure, thus resulting in different properties and functions of these compounds (Glicksman, 1987).
Formaldehyde is usually added to the brown seaweed, post harvest, to prevent microbial growth during its storage and to fix the polyphenols in the algae before the alginate extraction. Knowing that formaldehyde is toxic, allergenic and possibly carcinogenic, the salt addition in *Ascophyllum nodosum* (*Phaeophyceae*) was tested as an alternative by Moen and colleagues (1999). The algae were harvested on the coast of Norway, where the salinity is about 30 ‰ from late autumn to early spring, and stored at 22 + / - 2 ° C under certain conditions. If the algae samples are stored without treatment loses quality well as the material for alginate production by up to 14 days. Treatments with formaldehyde (2% weight / weight) or 20% NaCl preserve algae for up to 46 days, but the technique is less aggressive with NaCl, it is still inconvenient and costly for the alginate industry, it is necessary controlling the temperature, pH, oxygen, and salt (Moen et al., 1999).

3.2 Bacterial alginate

The alginic acid structure consists of linear chains of residues β-D-mannuronic acid (M) joined by links type (1 → 4) and of its epimer, the α-L-guluronic acid (G) in various proportions. These residues are arranged in the form of blocks mannuronic acids (M) or guluronic (G), connected so that the sequence of these residues in the molecule is alternating. The polymer molecule is composed of homopolymer blocks, M-and G-and MG-block heteropolymer (Figure 1).
The physicochemical alginates properties depend on the molecular weight, the monomers M: G proportion along the chain and also the acetylation degree. The alginates are produced by bacteria and brown seaweed, and mannuronate residues of bacterial alginate O-acetylated in positions 2 and / or O-3.
Although seaweeds are usually the commercial alginates source, those of bacterial origin have been suggested as possible substitutes for the alginates from algae. For this purpose, studies initially focused on the opportunistic pathogenic bacterium *Pseudomonas aeruginosa* and then, three species non-pathogenic strains of *Pseudomonas*, including *P. mendocina*, *P.*

putida and *P. fluorescens*, and yet, the soil bacterium *Azotobacter vinelandii* (Brivonese & Sutherland, 1989). Most knowledge about the alginate biosynthesis comes from studies of *Pseudomonas aeruginosa*, mainly because of the medical relevance of this bacterium as an important opportunistic pathogen microorganism to humans, in patients suffering from cystic fibrosis (Alkawash et al., 2006; Govan & Harris, 1986; May & Chakrabarty, 1994). Herein, the alginates have an important role as a virulence factor. The reason for this seems to be alginate biofilm formation, which facilitates colonization of the lung (Gacesa & Russell, 1990).

A. vinelandii and *P. aeruginosa* produce alginate as an extracellular polysaccharide in vegetative cells, whereas the alginate production by *A. vinelandii* is involved in a differentiation process called "cyst" (Sadoff, 1975). This cyst is formed by the intracellular accumulation of polyhydroxybutyrate delimited within the cytoplasm by a membrane lipoprotein double wall when the bacterial cell is in an environment where, in general, there are large amounts of carbon source and nitrogen, phosphorus or oxygen limitation. When there is carbon source exhaustion, these cysts oxidize quickly (through the activation of the PHB depolymerases enzymes) and are used as energy sources.

The potential of alginate produced by bacteria, such as industrial polymers, is still a controversial subject. However, the possibility of using raw materials free of seasonal and geographical variations, and also selected strains under carefully controlled operating conditions, so as to meet specific applications in biotechnology and biomedicine may be sufficient to compensate for the relatively low production, and acetylation relatively high, the bacterial polymers (Clementi et al., 1999).

3.3 Bacterial alginate applications

The alginate gels are capable of forming micro-beds and incorporate whole living cells or enzymes, and this application has aroused interest in the food industry, biotechnology and biomedical sector (Smidsrød & Skjåk-bræk, 1990; Sabra et al., 2001).

The alginates are widely employed in the fruit analogues area or type imitation products. In 1946, Peschardt patented a process for making artificial cherries using a colored and flavored alginate solution with sugar, which was added in the form of drops in coagulant solution made with calcium salts soluble. A film of insoluble calcium alginate is formed immediately around the surface of the droplets. After dipping them in calcium salts solution, the ions penetrated by diffusion inside to gelation. Various textures types could be obtained through proper control of the type of calcium salt, concentration, time and temperature, etc. These cherries produced were not affected by heat and can be perfectly used in bakery products. Furthermore, had the advantage of providing uniformity in size, weight and quality. Similar products were developed using cherry puree in a system of alginate gelation, sold under the name "cherry shaped balls." Other type imitation products commercially found are: peppers imitation for stuffing olives, onion rings, caviar, meat and fish imitation, etc. (apud Glicksman, 1987).

3.4 Bacterial alginate biosynthesis

The alginate has an essential biological role in *Azotobacter vinelandii*, that when the vegetative cell becomes mature cysts layers of exine and intine present 32 and 13% of the dry weight of alginate, respectively. The mutant varieties of bacteria do not produce alginate and are unable to form mature cysts. The alginate extracellular accumulation acts as a

barrier to oxygen diffusion or heavy metals or as protection against other environmental insults (Segura et al., 2003).

In bacterium *A. vinelandii* the alginate is synthesized from fructose-6-P, which is converted by the phospho-mannose isomerase (PMI) in mannose-6-P and this, in turn, becomes mannose-1-P by the enzyme phospho-mano-mutase (PMM) action. The next step is the mannose-1-P activation by GDP-mannose-pyrophosphorylase (GPMP) resulting in GDP-mannose formation, which is oxidized to GDP-mannuronic acid by the enzyme GDP-mannose dehydrogenase (GMD). The GDP-mannuronic acid is the substrate that is polymerized in the inner membrane to form polymannuronic acid. In periplasm some mannuronic residues of the polymannuronic acid are acetylated by acetylase. The polymer is secreted outside the cell where some manurônic residues not acetylated are epimerized to guluronic residues by multiple extracellular epimerases resulting in alginate. (Fig. 3).

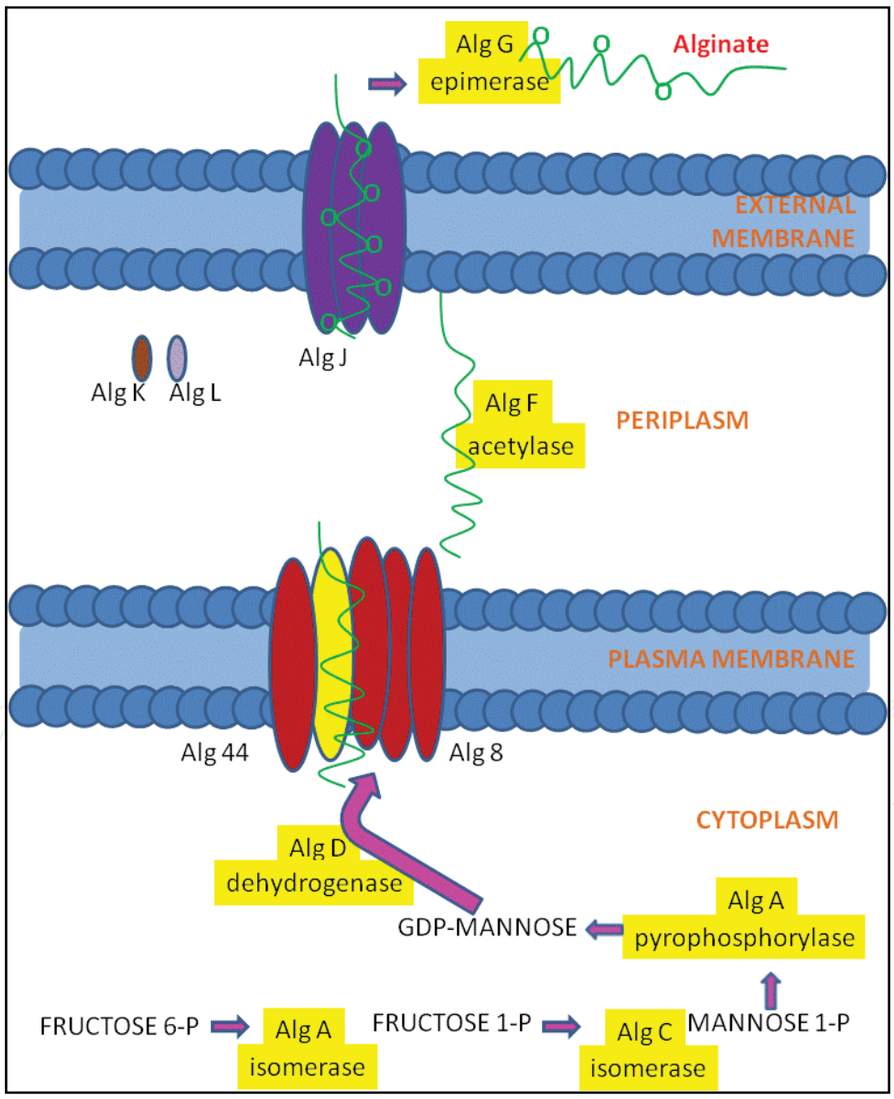


Fig. 3. Alginate biosynthesis by *Azotobacter vinelandii*. Adapted from Trujillo-Roldan et al., (2003).

The genes that encode enzymes involved in alginate synthesis, modification and excretion, were identified in *A. vinelandii*. All structural genes are clustered on the chromosome and are transcribed from multiple promoters, except algC gene that encodes the enzyme phospho-mano-mutase. The algD gene, which encodes GDP- mannose dehydrogenase (GMD) is transcribed from three promoters. Genes alg8, alg44, algK algJ which are located below the algD are organized into a transcription unit and their products participate in the alginate polymerization and excretion, the product of the alg8 is a glycosyl transferase, which has been proposed as responsible for polymerase activity, the alg44 encodes another inner membrane protein, which is responsible for part of the complex polymerization and also transports the polymer to the periplasm, the algJ encodes a protein of the outer membrane and present activity in the ion channel that is essential for the alginate excretion and algK gene product is a periplasmic protein that can participate in the AlgJ incorporation in the outer membrane. The algG gene encodes the epimerase and algL the enzyme alginase. Genes algX, algV, algI and algF are responsible for acetylation of mannuronic residues in the periplasm and as the algA product form bifunctional enzyme that catalyzes the first and third step of the biosynthesis (Gaona et al., 2004; Steigedal et al., 2008).

3.5 Bacterial alginate production

Horan and colleagues (1983) noted that when *Azotobacter vinelandii* mutant was grown in continuous culture, the polysaccharide amount produced depended on the dissolved oxygen concentration and also the carbon source. Changes in specific activity of key enzymes in the alginate biosynthesis (phosphomannose isomerase and GDP-mannose pyrophosphorylase), measured in cells extracts grown within a range of values of dissolved oxygen concentration were reflected by changes observed in the alginate production, whereas that the activity of GDP-mannose dehydrogenase remained unchanged. A similar correlation between the specific activities of these enzymes and the rate of alginate production was observed in this study during the transition of sorbitol to sucrose as sole carbon source, but the activity of GDP-mannose dehydrogenase also increased with increased alginate production. After prolonged continuous cultivation in sucrose the mutant lost the ability to produce alginate. The key enzymes in the alginate biosynthesis was not detected in extracts of this strain does not produce alginate, which had also lost the ability to form cysts. These results support the view that the alginate formation is controlled by the key enzymes induction in the alginate biosynthesis and the alginate has an important role in cyst formation by bacteria (Horan et al., 1983).

Some factors that influence the alginic acid production by *Azotobacter vinelandii* were investigated in batch culture by Brivonese & Sutherland (1989). We observed that the highest alginate yields (6.0 to 7.5 mg mL^{-1} supernatant) occurred in rich medium in nitrogen and phosphate, and glucose as carbon source, by aerating the medium with agitation at 280 rpm. The oxygen importance was evident when, at 120 rpm, the alginate production decreased to 1.4 mg mL^{-1}. A 120 rpm intracellular polyhydroxybutyric acid (a polymer whose intracellular accumulation is associated with the limitation of oxygen) accumulation was 40% versus 30% at 280 rpm. The inorganic phosphate presence was considered important for the growth, because in medium with low salts concentration resulted in decreasing production, which was not improved by the nitrogen sources addition like nitrate and glutamate. The glucose replacement by sucrose also reduced the alginate production.

In a similar study, Savalgi & Savalgi (1992) found that the largest alginic acid amounts produced by *Azotobacter vinelandii* NCIB 9068 (5.5 - 6.2 mg mL^{-1} supernatant) in flasks, were obtained when growth was conducted in a medium rich in nitrogen-limited phosphate, and sucrose as carbon source under shaking at 240 rpm. Also observed reduction in the alginate production of to 1.6 mg mL^{-1}, with the decrease in agitation (140 rpm). In contrast to Brivonese & Sutherland (1989), the sucrose replacement by glucose reduced the growth and the alginate production.

The authors Peña and colleagues (1997) had a higher alginate concentration under low aeration conditions (conventional Erlenmeyer flasks). This phenomenon may be related to oxygen sensitivity by some enzymes involved in the alginate biosynthesis by *A. vinelandii*. The results are consistent with those observed by Chen and colleagues (1985), which indicated that the alginate concentration was increased to 170 rpm (for values between 110 and 200 rpm) for *A. vinelandii* culture conducted in shake flasks. An increase in stirring speed (ie greater aeration) was unfavorable for the alginate production.

The culture broth viscosity was greater at lower aeration, suggesting an important effect of oxygen on the molecular characteristics (ie molecular weight) polymer. It is known that the alginate solutions viscosity is strongly influenced by the molecular weight distribution, but not both, content and distribution of the two monomers of the alginate molecule (Martinsen et al., 1991). However, no previous data published on the culture broth viscosity under different growing conditions. Peña and colleagues (1997) observed that the alginate acetylation degree produced by *A. vinelandii* was independent of the aeration conditions.

Clementi et al (1995) observed that the rate of alginate synthesis of drops rapidly to zero as a result of a drastic drop in pH in the *A. vinelandii* culture. However, the study by Peña and colleagues (1997), the alginate formation rate was not affected by pH drop experienced in the stationary phase of growth.

4. Bioplastics or biodegradable plastics

Biodegradable plastics are degraded by microorganisms when discarded in soil and landfills. This degradation results primarily from the microorganisms action such as fungi, bacteria and algae, generating CO_2, CH_4, cellular components and other products, as stipulated in "American Standard for Testing and Methods" (ASTM D-833). Thereby, biodegradable plastics are materials which degrade into carbon dioxide, water and biomass, as a result of the living organisms or enzymes action (Franchetti & Marconato, 2006; Suriyamongkol et al., 2007; Van-Thuoc et al., 2008).

The intracellular PHAs are polymers that can be synthesized by many bacteria in bioreactors from sugars in growth conditions characterized by carbon source excessive and other nutrients limitation such as nitrogen and phosphorus (Franchetti & Marconato, 2006; Albuquerque et al., 2007; Suzuki et al., 2008). Although many microorganisms have the ability to accumulate this polymer, a limited number is considered good candidates for industrial PHB production. Among them, *Cupriavidus necator* (formerly *Ralstonia eutropha*), *Alcaligenes latus*, *Azotobacter vinelandii* and recombinant *Escherichia coli* have demonstrated the highest polyester accumulations (about 70-90% of dry cell mass). The microorganism *Cupriavidus necator* requires limiting nutrients during fermentation to then produce PHB. This microorganism has accumulated more PHB during the stationary phase of growth. The other three bacteria do not require nutrient limitation for initiating the PHB synthesis despite the nutrient limitation in the culture medium promote greater accumulation of this

polymer within the cells. In some of these cases, these complex nitrogen sources such as yeast extract or fish peptone may be used to enhance cell growth and, in turn, the volumetric productivity (Quillaguamán et al., 2008).
Previous studies showed that enterobacteria did not accumulate PHAs naturally, requiring the cloned genes introduction. However, Lugg and colleagues (2008) studied enterobacteria of natural occurrence, *Serratia* sp, and observed that it was able to accumulate PHB (about 50% of cell dry weight) in medium containing excess carbon source and a deficiency in nitrogen.
The synthesis and incorporation of different monomers depend on the provision of a suitable substrate that can be converted into the desired hydroxyacyl-CoA through metabolic reactions in the bacterial cell. Furthermore, it is necessary that the bacterial cell contains an enzyme called PHA synthase capable of incorporating the hydroxyacyl-CoA synthesized a polymer chain.

4.1 Polyhydroxyalkanoate

The inclusions presence in the cytoplasm, like lipid inclusions, which were soluble in chloroform were first observed in *Azotobacter chroococcum* by 1900. The chemical inclusions composition found in similar bacterium *Bacillus megaterium* was identified in 1926 as the acid-poly 3-hydroxybutyrate (P(3HB)), by Lemoigne. In the late '50s, studies of the *Bacillus* genus suggested that the P(3HB) had the intracellular function of carbon reserves and energy for this bacterium. In 1974, Wallen and Rohwedder identified other polyhydroxyalkanoate beyond the P(3HB), extracted from activated sludge.. An interesting work was also done by Witholt and coworkers in 1983, when cultured *Pseudomonas oleovorans* on n-octane. An elementary analysis of the polymer showed that intracellular bacteria mainly accumulated 3-hydroxyoctanoate and small amounts of 3-hydroxyhexanoate (apud Sudesh et al., 2000, p.: 1504).
The copolymer P(3HB-co-3HV) was produced commercially by Zeneca Bioproducts, England, from glucose and propionic acid, using a mutant strain of *Ralstonia eutropha*. The polyester obtained from this process was put on the market, with the name Biopol (Byrom, 1992). The Biopol is used since 1990 in Germany in the shampoo bottles production for the cosmetics industry Wella (Braunegg et al., 1998). Other products such as disposable razors, also made by Biopol, were tested in Japan. Cups made of pure PHA or internally coated with a Biopol film, as sealant, have been used. The PHA blends with conventional plastics such as polypropylene, were also tested and currently Monsanto (USA) has the right to patent this product (Byrom, 1992).
PHB has properties similar to polypropylene with three unique features: thermoplastic processability, 100% water resistance and 100% biodegradability (Hrabak, 1992). Boom, Giacino and Selker (1994) established that PHB is an aliphatic homopolymer with melting point of 179 ° C and highly crystalline (80%). The molecular weight of PHB decreases by about half the original value when the latter is maintained at 190 º C for 1 hour. The PHAs have physical properties ranging from brittle and thermally unstable until soft and tough, depending on their composition. The PHB physical properties, for example, crystallization and high tensile strength depends on the molecular weight, which is influenced by the species of microorganism used, growth conditions and purity of the samples (Punrattanasin, 2001; Heo et al., 2008).

4.2 Polyhydroxyalkanoate applications

An international standard ISO 10993-3 (1982) lays down specific requirements for biocompatibility, including tests based on the nature of contact and residence time of the implanted biomaterial. The standard requires that all materials that remain in contact with mucous membranes, bone or dental tissues, in which the contact exceeds 30 days, and all implantable devices in the contact exceeds 24 hours, should be tested for genotoxicity. Based on the ISO standard 10993-3 (1982) and ASTM (1987) recommendations, many test methods are needed to determine the genotoxic and mutagenic activities of the implant. These include tests for toxicity, mutagenesis, chromosomal aberrations, etc (Ali et al., 2008).

Although the P(3HB) degradation product, D(-)-3-hydroxybutyric acid, is an intermediate metabolite of many organisms (Lafferty et al., 1988; Lee, 1996), Ali and colleagues (2008) performed the mutagenicity study of polyhydroxybutyrate test using the *Salmonella*/microsome and found that the PHB is not genotoxic and does not alter the expression of proto-oncogenes and anti-apoptotic gene considered in the study. Thus, it is plausible that there animal biocompatibility with the P(3HB) and can therefore be deployed in their tissues without any toxicity.

Some possible PHAs applications include: biodegradable carriers that have the function of drug release for a given time within the individual's body; surgical needles; sutures; bone tissue replacement; etc. The biodegradable plastics advantage is not requiring surgical removal (Punrattanasin, 2001).

Another advantage of using PHB in drug delivery systems is that the drug concentration in patient blood and / or tissue can be maintained at a desired level for a long time. The main requirement for the drugs manufacture with a release system and prolonged action is the availability of a suitable capsule material, which should be absolutely harmless to the body and possess the physical and mechanical properties and biomedical necessary, including their degradation in the middle biological. Other materials are widely used in controlled drug, such as gelatin, proteins, etc. The PHAs are available in chemically pure form and their degradation rate is low when compared with the compounds mentioned above, indeed it is very important in prolonged treatments. By varying the PHAs chemical structure or their mixture with different materials, you can control the porosity and degradation rate of polymer matrix and, consequently, the rate of drug release. Currently, biodegradable carriers are used to carry anti-depressants, contraceptives, antineoplastic and antiinflammatory (Shishatskaya et al., 2008; Suzuki et al., 2008).

The PHAs are biodegradable in soil and can be used as porters to release pesticides, herbicides or fertilizers for a given time in the soil, such as containers and packaging, as biodegradable matrix for drug delivery in veterinary medicine, etc.

Possible PHAs applications include plastic films, bags, containers, razors, household items and a variety of products found in everyday life.

4.3 Polyhydroxybutyrate biosynthesis

The PHB production in the bacterium *A. vinelandii* involves three enzymes. At the beginning process is the condensation of two acetyl-CoA molecules by the enzyme β-ketothiolase to generate acetoacetyl-CoA, which is reduced by acetoacetyl-CoA reductase using NADPH, producing D(-)-β-hydroxybutyryl-CoA, which is finally polymerized by PHB synthase resulting in PHB (Segura et al., 2003).

In general, bacteria producing PHB present the accumulation of this polymer in response to interference in its growth, mainly due to lack of nutrients such as nitrogen, phosphorus, magnesium and oxygen, and the presence of an excess carbon source (Burns et al., 2007).
The control point is the major activity of the enzyme β-ketothiolase. This enzyme is activated when the acetyl-CoA concentration is high, a situation that results from the accumulation of NADH or NADPH in response to low oxygen concentration in the medium. With these metabolites, occurs enzymes inhibition (citrate synthase and isocitrate dehydrogenase) of the Krebs cycle by reducing the carbon flow into the cycle and leading to an increased of acetyl-CoA concentration, a fact that stimulates the β-ketothiolase activity. These conditions also favor the enzyme activity (acetoacetyl-CoA reductase) that catalyzes the second step of the process (Page et al., 1992).
Many researchers have identified the structural genes responsible for PHB synthesis in *A. vinelandii*. It was observed that the phbA gene encoding the β-ketothiolase and phbC and phbB genes codify the second and third enzyme, respectively, the step of PHB biosynthesis (Segura et al., 2003). (Fig. 4).

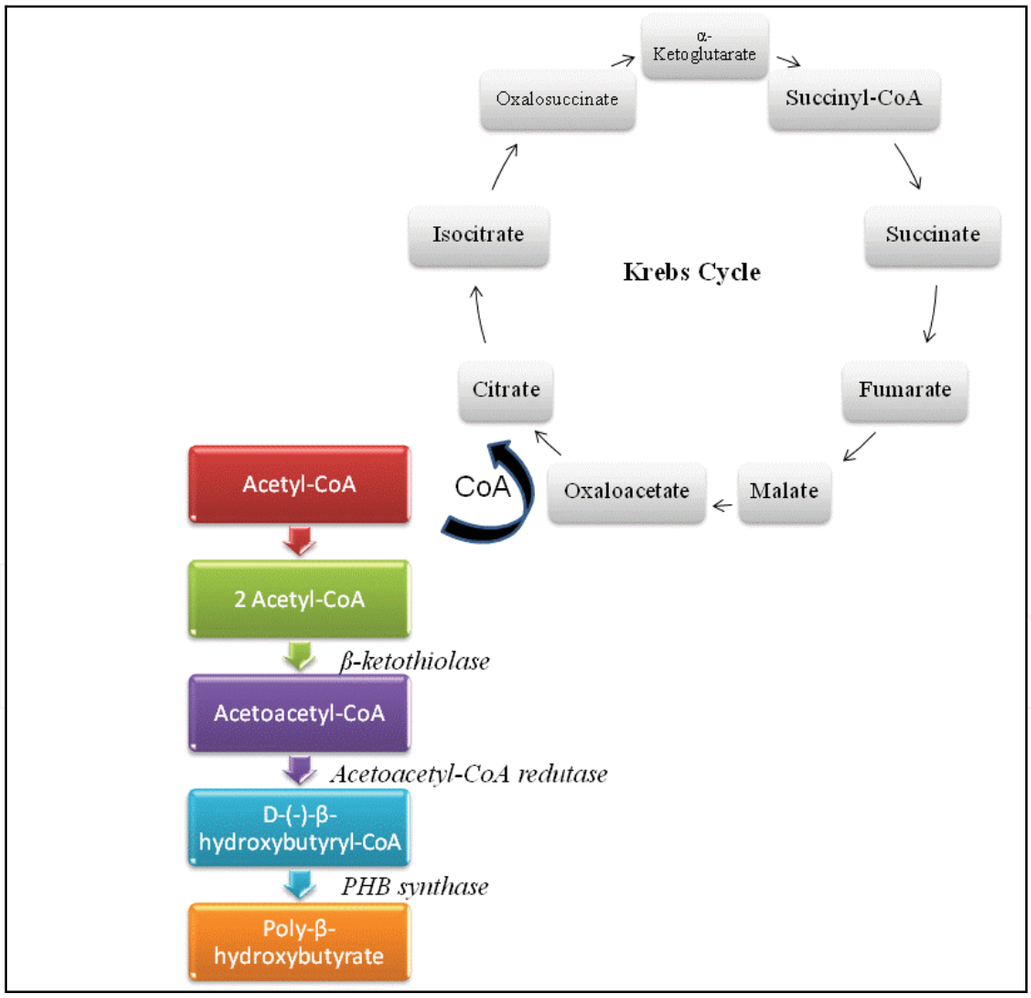


Fig. 4. Polyhydroxybutyrate biosynthesis and Krebs Cycle by *Azotobacter vinelandii*. Adapted from Segura et al., (2003).

4.4 Polyhydroxyalkanoate production

The PHA production using bacteria involves the following steps: maintaining the bacterial culture in appropriate medium, pre-fermentation and/or fermentation. During the pre-fermentation and fermentation, the microorganism is cultivated in medium containing mineral salts and one or more carbon sources. The carbon sources can be refined sugar like sucrose and glucose or more complex sources such as beet molasses, sugar cane molasses, whey and others agro-industrial residues (Page & Knosp, 1989).

5. Purpose of the study

The alginate is a polysaccharide extracted from cell walls of brown seaweed used in food, pharmaceutical and biotechnology industries. The production is concentrated in the brown seaweed cultivation, but several bacteria, *Pseudomonas* and *Azotobacter* genus, produce alginate. The chemical alginate structure by algae produced is similar to those synthesized by *A. vinelandii*. Thus there is the possibility of using raw materials free of seasonal and geographical variations, and also selected strains under carefully controlled operating conditions, so as to meet specific applications in biotechnology and biomedicine. This bacterium also produces intracellular polymers such as polyhydroxybutyrate (PHB), known as bioplastic. The PHB has properties similar to polypropylene with three unique features: thermoplastic processability, 100% water resistance and 100% biodegradability. The *Azotobacter vinelandii* advantage is the PHB production during growth using a wide sugars variety like those found in cane sugar molasses, beet molasses and corn syrup, for example. Another advantage is the easy attainment of this bacterium, because *A. vinelandii* is found in soil and also in freshwater

6. Materials and methods

6.1 Microorganism

The microorganism used was the *Azotobacter vinelandii* CCT 2841 obtained from the Research and Technology André Tosello Foundation, Campinas – SP, Brazil.

6.2 Culture medium maintenance

Azotobacter vinelandii CCT 2841 was maintained in agar Yeast Medium (YM) whose formula is given below (in grams per liter): yeast extract 3.0, malt extract 3.0, peptone 5.0, dextrose 10.0, agar 20.0 and 1.0 L distilled water.

6.3 Medium fermentation

For the polyhydroxybutyrate and alginate production was used minimal or basal medium adding cane sugar molasses as carbon source.

6.4 Minimal or basal medium

The minimal medium used was described by Garcia and colleagues (2002), composed for the following components (in grams per liter): KH_2PO_4 0.16, K_2HPO_4 0.64; $MgSO_4.7H_2O$ 0.4, NaCl 0.2, $CaSO_4.2H_2O$ 0.05; $FeSO_4.7H_2O$ 0.0025 and $Na_2MoO_4.2H_2O$ 0.001. The pH was adjusted according to the experimental design and sterilized in autoclave at 121 °C for 20 minutes. The minimal medium was sterilized separately the cane sugar molasses solutions at 121 °C for 20 minutes. Cane sugar molasses was diluted to 30 Brix with distilled water

and subsequently clarified. After cool and sit for 24 h was used a refractometer to adjust the desired soluble solids concentration with sterile distilled water. Next, the flasks containing solution of cane sugar molasses in desired concentrations were sterilized in autoclave at 121 °C for 20 minutes. The basal or minimal medium was added to the fermentation stage.

7. Fermentation conditions

7.1 Pre-inoculum preparation

Azotobacter vinelandii CCT 2841 from the stock culture was transferred for tubes containing Plate Count Agar (PCA) inclined, which were incubated in an oven at 30 ^{o}C for 24 hours.

7.2 Pre-fermentation

With the pre-inoculum previously obtained, was realized the bacterial cells suspension by adding 5.0 mL of nutrient broth contained in the flask (total of 50.0 mL nutrient broth), and then moved the suspension to the same flask. The vials were incubated in an orbital shaker rotating at 30 °C for 24 h and 225 rpm (Page & Knosp, 1989; Page et al., 1992).

7.3 Polyhydroxybutyrate and alginate production

In a bucket spectrophotometer (Cintra 5 UV-VIS Doublebar "), was added 3.0 mL of minimal or basal medium and culture medium drops obtained in the pre-fermentation to reach an optical density of 0.9 at 620 nm wavelength. For all experiments, the *Azotobacter vinelandii* CCT 2841 inoculum was standardized in 0.74 mg dry weight/mL, corresponding to 0.9 absorbance of the suspension at 620 nm. Soon after, the flasks were incubated in an orbital shaker rotating at 120 rpm, temperature and incubation time predetermined by the experimental design.

7.4 Fermentation parameters optimization

For the PHB (Y1 = polyhydroxybutyrate) and alginate (Y2 = alginate) optimization production were carried out two experimental design to determine the best production area of both compounds. Thus, in the first experiment was performed a statistical fractional factorial design 2^{6-2} and the independent variables were: X1 = soluble solids concentration (%), X2 = pH, X3 = incubation temperature (^{o}C), X4 = ammonium acetate (mmol L^{-1}), X5 = ammonium citrate and iron (III) (mol L^{-1}) and X6 = incubation time (h), resulting in 16 experiments plus 2 replicates at the central point (Table 1).

Variables	Levels		
	-1	0	+1
X1 – Soluble solids concentration (%)	1.0	3.0	5.0
X2 – pH	6.0	7.0	8.0
X3 – Incubation temperature (^{o}C)	25.0	32.5	40.0
X4 – Ammonium acetate (mmol L^{-1})	15.0	37.5	60.0
X5 – Ammonium citrate and iron (III) (µmol L^{-1})	30.0	60.0	90.0
X6 – Incubation time (h)	48.0	72.0	96.0

Table 1. Independent variables in the first experimental design 2^{6-2}.

With the prospect approaching the optimum production region was conducted a second experimental design for sugar cane molasses by means independent variables: X1 = carbon source; X3 = incubation temperature and X6 = incubation time, resulting in a full factorial statistical design 3^{3-0} (Table 2).

Variables	Levels		
	-1	0	+1
X1 - Soluble solids concentration (%)	5.0	15.0	25.0
X2 - Incubation temperature (°C)	40.0	50.0	60.0
X3 - Incubation time (h)	12.0	30.0	48.0

Table 2. Independent variables in the second experimental design 3^{3-0} for sugar cane molasses.

The second experiment for sugar cane molasses was made with the variables fixed: initial pH = 7.0, ammonium acetate = 60.0 mmol L^{-1}, ammonium citrate and iron (III) = 90.0 mmol L^{-1}.

7.5 Standard curve for biomass determination

To construct the calibration curve of cell concentration was used *Azotobacter vinelandii* CCT 2841 suspension obtained after the pre-fermentation. In addition, absorbance readings were made at 620 nm (spectrophotometer Cintra 5 UV-VIS "DoubleBeam") with samples of the original cell suspension and the diluted corresponding to cell dry weight of each sample for built up a calibration curve (relating the absorbance and biomass in mg mL^{-1}).

7.6 Biomass determination

The cell mass was determined by measuring in a spectrophotometer at 620 nm a cell suspension in distilled water, separating it by centrifugation at 7077g, 15 minutes at 4 °C. Previously, was obtained a correlation plot of absorbance versus cell concentration. All tests were performed in triplicate.

7.7 Polyhydroxybutyrate and alginate extraction

The cells were separated from the fermentation medium by centrifugation at 7077g, for 15 minutes at 4 °C. After this procedure, the PHB intracellular was extracted of the cell mass with chloroform and the solution was filtered with paper filter. Next, was added three times the absolute ethanol volume for PHB precipitation. The supernatant obtained on centrifugation was used to obtain the alginate. Thus, added three times the absolute ethanol volume for alginate precipitation. Both were dried in vacuum oven at 45 °C to constant weight.

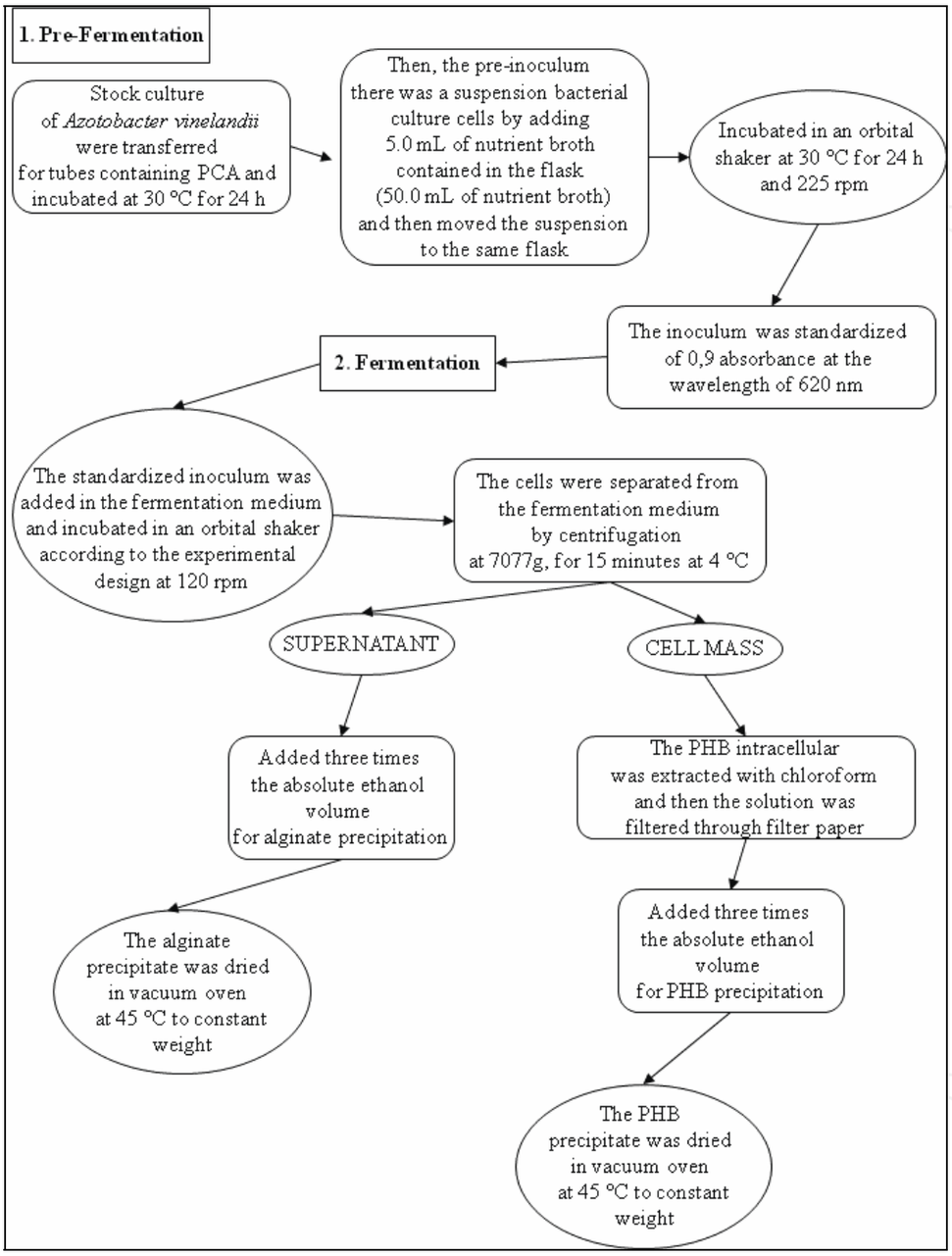
7.8 Polyhydroxybutyrate and alginate purification

The purification was performed by dissolving PHB in sodium hydroxide 1 N and precipitated again with absolute ethanol (Lin & Sadoff, 1968; Pouton & Akhtar, 1996). The alginate dried was dissolved in distilled water and precipitated again with absolute ethanol. Both procedures were repeated three times.

7.9 Polyhydroxybutyrate purity determination

The PHB purity determination was performed the Law & Slepecky (1961) method based in hot reaction, the polymer obtained (PHB) with concentrated sulfuric acid of high purity analytical results in crotonic acid, whose absorbance is measured in 235 nm.

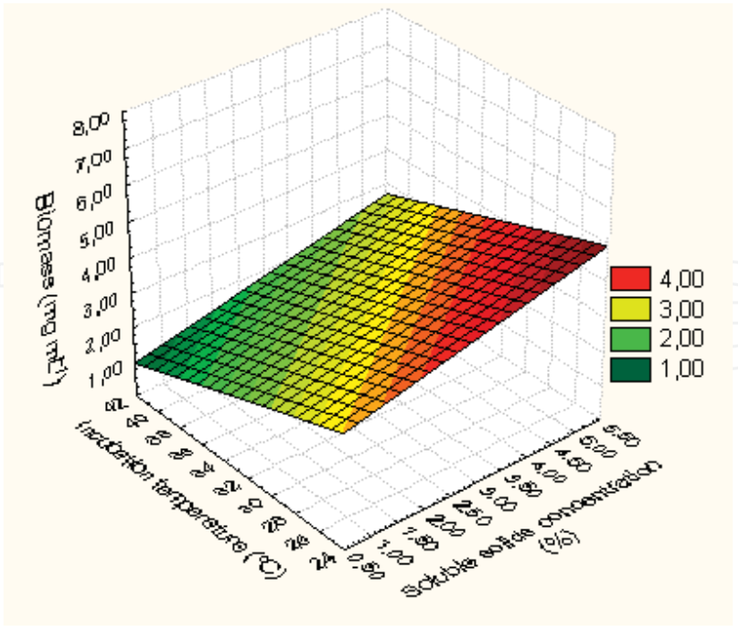
8. Production flowchart



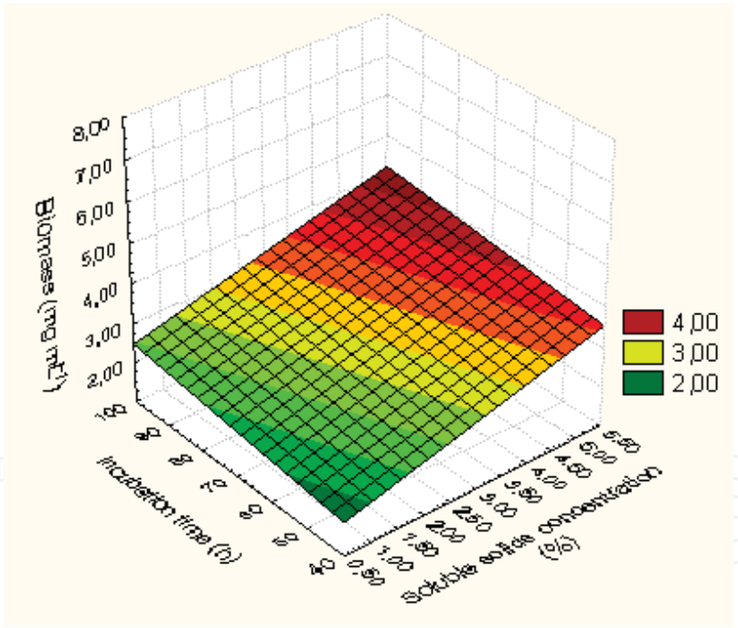
9. Results and discussion

9.1 First experimental design

With the help of the response surface methodology can be observed that the biomass (5 mg mL^{-1}) was favored by higher soluble solids concentrations. The lower incubation temperature (24 °C) and the longest incubation time favored cell growth, this is due to the need for more time to adapt to this fermentation medium, since the molasses is a medium nutritionally complex. (Fig. 5).



(a)

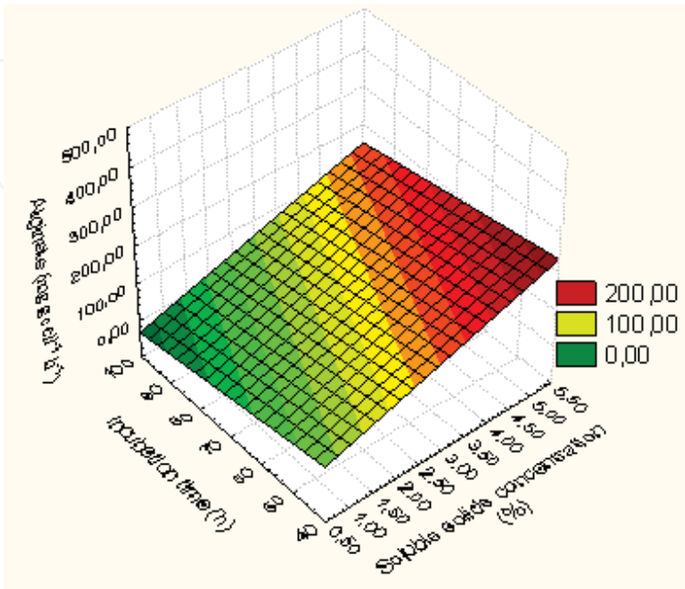


(b)

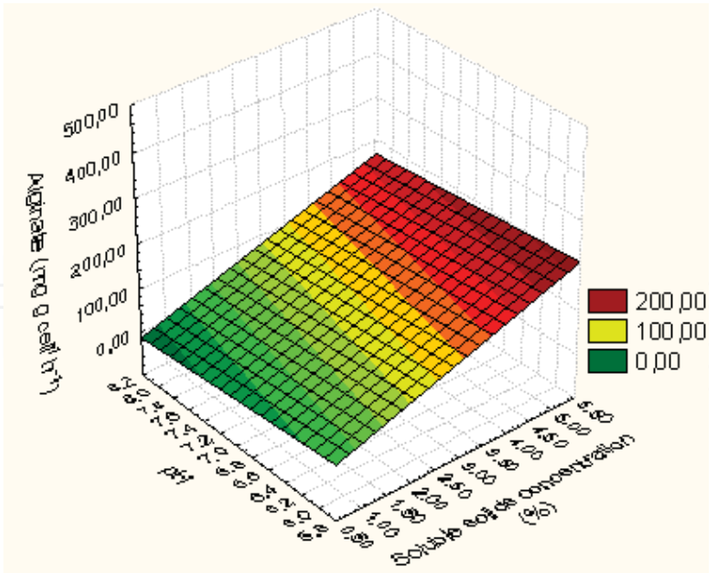
Fig. 5. (a) Surface response for biomass (mg mL^{-1}) around the optimal values of soluble solids concentration (%) and incubation temperature (^oC) (equation 6.17 + 0.34 * x – 0.13 * y), (b) biomass response areas (mg mL^{-1}) around the optimal values of soluble solids concentration (%) and incubation time (h) (equation 1.23 + 0.34 * x + 0.011 * y).

The temperature had a positive effect on the alginate production, with a higher exopolysaccharide synthesis by bacteria in response to high temperature (40 - 42 °C), functioning as a defense to the environment with a high temperature. The productivity was

also higher with soluble solids concentration increasing. Already, the shorter the incubation time (40 - 50 h) and pH (5.8 - 7.4) increased the alginate production. (Fig. 6).
The maximum alginate yield obtained using sugar cane molasses as carbon source was between 200 - 280 mg g cell^{-1} h^{-1}.



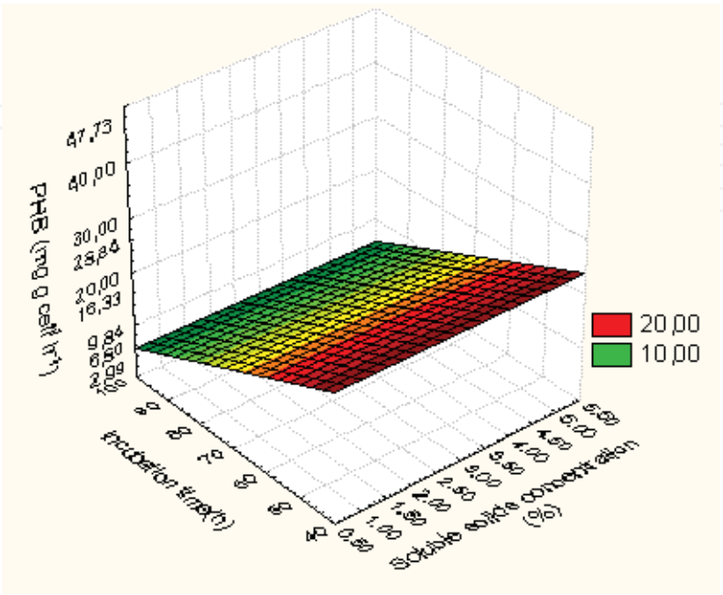
(a)



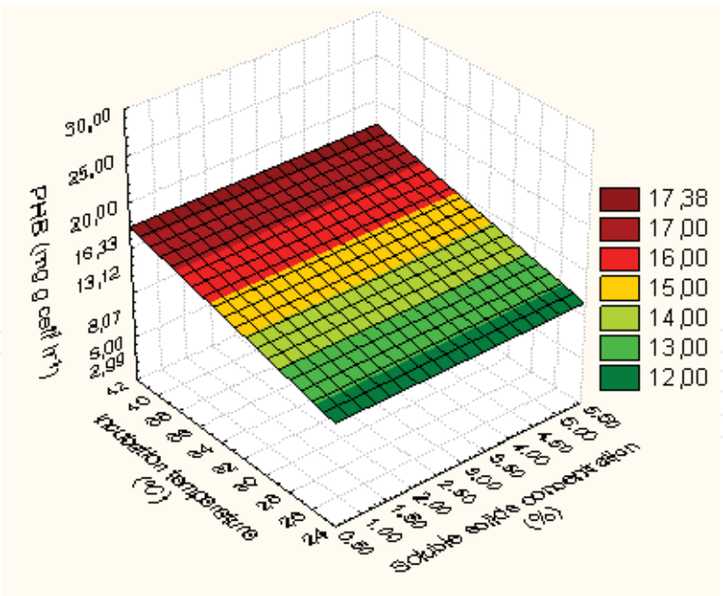
(b)

Fig. 6. Response surfaces for: (a) alginate productivity (mg g cell^{-1} h^{-1}) around the optimal values of soluble solids concentration (%) and incubation time (h) (equation: 88.73 + 36.68 * x – 1.39 * y), (b) alginate productivity (mg g cell^{-1} h^{-1}) around the optimal values of soluble solids concentration (%) and pH (equation: 102.06 + 36.68 * x – 16.26 * y).

The PHB production was maximum (PHB = 27 mg g cell^{-1} h^{-1}) in the shortest incubation time (40 h) and higher incubation temperature (42 ^{o}C). Since the sugar cane molasses concentrations tested were not significant for PHB production as shown in Figure 7.
The presented purity PHB extracted between 93.0 and 95.0%.



(a)

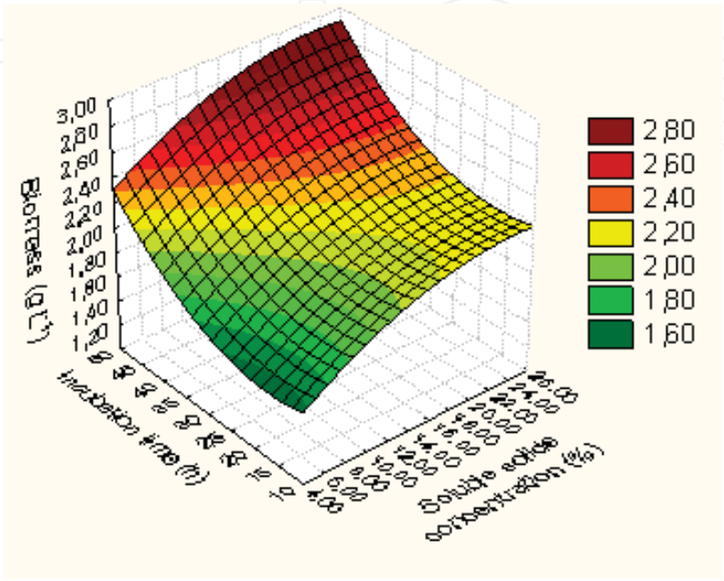


(b)

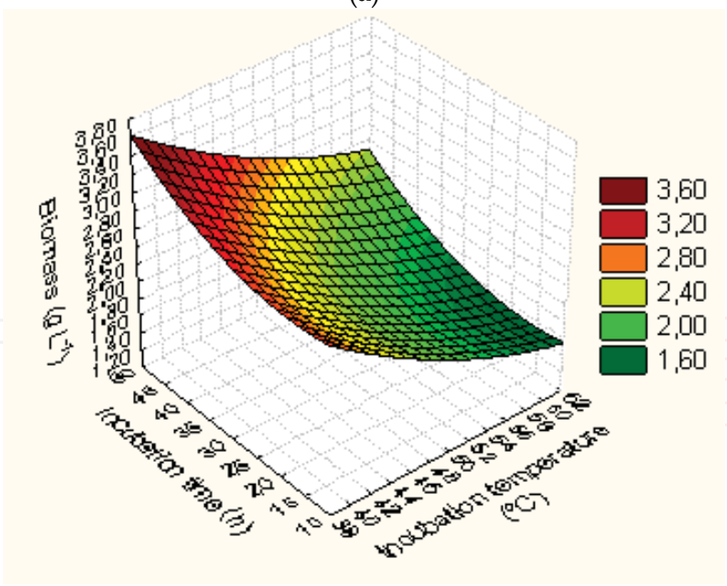
Fig. 7. Response surfaces for: (a) PHB yield (mg g cell^{-1} h^{-1}) around the optimal values of soluble solids concentration (%) and incubation time (h) (equation: 36.43 -0.0053 * x – 0.31 * y), (b) PHB yield (mg g cell^{-1} h^{-1}) around the optimal values of soluble solids concentration (%) and incubation temperature (^{o}C) (equation 3.57 -0.0053 * x + 0.33 * y).

9.2 Second experimental design

With the second experimental design (3^{3-0}) using the soluble solids concentration, temperature and incubation time was possible to prove that the biomass increase (2.8 - 3.6 mg mL-1) was accompanied by the soluble solids concentration (12 - 26%). Noted in the first experimental design that bacterial growth was less pronounced in the incubation temperature tested (38 – 40 °C) and the longest incubation time measured (45 – 50 h). (Fig. 8).



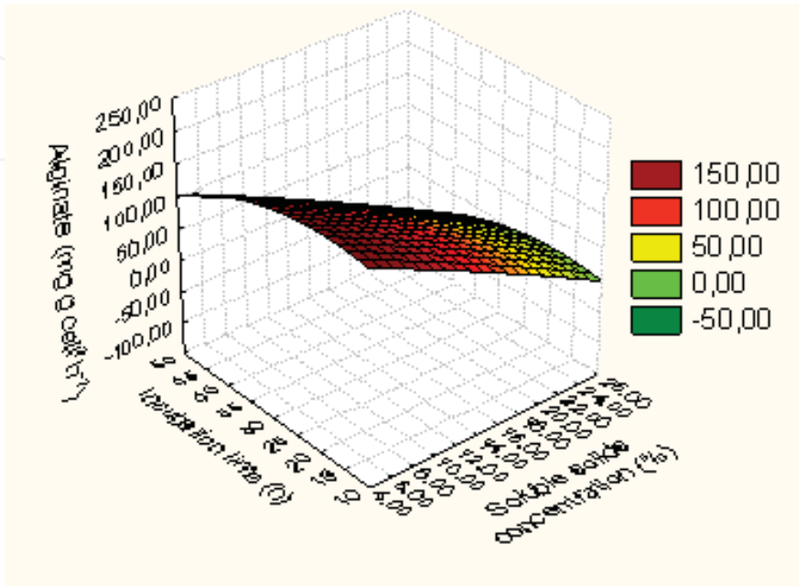
(a)



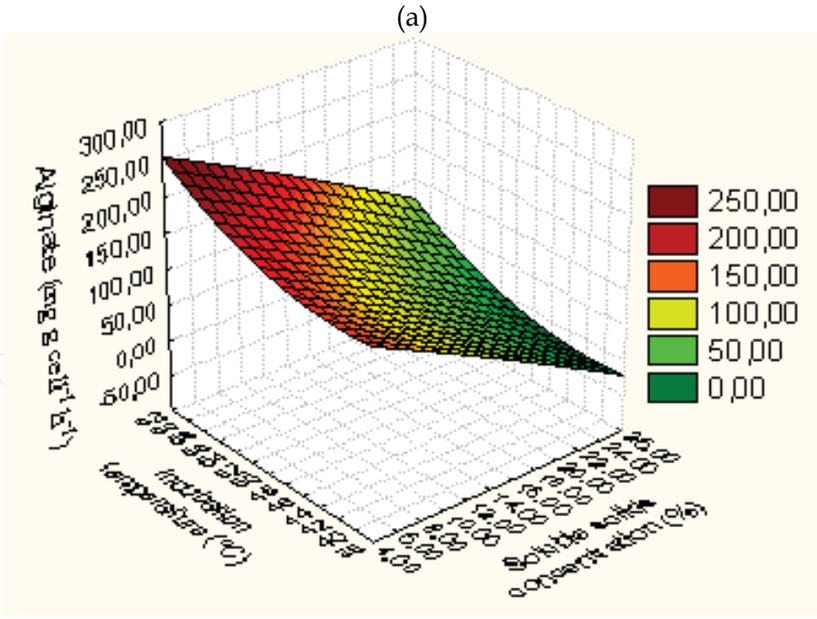
(b)

Fig. 8. (a) Surface response for biomass (mg mL^{-1}) around the optimal values of soluble solids concentration (%) and incubation time (h) (equation $7.32 + 0.072 * x - 0.0015 * x^2 - 0.025 * y + 0.00072 * y^2 - 5.83$), (b) biomass response areas (mg mL^{-1}) around the optimal values of incubation temperature (°C) and incubation time (h) (equation: $7.32 - 0.18 * x + 0.0012 * x^2 - 0.025 * y + 0.00072 * y^2 + 0.75$).

During the study of alginate productivity (maximum 200 - 250 mg g cell^{-1} h^{-1}) can be observed that the optimal soluble solids concentration was between 4.0 and 8.0%, a fact consistent with the behavior of first experiment for molasses, in which the optimal concentration was 4.5 - 5.5%. The ideal temperature is between 58 - 62 °C, at high incubation temperatures and shorter incubation (10 - 40 h) had the highest alginate synthesis, as shown in Figure 9.



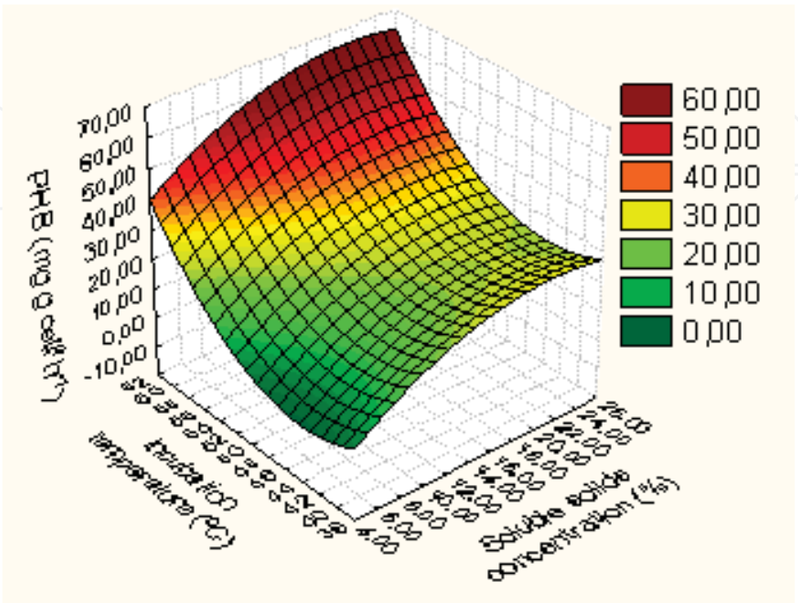
(a)



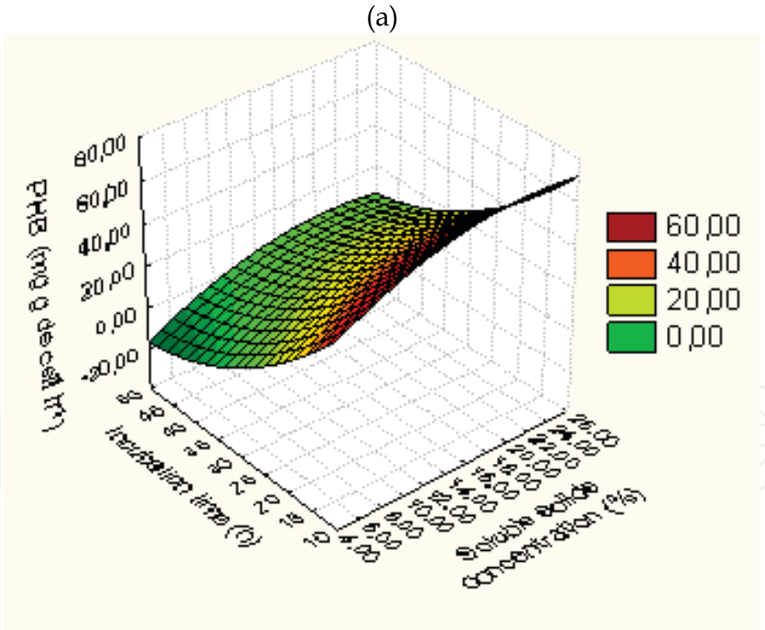
(b)

Fig. 9. Response surfaces for: (a) alginate productivity (mg g cell^{-1} h^{-1}) around the optimal values of soluble solids concentration (%) and incubation time (h) (equation: 544.83 – 7.40 * x – 0.028 * x^2 + 3.01 * y – 0.083 * y^2 – 354.12), (b) alginate productivity (mg g cell^{-1} h^{-1}) around the optimal values of soluble solids concentration (%) and incubation temperature (°C) (equation: 544.83 – 7.40 * x – 0.028 * x^2 -18.01 * y + 0.22 * y^2 + 15.33).

The Figure 10 shows an increase in the PHB yield from 27 to 100 mg g cell^{-1} h^{-1} between the first and second experimental results for the molasses and it was noticeable that the soluble solids concentration was ideal in the range of 12 – 26 %, mostly incubation temperature (60 - 62 °C) and shorter incubation (10 - 15 h).



(a)



(b)

Fig. 10. Response surfaces for: (a) PHB yield (mg g cell^{-1} h^{-1}) around the optimal values of soluble solids concentration (%) and incubation temperature (C) (equation: 317.71 + 3.52 * x – 0.082 * x^2 – 11.50 * y + 0.13 * y^2 – 77.04), (b) PHB yield (mg g cell^{-1} h^{-1}) around the optimal values of soluble solids concentration (%) and incubation time (h) (equation: 317.71 + 3.52 * x – 0.082 * x^2 – 3.52 * y + 0.032 * y^2 – 250.19).

The presented purity PHB extracted between 93.4 and 95.1%.

10. Conclusion

The highest PHB yield (100 mg g cell^{-1} h^{-1}) using sugar cane molasses occurred in the incubation time of 10 h, 60.0 ^{o}C and the soluble solids concentrations between 14.0 – 25.0%. To alginate yield was observed that, using molasses, yield was greater (250 mg g cell^{-1} h^{-1}) also in the incubation time of 10 h, temperature of 60.0 ^{o}C and the soluble solids concentration between 4.0 - 6.0%. The PHB purity was between 93.0 to 97.5%. Thus, Cane sugar molasses was very promising for the alginate and PHB production.

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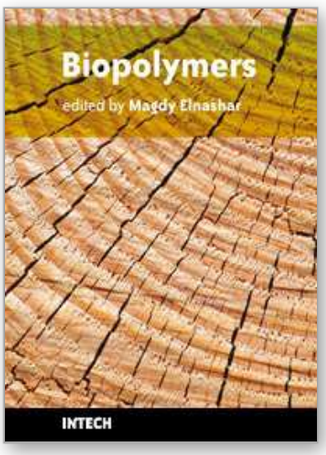
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Biopolymers are polymers produced by living organisms. Cellulose, starch, chitin, proteins, peptides, DNA and RNA are all examples of biopolymers. This book comprehensively reviews and compiles information on biopolymers in 30 chapters. The book covers occurrence, synthesis, isolation and production, properties and applications, modification, and the relevant analysis methods to reveal the structures and properties of some biopolymers. This book will hopefully be of help to many scientists, physicians, pharmacists, engineers and other experts in a variety of disciplines, both academic and industrial. It may not only support research and development, but be suitable for teaching as well.

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