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(71) Applicant *(for all designated States except US)*: **CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL** [MX/MX]; Av. Instituto Politécnico Nacional no., 2508, Col. San Pedro Zacatenco, México, D.F. 07360 (MX).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **PÉREZ GUEVARA, Fermín** [MX/MX]; Pasegua no. 1235, Depto. 4, Col. San, Pedro Zacatenco, México, D.F. 07360 (MX). **LÓPEZ CUELLAR, María del Rocío** [MX/MX]; Montes Urales no. 103, Fraccionamiento, Lomas de Vista Hermosa, Pachuca, Hidalgo 42026 (MX). **GRACIDA RODRÍGUEZ, Jorge Noel** [MX/MX]; Privada los Pinos no. 18, Col. Ricardo, Flores Magón, Estado de México, 54607 (MX).

(74) Agent: **CARREÑO SÁNCHEZ, Luis Antonio**; Av. Instituto Politécnico Nacional no., 2508, Col. San Pedro Zacatenco, Edificio Administrativo, 1er. piso, Subdirección de, Vinculación Tecnológica, México, D.F. 07360 (MX).

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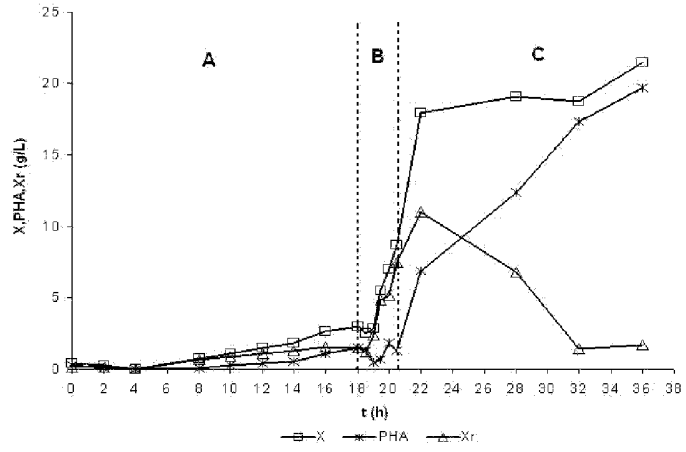
[Continued on next page]

(54) **Title**: METHODS FOR PRODUCING MEDIUM CHAIN POLYHYDROXYALKANOATES (PHA) USING VEGETABLE OILS AS CARBON SOURCE

FIGURE 2



(57) **Abstract**: The present invention describes efficient and low cost fermentation methods for the efficient production of medium chain poli-3-hydroxyalkanoates (PHAs), which comprise the growth of microorganisms producing PHAs using vegetable oils as carbon source, either in the presence or absence of saccharide. The methods of the invention comprise producing biomass in batch culture, subsequently producing biomass in fedbatch culture by means of the addition of saccharide and a nitrogen source to the culture medium and finally producing PHAs by means of the addition of vegetal oil as carbon source with limitation of nitrogen or phosphorus. By means of the method of the invention, at least 90% in dry weight of medium chain PHAs with respect to dry biomass is obtained from the culture medium, presenting from 5 to 10% of medium chain monomeric units in their structure.

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Methods for producing medium chain polyhydroxyalkanoates (PHA) using vegetable oils as carbon source

Field of the invention.

The present invention relates to biological methods for producing biodegradable plastics, specifically to fermentation methods for producing medium chain poly-3-hydroxyalkanoates (PHAs) by means of growing micro-organisms using vegetable oils as carbon source.

Background of the invention.

Population increase on a global scale has multiplied the demand of oil provoking a progressive increase in its price caused by the scarceness of this resource in the world market. The production of this natural resource will begin to diminish intensively provoking a serious problem of depletion in the year 2050. An important quantity of oil is destined for the production of plastics, therefore, and the shortage of oil reserves, will also cause the scarceness of plastics.

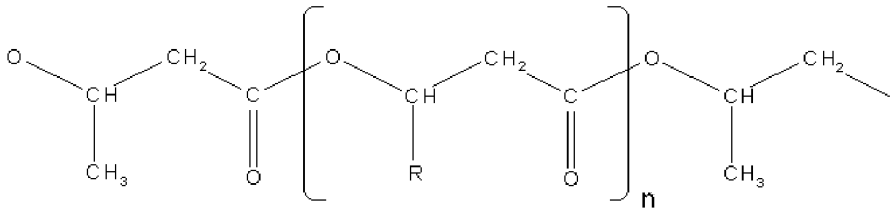
Plastics of chemical origin, have versatile qualities, being resistant to degradation and simultaneously light, which has caused them to become an essential input for most of the industries. Nevertheless, their useful life time of a most of them is very short; many are used to produce bottling and baling materials, provoking their rejection by the environment and causing serious problems of environmental contamination such as the accumulation of non degradable plastics which, once incinerated, provoke greenhouse gas emission to the environment (methane, nitrous oxide and carbon dioxide). As an alternative to avoid the problems caused by conventional plastics, biodegradable plastics are a viable option. In this sense, poly-3-hydroxyalkanoates (PHAs) represent the best alternative given their structural characteristics and their compatibility with the environment.

PHAs are polymers 100% biodegradable, thermoplastic, elastomer, insoluble in water, not poisonous and biocompatible. These kinds of polyster have characteristics similar to those of the polyethene and polyprophylen, and therefore they can be used instead of conventional plastics. Likewise they are degraded completely in aerobic and anaerobic conditions by soil, sea, lake water and residual waters microorganisms[1,2].

PHAs are classified based on the number of carbon atoms in their monomeric units dividing them in two groups, short chain PHAs (PHA_{scl}) with 3-5 carbon atoms and

medium chain (PHA_{mcl}) with 6-14 carbon atoms. The chemical structure of PHAs can be observed in formula 1:

Formula 1:



Where n can be 600 to 35,000. In table 1 we can observe the name of the generated PHA when R is replaced by the radical indicated in its monomeric unit.

Table 1. PHAs

R	PHA	Carbons per monomeric unit	Chain	Name
Hydrogen	Poly-3-hydroxypropionate	3	Short	PHP
Methyl	Poly-3-hydroxybutyrate	4	Short	PHB
Ethyl	Poly-3-hydroxyvalerate	5	Short	PHV
Prophyl	Poly-3-hydroxyhexanoate	6	Medium	PHH
Penthyl	Poly-3-hydroxyoctanoate	8	Medium	PHO
Nonyl	Poly-3-hydroxydodecanoate	12	Medium	PHDD

The PHAs are biodegradable and have thermal and mechanical properties similar to thermoplastics such as polyethylene and polyprophylene, therefore they have a big potential in medical and industrial applications[3].

The PHAs family has a wide variety of mechanical properties as the incorporation of monomeric units of 6 to 14 carbons results in a major molecular weight and improves its physical characteristics since they are semicrystalline elastomers with a low melting point, a low tension force and a high extension to rupture, in addition of having a major biodegradability[4]. PHAs of medium chain (PHA_{mcl}), have a much lower percentage of crystallinity and they are more elastic, since their resistance to impact increases whereas the Young module diminishes, which gives them a wider range of applications compared to PHAs of short chain[5] (see table 2).

Table 2. PHAs and polyprophylene properties

Parameter	PHB	P(3HB-HV)	P(3HB-4HO)	P(3HO-3HH)	Polyprophylene
Tm (°C)	179	145	150	61	176
Tg (°C)	-4	-1	-7	-36	-10
Crystallinity (%)	70	56	45	30	60
Rupture extension (%)	5	50	444	300	400
Young Module (GPa)	3.5	2.9	--	--	1.7

Tm Melting temperature.

Tg Vitreous transition temperature.

PHAs are non toxic, biocompatible and biodegradable plastics which be produced from renewable resources. It has been reported that they have a high grade of polymerization, with a grade of crystallinity in the interval of 60 to 80%, they are active optically and isotactic (that is to say, regularly stereochemical in repeated units), piezoelectric and insoluble in water. These characteristics make them highly competitive against polyprophylene and plastic derivatives of oil[3,6].

PHAs initially were used in packaging film mainly in bags, containers and paper packings[6]. These films can also be used to make sheets together with other polymers such as polyvinilic alcohol[5].

Other applications of PHAs it is to use them as implements, hygienic feminine products, cosmetic containers and shampoo packing. In addition to their potential as plastic material, PHAs are also used as stereo regular components that can serve as chiral precursors for the chemical synthesis of optically active components[6]. Also they are used as biodegradable carriers for the dosage of medicines, hormones, insecticides and herbicides, and as synthetic bone materials in the growth stimulation of bones, due to its piezoelectric properties, in bone badges, surgical structures and replacement in blood vessels[3,6].

PHAs are hydroxyalkanoates polyesters synthesized by at least 75 different bacteria genus including both Gram negative and Gram positive, as for example *Wautersia eutropha*, *Bacillus megaterium*, *Klebsiella aerogenes*, *Pseudomona putida*, *P.*

oleovarans, *Sphaerotilus natans*, among others (see table 3). These polymers intracellularly accumulate in the bacterium as inclusions under stress conditions, such as the limitation of phosphorus, nitrogen, oxygen and a non optimum pH culture medium, to the point that PHAs can represent up to 90% of the dry weight of the dry biomass obtained in the fermentation[3].

Table 3. PHAs produced by bacteries

Microorganism	Carbon source	PHA produced	PHA contents*
Wautersia eutrophia	Fructose	PHB	46-90
	Glucose	PHB	46-85
	Propionate	PHB	26-36
	Octanate	PHA	38-45
Bacillus megaterium QMB1551	Glucose	PHB	20
Klebsiella aerogenes recombinant	Molasses	PHB	65
Pseudomona putida	Palm oil	PHA	37
	Lauric acid	PHA	25
	Myristic acid	PHA	28
	Oleic acid	PHA	19
P. oleovarans	Gluconate	PHB	1.1-0.5
	Octanoate	PHB	50-68
Sphaerotilus natans	Glucose	PHB	40

* % dry weight.

The PHAs produced by bacteria have showed a sufficiently high molecular weight as to asume characteristics similar to those of conventional plastics, such as polypropylene.

It is known that the PHAs produced by microorganisms can have a variety of compositions and structures depending on the type of microorganism used for their production, as well as the composition of culture media and culture conditions.

For example, it has been reported that *Wautersia eutropha* H16, ATCC no. 17699 and its mutant strains produce copolymers of 3-hydroxybutyrate acid (3HB) and 3-hydroxyvaleric acid (3HV) in diverse proportions when the sources of carbon change in the culture.

It has been described[7,8], the production of binary copolymers of 3HB and 3-hidroxyhexanoic acid (3HH) by means of the culture of *Aeromonas caviae* with oleic acid or olive oil as carbon source.

Baanaado[9] describes the production of PHAs, having as monomeric units 3-hydroxyalkanoate, units from 6 to 12 carbons using as carbon source aliphatic hydrocarbur(s) and using *Pseudomonas oleovorans* ATCC 29347.

It has been described[10] that *Pseudomonas resinovorans* produce a polyester with monomeric units of 3-hydroxybutyric acid, 3-hydroxyhexanoic acid, 3-hydroxyoctanoic acid and 3-hydroxydecanoic acid in a ratio of 1:15:75:9 using octanoic acid as the only carbon source. Polyester also is produced with 3-hydroxybutyric acid, 3-hydroxyhexanoic acid, acid 3-hydroxyoctanoic and 3-hydroxydecanoic acid units in a quatitative ratio of of 8:62:23:7 using hexanoic acid as the only source of carbon.

It has been described[11] that *Pseudomonas sp.* strain 613 produces a polyester of 3-hydroxyalkanoic acids, such as 3-hydroxybutyric acid, 3-hydroxyhexanoic acid, 3-hydroxyoctanoic acid, 3-hydroxydecanoic acid and 3-hydroxydodecanoic acid, as well as of 3-hydroxyalkenoic acids, such as 3-hydroxy-5-cis-dodecanoic acid and 3-hydroxy-5-cis-dodecanoic acid, using sodium gluconate as the only carbon source.

Wautersia eutropha (before *Ralstonia eutropha*) is a Gram negative bacteria, aerobic obligated, chemo-organotrophic, capable of accumulating from 8 to 13 granules with PHAs in the cell with a diameter that ranges from 0.2 to 0.5 μm. The hydrophobic inclusions, suspended in the cell cytoplasm, contain 5-10% in water weight and they are largely in an amorphous state. After a cellular destruction, when the PHA is extracted, a crystallization happens rapidly.

Among the PHAs synthesized by *Wautersia eutropha*, the polyhydroxybutyrate (PHB) it is the more widely studied, which has a molecular weight in an interval of 10^4 - 10^6 Da and a polydispersity of 2. It presents a vitreous transition temperature (Tg) of -4°C, whereas the melting temperature appears at 180°C, similar to that of the polyprophylene; nevertheless it presents an extension percentage to rupture of only 5%. Its relatively high melting point near to its thermal decomposition temperature, determines its limits of processabilitly.

The price of PHA depends on the cost of the substratum, yield and the efficiency of the process; this involves high levels of PHAs as percentage in dry weight and high productivity, in terms of gram of product per unit of time volume[6], therefore

improvements in the PHA yields and the substratum selection, impact the costs of the process.

Choice of a suitable carbon source is an important factor in the optimization of the PHAs production. The nature of the carbon source not only determines the content of PHA, but also the composition, which subsequently affects the final properties of the polymer. Within the final cost of the materials used to produce PHAs, 80% corresponds to the carbon source used, therefore the price of PHAs can be decreased by using cheap substrates.

In this sense, to produce short chain PHAs of low cost as PHB, simple sources of carbon as glucose or fructose are used, in addition to organic volatile acids such as acetic, propionic and butyric acids. On the other hand, with valeric acid it is possible to obtain a copolymer (PHB-HV) in different proportions[12].

Pradella[13] for example, describes a production process of short chain PHAs using soybean oil or the greasy acids originated by the latter, palm, cotton, corn, sunflower or castor seed oils like carbon source. The process consists of four stages, for which use the bacterial strains *Wautersia eutropha* IPT026 or IPT027 and the strains of *Burkholderia cepacia* IPT048, 064, 066, 074, 110 or 189. The stage of growth of the biomass (stage II) is carried out by means of a feedback system (feed batch). The weight fraction of PHAs in the cells obtained by means of this method is 90% of dry biomass with a concentration of bacterial biomass of 200 g/L of fermentation medium and a conversion of carbon mass to PHA mass of 0.6 Kg/Kg.

Bright[14] for example describes the production of PHAs by means of cells of plants producing vegetable oils, canola, soybean and sunflower that contain one or several genes of enzymes which are involved in the PHAs biosynthetic pathway of *Wautersia eutropha*, which synthesizes PHA in a natural way. It is important to notice that in order carry out this type of process it is necessary to use recombinant DNA techniques.

There exist diverse and multiple works describing processes for the obtention of PHAs with very specific characteristics, by means of recombinant DNA techniques. In this sense, Nagaoka[15] describes the creation of a new strain of *Wautersia eutropha* to produce polyesters particularly containing monomeric P(3HB-co-3HH) units by means of inserting the gene of the enzyme polyhydroxyalkane acid synthetase, which participates in the PHAs synthesis, obtaining thereby a PHA with special characteristics regarding its hardness and flexibility.

Although the use of molecular biology techniques and recombinant DNA are of great utility to direct the production of PHAs to a very specific product, this type of methodologies increase the price of PHAs production, while on the other hand with the manipulation of fermentation methods and the energy supply to PHAs producing microorganisms, the above mentioned polymers with the desired characteristics, can be obtained at lower costs.

In contrast to the synthesis of short chain PHAs, the synthesis of medium chain PHAs requires organic acids as substrates such as octanoic, dodecanoic, lauric and miristic acids that can incorporate monomeric units of 8 up to 14 carbons due to the different carbon content of each acid[16]. Nevertheless the cost of purification of these acids can be high, which affects the production costs of PHAs when using them as carbon source in fermentation processes.

On the other hand, fermentations using short chain organic acids as carbon source (butyric and valeric acids), have been thoroughly studied since these two acids can be toxic to cells or can inhibit the cellular growth depending on the pH and on its concentration in the culture medium.

Although the use of *Wautersia eutropha* bacteria to obtain PHAs is of great use, the only way to make it grow is by using such carbon sources as glucose, propionate, valerate, methanol, sucrose and prophanol to obtain only short chain copolymers of hydroxyalkanoates as PHB-HV[17].

Therefore production methods are necessary to produce medium chain PHAs at low cost, in order to obtain biodegradable plastics with better physical properties and biodegradability.

Objectives of the invention.

Therefore, one of the objectives of the present invention is to provide methods for the production of medium chain PHAs by means of microorganisms grown in culture media containing vegetable oils as carbon source.

Another objective of the invention is to provide methods for the production of medium chain PHAs by means of microorganisms grown in culture media containing vegetable oils and fructose as carbon sources.

Another objective of the invention is to provide methods for the production of medium chain PHAs by means of microorganisms grown in culture media containing canola oil as carbon source.

Another objective of the invention is to provide methods for the production of medium chain PHAs by means of microorganisms grown in culture media containing canola oil and fructose as carbon sources.

Another objective of the invention is to provide methods for the production of medium chain PHAs by means of the bacteria *Wautersia eutropha* grown in culture media containing canola oil and fructose as carbon sources.

Another objective of the invention is to provide medium chain PHAs obtained by means of microorganisms grown in culture media containing canola oil and fructose as carbon sources.

Another objective of the invention is to provide medium chain PHAs that comprise PHO and PHDD monomers as repeated units in their structure.

Brief description of the figures.

Figure 1. Shows the development of total biomass (X), residual biomass (Xr) and PHAs in cultures performed in flasks using canola oil as carbon source. A cellular growth is observed with fructose (A) and the addition of vegetable oil (B).

Figure 2. Shows the development of total biomass (X), residual biomass (Xr) and PHAs in cultures performed in a bioreactor with canola oil as the only carbon source. Batch culture (A), fedbatch culture (B) and PHAs production (C) are observed.

Figure 3. Shows the development of fructose concentration during fermentation using canola oil as the only carbon source. Batch culture (A), fedbatch culture (B) and PHAs production (C) are observed.

Figure 4. Shows the ammonium concentration profiles during fermentation using canola oil as the only carbon source. Batch culture (A), fedbatch culture (B) and PHAs production (C) are observed.

Figure 5. Shows the concentration profile of canola oil in phase 3 of the fermentation with canola oil.

Figure 6. Shows the concentration profiles of total biomass (X), residual biomass (Xr) and PHAs using canola oil and fructose as carbon sources. Batch culture (A), fedbatch culture (B) and PHAs production (C) are observed.

Figure 7. Shows the concentration profile of fructose during fermentation using oil and fructose as carbon sources. Batch culture (A), fedbatch culture (B) and PHAs production (C) are observed.

Figure 8. Shows the concentration profile of oil during phase 3 of fermentation with canola oil and fructose.

Figure 9. Shows the concentration profile of ammonium during fermentation using oil and fructose as carbon sources. Batch culture (A), fedbatch culture (B) and PHAs production (C) are observed.

Figure 10. Shows the MNR ^{13}C (A) and MNR ^{1}H (B) spectra of polyhydroxybutyrate (PHB).

Figure 11. Shows the MNR ^{13}C at 500 MHz spectrums of the obtained PHAs by the invention method, using canola oil as the only carbon source (A) or canola oil and fructose as carbon sources (B). Hydroxybutyrate (HB), hydroxyvalerate (HV), hydroxyoctanoate (HO) and hydroxydodecanoate (HDD) are clearly seen.

Figure 12. Shows the MNR ^{1}H at 125 MHz spectrums of the obtained PHAs by the method of the invention, using canola oil as the only carbon source (A) or canola oil and fructose as carbon sources (B).

Figure 13. Shows the FT-IR spectrum in the region 4000 to 650 cm^{-1} of the PHAs obtained by the method of the invention, using canola oil and fructose as carbon sources (a), using canola oil as the only carbon source (b) and PHB (c).

Figure 14. Shows the FT-IR spectrum in the region 3600 to 2600 cm^{-1} of the obtained PHAs by the method of the invention, using canola oil and fructose as carbon sources (a), using canola oil as the only carbon source (b) and PHB (c).

Figure 15. Shows the FT-IR spectrum in a region 1850 to 1600 cm^{-1} of the PHAs obtained by the method of the invention, using canola oil and fructose as carbon sources (a), using canola oil as the only carbon source (b) and PHB (c).

Figure 16. Shows the thermograms of the PHAs obtained by the method of the invention, using canola oil and fructose as carbon sources (A), using canola oil as the only carbon source (B) and PHB (C).

Detailed description of the invention.

The present invention provides efficient and low cost fermentation methods to produce medium chain PHAs comprising the growth of microorganisms in culture media containing vegetal oils as substrate or vegetal oils and fructose.

For effects of the invention, efficient and low cost fermentation methods can be achieved using vegetal oils as carbon source which contain a large variety of fatty acids in their composition, e.g. canola oil is basically constituted by seven fatty acids in different proportions; typical composition is shown in table 4.

Table 4. Canola oil composition.

Fatty acid	**% w/w***
Palmitic acid	5.2
Estearic acid	2.2
Araquidonic acid	0.56
Palmitoleic acid	0.2
Oleic acid	60.03
Linoleic acid	21.22
Linolenic acid	7.81

* Average weight.

For the effects of the invention, it is possible to use vegetable oils which are normally produced in big quantities due to their low cost of production, for example those destined for human or animal consumption, such as sunflower, soybean, corn, olive tree, safflower, or canola oil as well as mixtures of them. However, it is preferred to use canola oil due to the PHAs yields produced by this oil.

The present invention provides PHAs copolymers with monomeric units of medium chain produced by microorganisms at very low cost. Such copolymers can be useful in the industry due to the physical characteristics. The PHAs of the present invention contain monomeric units with more than five carbons that those previously obtained by means of microorganisms using vegetable oils as carbon sources or with other carbon sources.

The PHAs of the present invention exhibit melting temperatures of 130ºC to 150ºC, more convenient than known those of PHAs obtained by present industrial processes where melting temperature is an important factor, as for example in molding processes at medium temperatures.

The present invention provides methods to produce medium chain PHAs comprising the culture of one or more microorganism capable of producing PHAs in such conditions that PHAs could be accumulated, for example by means of limitation of some nutrient in the culture medium, for example, nitrogen, oxygen or a source of phosphorus, which are needed for growing the microorganism but not for the accumulation of PHAs. During the stage of accumulation of PHAs, vegetable oil is added to the culture medium in presence or absence of saccharides, and used to produce PHAs into the microorganism to be later recovered.

The process of the invention also comprises an earlier stage to the production of PHAs that allows the build up of biomass. This stage makes possible to increase the yields of medium chain PHAs, which can reach at least 90% of the dry weight of the biomass obtained in the fermentation.

For obtaining the PHAs here described, it is possible to use native or genetically transformed microorganisms to produce them, which eliminates the need to use genetically transformed strains for this intention and reduces the costs of production.

According to the method of the present invention, the microorganism is cultured in a process comprising three stages (batch, fedbatch, and PHAs production) in a medium added with vegetable oil prior or within the PHAs production stage where the microorganism produces and accumulates polymers which comprises the monomeric units PHB, PHV, PHO and PHDD. In the same sense, when vegetable oil is used as the sole carbon source in the PHAs production stage the polymers formed contain at least 5% of medium chain monomeric units, i.e. PHO and PHDD. The use of both vegetable oil and saccharide as carbon sources in the PHAs production stage, gives a polymer containing 10% of medium chain monomeric units, i.e. PHO and PHDD.

The change of the carbon source according to the methods of the invention makes possible the production of PHAs with increased content of medium chain monomeric units. Such polymers can be produced with different and advantageous properties, for example different melting points. Likewise, the use of vegetable oil as carbon source to produce PHAs in the methods of the invention, allows the production of the mentioned biopolymers at a considerably lower cost.

The microorganism which may be used in the method of the invention are those capable of producing short chain PHAs, which are selected from the group comprising the genera *Wautersia*, *Klebsiella*, *Zoogloea*, *Bacillus*, *Aeromona*, *Azotobacter*,

Clostridum, *Nocardia*, *Halobacterium*, *Burkholderia*, *Sphaerotilus* y *Pseudomonas*, although bacteria within the genus *Wautersia* are preferred to use.

The essential nutrients required for the microbial growth comprise elements, which are normally present in the culture medium in an easily assimilable way, such as nitrogen, phosphorus, sulfur, potassium, sodium, magnesium, calcium and iron, together with traces of manganese, zinc and copper. Whereas it is possible to induce the accumulation of medium chain PHAs by limiting the supply of oxygen to the culture medium, the restriction of one or more of the essential nutrients is preferred, in particular nitrogen or phosphorus limitation are preferred.

As sources of nutrients added to the culture medium, saccharides can be used as carbon source, which are selected of the group comprises arabinose, glucose, mannose, fructose, galactose, sorbitol, manitol and inositol, although it preferred to use fructose. It is possible to use nitrogen sources chosen from the group comprising ammonia, ammonium salts, as for example ammonium sulphate, nitrates and/or organic nitrogen contained in such compounds as urea, casein, peptone, yeast extract and meat extract.

The carbon source may be used in such a quantity to generate the medium chain PHAs of the invention but without inhibit the growth of the microorganism. Concentrations within the range of 2 to 50 g/L are generally used, but preferably within the range of 8 to 12 g/L are used in the culture medium. As for the nitrogen source, this can be used in concentrations within the range of 0.2 to 20 g/L, preferably of 1 to 2 g/L in the culture medium. In the case of the vegetable oils, concentrations within the range of 40 to 80 g/L are generally used, but preferably within the range of 50 to 80 g/L are used in the culture medium.

For effects of the present invention, the culture temperature can be 20ºC to 35ºC, preferably 31ºC, whereas the pH can range 6.5 to 8, preferably 6.7 to 7.5.

The recovery of the PHAs of the present invention can be achieved by any suitable method known in the art. Generally the cells containing PHAs can be separated from the fermentation broth by centrifugation, subsequently broken and afterwards the PHAs extracted with an organic solvent, preferably chloroform. Nevertheless, in environments where the use of an organic solvent is inadequate, it is possible to apply methods to eliminate the cell components through a treatment with tensoactives such as sodium dodecyl sulfate (SDS), enzymes as lyzosome, and chemical agents such as ethylene

diamine tetraacetic acid (EDTA), sodium hypochlorite, ammonia and other similar can be used.

The culture medium can be varied in the quantity and sources of the different elementary nutrients, but should provide the necessary nutrients for growth of the microorganisms and the production of the PHAs of the invention. In one embodiment of the invention, methods are provided to culture such microorganisms in culture media which supply all the required essential nutrients, that is, the nutrients are present in such a concentration to allow the growth of the PHAs producing microorganism.

The method of the present invention aims to the production of PHAs with better thermal and mechanical properties, by means of two strategies, each of them comprising fermentations of three stages.

I. Medium chain PHAs production using vegetal oil as the only carbon source.

First stage. Batch culture.

In this stage the microorganism is grown in batch culture in presence of at least one saccharide as carbon source in a concentration from 2 to 18 g/L, preferably of 8 to 12 g/L. The growth takes place in presence of at least one nitrogen source, in a concentration of 0.5 to 10 g/L, preferably from 1 to 5 g/L. In this stage the fermentation continues up to exhaustion of the saccharide in the culture medium until reaching a consumption of at least 80% of its initial concentration.

Second stage. Fedbatch culture.

The cell biomass is increased in the broth by the controlled addition of one or more substrates to the culture (fedbatch culture). In this stage, after the saccharide is consumed according to the first stage, the same saccharide is added as carbon source to the culture in such a way that its maximum concentration does not exceed 5 g/L, preferably up to 2 g/L in the fermentation broth. In this stage the total biomass increases at least 130% with regard to the total biomass obtained at the end of the first stage. Likewise, at the beginning of former stage the nitrogen source is added in such a way that it's maximum concentration should not exceed 0.91 g/L.

During this stage, the biomass grows quickly (see figure 2), stopping its growth when the addition of nitrogen source is stopped; likewise the saccharide is used for microbial growth at this stage; therefore the microorganism does not use the saccharide for PHAs production.

Third stage. PHAs production.

Medium chain PHAs are produced by adding vegetal oil as the only carbon source to the culture. During this stage, the concentration of the nitrogen source in the culture medium is below 0.5 g/L; therefore the microorganism encounters nitrogen limitation, such condition induce the production of PHAs from the only carbon source available in the culture (vegetable oil). After the vegetable oil is added to the culture in a concentration from 50 to 80 g/L, the accumulation of medium chain PHAs is approximately 90% of the biomass dry weight. Therefore, vegetable oil is an excellent carbon source for the production of medium chain PHAs.

Once finished the fermentation, the PHAs are obtained from the microbial cells by means of the extraction methods mentioned above.

The PHAs obtained with this method, incorporate into their structure at least 4% of medium chain monomers, preferably at least 5% of medium chain monomers.

Fedbatch fermentations are commonly used to reach a high cellular density, at high productivity, while inhibition by substrate is avoided or to induce some kind of nutrient limitation to stimulate the synthesis of microbial products. During the development of the fedbatch culture the reaction volume increases progressively in the fermenter. The concentration of the different components in the culture medium changes continuously in the broth, as a result of volume changes, but also by the consumption of nutrients by the cells and the continuous feeding of nutrients during the fedbatch stage. This affecting all the reagents concentrations, reason why the feeding depends on the flow like of the reagent concentration in the fed solution; due to this it is advisable to refer the feeding magnitude to the residual biomass. Fedbatch culture contributes to increase the cellular density and to avoid catabolic repression by high substrate concentrations.

As mentioned before, the composition, structure and length of the medium chain PHAs are related to the used carbon source. For this reason, in the present invention is advisable to use preferably fructose in the fedbatch stage of biomass build-up (stage two) and canola oil like carbon source in the stage of PHAs production (stage 3).

II. Medium chain PHAs production using vegetal oil and saccharide as carbon sources.

First stage. Batch culture.

This stage allows the growth of the PHAs producing microorganism in the presence of at least a saccharide as carbon source and at least a nitrogen source. During this stage

the microorganism consumes at least 80% of carbon and nitrogen sources initially supplied to the culture medium.

Second stage. Fedbatch culture.

The saccharide is added gradually to the culture up to reach a bulk concentration of 30 to 50 g/L and the nitrogen source up to 0.2 g/L (fedbatch culture). The total biomass generated in this stage is increased in at least 400% with respect to the biomass obtained in the first stage. As it can be observed, during this phase the carbon and nitrogen sources added are used solely for the generation of total biomass.

Third stage. PHAs production.

Unlike the third phase of the previous method, in the beginning of this phase the saccharide maximum concentration is 30 to 40 g/L, consequently one saccharide carbon source is present in the culture medium. For the production of PHAs, vegetal oil as carbon source is added to the culture at a concentration of 40 to 80 g/L jointly with a nitrogen source at a minimal concentration of 1.5 g/L. The PHAs are produced in the presence of two carbon sources (saccharide and vegetal oil) and while a nitrogen source is added. At the end of this stage, the PHAs obtained represents 90% of the biomass dry weight, being these of medium chain length.

Finished the fermentation, the PHAs are obtained from the microbial cells by means of the mentioned extraction methods previously.

For biomass production (stage two), the saccharide is preferably fructose whereas canola oil and fructose are the preferred carbon sources for the production stage of PHAs (stage 3).

The PHAs obtained with this method, incorporate in their structure at least 8% of medium chain monomers, preferably at least 10% of medium chain monomers.

In the previous two methods of the invention, the carbon and nitrogen sources are continuously supplied to the culture medium during the second stage, approximately at 0.8 to 5 $g_{carbon\ source}$ /h per $g_{residual\ biomass}$ and 0.2 to 5 $g_{nitrogen\ source}$ /h per $g_{residual\ biomass}$ respectively. Likewise the carbon/nitrogen molar relation (C/N) in the culture medium is fixed at C/N=14 during the beginning of the first stage, whereas in the third stage it continually changes as the carbon source is added, but remains above of C/N=120.

According to the methods of the present invention, it is possible to increase the medium chain PHAs' yields (dry weight) using a fermentation strategy in three stages (batch, fedbatch and PHAs production).

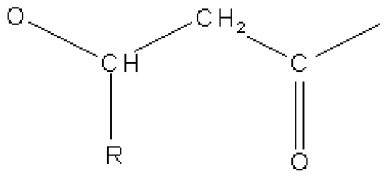
The methods of the invention use vegetable oils as carbon source with or without fructose. The methods carried out in a bioreactor generate a biomass containing up to 90% dry weight of PHAs. Consequently, allow high yields at low production costs in reasonable time.

By means of the method of the invention it is possible to produce medium chain PHAs that incorporate in their structure from 4 to 10% of medium chain monomers, preferably with 6 to 12 carbons in their monomeric unit. Likewise by means of the method of the invention, PHAs having different monomers can be obtained, preferably monomers with 4 to 12 carbons in their monomeric unit, and more preferably monomers selected from the group comprising PHB, PHV, PHO and PHDD. The mentioned monomers in different proportions are components of the PHAs of the present invention, particularly the medium chain monomers (PHO and PHDD) are found in proportions from 4 to 10%, while the short chain monomers (PHB and PHV) form the remaining percentage.

The PHAs of the invention are polymers formed by diverse monomeric units and which have the structure of formula 1, where R is an alkyl group of 1 to 9 carbons, where preferably monomeric units form the PHAs where R is methyl, ethyl, penthyl and nonyl.

Likewise, the PHAs of the invention contain repeated monomeric units of formula 2 which comprise 4 to 10% of monomeric units where R is an alkyl group of 3 to 9 carbons and of 90 to 95% of monomeric units where R is an alkyl group of 1 to 2 carbons.

Formula 2



This means that the PHAs of the present invention, contain 4 to 10 % of medium chain monomeric units, where they are preferably formed by monomeric units where R is penthyl and nonyl, that is, 3-hydroxyoctanoate and 3-hydroxydodecanoate; whereas the remaining percentage is formed by short chain monomeric units, where they are preferably formed by monomeric units where R is methyl and ethyl, that is, 3-hydroxybutyrate and 3-hydroxyvalerate.

As it can be observed, the methods of the invention allow obtaining medium chain PHAs with high quality and better physical-chemical characteristics, as a result of the incorporation of 4 to 10% of monomeric units having 6 to 12 carbons in the polymeric structure of the PHAs.

The following examples illustrate the present invention in its preferred form, but these are given without the intention of limiting its scope.

Example 1. PHAs production using vegetable oils as substrates.

A flask batch culture was used for probe the production of PHAs, using fructose as carbon source for bacterial growth and the later addition of diverse vegetable oils to the culture medium.

An inoculum of *Wautersia eutropha* ATCC 17699 was prepared in a Erlenmeyer flask of 200 mL with 100 mL of modified Luria-Bertani liquid medium (LB). Inoculum development was at 200 rpm and 30°C. Said inoculum was later added to 250 mL flasks containing 50 mL of production medium. The production medium containing per liter: 10 g of fructose, 1.57 g of NH_4SO_4, 5.66 g of $NaHPO_4.12H_2O$, 1.5 g of KH_2PO_4, 0.2 g of $MgSO_4.7H_2O$, 10 mg of $CaCl_2.2H_2O$, 20 mg of $FeSO_4.7H_2O$, 1mL of microelements solution (0.3 g of H_3BO_3, 0.2 g of $CoCl_2.6H_2O$, 0.1 g of $ZnSO_4.7H_2O$, 30 mg of $MnCl_2.4H_2O$, 30 mg of $Na_2MoO_4.2H_2O$, 20 mg of $NiCl_2.6H_2O$ and 10 mg of $CuSO_4.5H_2O$, in a solution of HCl 0.1 N); LB modified culture medium was used to inoculate the production medium at 10% v/v. The flasks were incubated at 200 rpm and 30ºC. During the fermentation process (30 h), a culture sample was taken every 2 h, adding 0.250 mL of oil to every flask at 15 h.

The concentration of the residual biomass (Xr) and of PHAs before adding the oil tested was respectively 4.9 and 1.4 g/L, whereas the resulting concentration at the end of the fermentation process is shown in table 5.

Table 5.

Comparison of residual biomass and PHAs obtained using different vegetal oils

Oil	Xr (g/L)	PHA (g/L)	PHA/Xr
Canola	3	11.1	3.7
Soy	4.1	6.1	1.4
Olive	3.4	6.1	1.79
Corn	3.8	5.4	1.42
Safflower	3.66	4	1.09
Sunflower	3.5	3.2	0.91

Table 5 shows that the PHAs concentration was approximately 2 times bigger when canola oil was used as oil source (11 g/L) compared to the PHAs production obtained

with soy, olive or corn oil (5-6 g/L), whereas it was 3 times greater compared to the PHAs concentration produced with safflower or sunflower oil (3-4 g/L). It was additionally observed that the measurable residual biomass (Xr, biomass faction that is metabolically active) varied from 3 to 4 g/L depending on the used carbon source.

As observed in figure 1, when oil is added for the PHAs production, it is effectively consumed for the production of such biopolymers.

Example 2. PHAs production at bioreactor level using canola oil as substrate.

Due to the results obtained in example 1, canola oil was chosen to obtain PHAs in a bioreactor using two different production strategies. The first used canola oil as the only carbon source whereas the second used canola oil and fructose as carbon sources.

In whichever two strategies, PHAs production was carried out in a 6 L BIOFLO 3000 (New Brunswick Scientific) bioreactor. Previously the bacteria was grown in LB culture medium and used to inoculate the reactor at 10% v/v. The medium used for production was the same as in example 1.

For all temperatures, temperature was controlled at 30ºC and the pH at 7 using H_3PO_4 0.47 M and NaOH 2 M solutions with an air flow of 5 L/min. Samples were taken every 2h.

Fermentations were carried out in three stages:

Stage 1: Biomass production in batch culture, at initial C/N=14, and 3 L volume.

Stage 2: Biomass production in fedbatch culture with addition of concentrated fructose solution, and

Stage 3: PHAs production with the addition of vegetal oil (previously selected at flask level), as carbon source, with nitrogen limitation, at C/N above 120.

- PHAs production with canola oil as the only carbon source.

Wautersia eutropha was cultured in a fermenter using a production system in three stages. The first stage was in batch culture for growing the bacteria, the second stage was a fedbatch culture in which the total biomass further increases, and the third stage was with the addition of vegetable oil to the culture medium to produce PHAs.

For the biomass production, a solution of fructose and ammonium sulphate was used to feed the fermenter with concentrations of 30 g/L and 9.37 g/L respectively, with a flow of 0.9 L/h at an agitation speed of 300 rpm. There was no control over the dissolved

oxygen in the medium. Once the fructose in the medium was consumed, oil was added for the production of PHAs.

Figure 2 shows the kinetics of biomass (X), residual biomass (Xr) and the poly-hydroxyalkanoates (PHAs). The production stage was a batch culture and began with a volume of 3 L and a concentration of 10 g/L of fructose. A 5 h lag phase can be seen. The biomass concentration reached 3 g/L at 18 h in the first fermentation stage, and 8 from the 10 g/L available of fructose were consumed at the beginning of the process, obtaining a yield of Yx/s = 0.42 g_x/g_s and an average sugar uptake rate of 0.4 g/h.

In the second stage and starting at the 18.5 h, fructose at 30 g/L was added at 0.9 L/h during 2 h. This addition was within the range from 0.8 to 4.5 $g_{substrate}$ /h per $g_{residual\ biomass}$. During this stage concentrations of total biomass and residual biomass of 8 and 7 g/L respectively were obtained with a fructose uptake of 3.01 g/h, which indicated that the carbon source was used for the production of residual biomass. The yield achieved during the feeding process was 0.35 g_{xr}/g_s. At the end of the feeding and before adding the canola oil (begin the third stage), we confirmed that the fructose added has been consumed (see figure 3).

The fructose was consumed following 5 h of fermentation and concentration declined to 2 g/L, a concentration that remained during the fedbatch culture with addition of fructose. Once the feeding of fructose was finished, the reactor was operated in batch culture, consequently the residual fructose was consumed (see figure 3). Thus, *W. eutropha* did not use fructose to produce PHAs in the third stage.

The concentration of ammonium was determined during the three stages of fermentation. Figure 4 shows that the ammonium declined rapidly from the initial concentration of 0.423 g/L to 0.03 g/L at the end of the first stage. The average consumption rate was 0.12 g/h. In the production stage, ammonium sulphate at 9.54 g/L was added at a flow of 0.9 L/h, simultaneously with the carbon source to reach a concentration of 0.25 g/L. Figure 2 shows the growth of biomass during the second stage, but the growth stopped soon after the end of the feeding.

During the production stage the concentration of ammonium was between 0.15 to 0.1 g/L. Yan[18] reports that *W. eutropha* remains sufficiently active to produce PHAs at 0.1 g/L of ammonium. This indicates that the bacteria met nitrogen limitations to continue with a growth metabolism, but it had the ideal conditions to produce PHAs with the only available carbon source.

Figure 5 shows the development of oil concentration. About 15% of the 74 g/L of added oil was consumed. The yield was $Ygc_{PHA}/gc_{substrate}$ = 0.68 $gc_{PHA}/gc_{substrate}$, the uptake rate was 0.72 g/Lh and the productivity was 0.45 g/Lh.

About 90% of the dry biomass weight was PHAs, greater than other values reported up to date and 10% more than the percentage reported by Fukui[19] when using olive oil. The results indicate that the vegetable oil used is an excellent carbon source for the PHAs biosynthesis.

Other reports in the art are not comparable with the present invention since no fermentations have been done using a system in three stages as the one proposed here.

- PHAs production with canola oil and fructose as carbon sources.

In this case a solution or separate solutions of fructose and ammonium sulphate were added to the fermenter during the second stage of the process. The concentration solutions were 120 g/L and 34.6 g/L respectively. The flow rate was in both cases 0.225 Lh^{-1}. This was equivalent to 1.23 to 4.3 $g_{substrate}$ /h per $g_{residual\ biomass}$. Operating conditions were stirring speed of 300 to 800 rpm, determining a minimum DO level of 40%.

Figure 6 shows the kinetics of a 5 h adaptation phase, the biomass concentration achieved in the first stage was 3 g/L, similar to the biomass obtained in the fermentation with canola oil only. However this maximum concentration was achieved in a less time (14 h). The yield obtained in this first stage was 0.4 g_x/g_s with a carbon source uptake rate of 0.09 g/Lh.

The second stage began when the fructose concentration in the medium was of 2.4 g/L. A fructose solution containing 117 g/L was added to the fermenter at a flow rate of 0.225 L/h. At the end of this stage the total biomass and residual mass concentrations achieved were 11.6 and 4.4 g/L respectively, the fructose uptake rate was 0.31 g/Lh. This indicated that the added substrate was used for the production of biomass; the yield achieved during the feeding was 0.9 g_{xr}/g_s.

The profile of fructose concentration in this fermentation can be observed in figure 7. At the end of this phase, the potassium phosphate addition that was used to regulate the pH was suspended.

To observe the effect of producing PHAs in the presence of two carbon sources at the same time, 74 g/L of canola oil was added to the fermenter when the fructose

concentration in the medium was still high, so that the C/N increased to 180 causing an excess in the carbon source and a low concentration of nitrogen.

As it can be seen in figure 7, after the oil addition there was a simultaneous consumption of fructose and oil, which indicates that both carbon sources were used to produce PHAs. The carbon yield in this fermentation stage was $Y_{p/s} = 0.3_{gC/gC}$.

The percentage of consumed oil in this fermentation was 40% (see figure 8), what is somewhat greater than the percentage of oil used in the fermentation added with canola oil only as carbon source.

The dissolved oxygen available in the medium might explain the high consumption of oil since it was controlled by the stirring speed to support a minimum DO of 40%. Therefore, in this fermentation oxygen was not limiting for the production of PHAs.

The ammonium concentration was determined during the three stages of the fermentation. In figure 9 one can observe that the ammonium is rapidly consumed during the first stage, being the initial concentration of ammonium 0.43 g/L and 0.074 g/L at the end of this stage, the uptake rate was larger than the observed in the fermentation with only canola oil (0.05 g/h). The larger uptake rate can be greater because the concentration of OD in the reactor was controlled well above of 40%, thus preventing limitation of oxygen during the culture. During the second stage ammonium sulphate was added to the reactor at a flow rate of 0.225 L/h, at concentration of 35 g/L. In the third stage ammonium sulphate addition to the reactor was continued, so the ammonium was maintained at concentrations above 0.4 g/L. In this stage, the stress to induce the synthesis of PHAs for *W. eutropha* was not caused by nitrogen nor by oxygen limitation in the culture medium.

The stress to stimulate the synthesis of PHAs was caused by the absence of phosphates, this because during the culture potassium phosphate is used to regulate the pH of the medium, nevertheless this one was not added afterwards to the medium, which indicates that *W. eutropha* only used the initial phosphate, consuming it up to reaching a limitation of phosphates.

The biomass produced in this case, as in the former example with canola oil only, contained al least 90% of PHAs on a dry weight basis.

The principal differences found in the PHAs produced with the two different fermentation strategies of the invention are reflected in the thermochemical characterization of the biopolymers obtained (see example 3).

Example 3. Characterization of the PHAs obtained with the method of the invention.

The results showed in the previous examples, indicated that *Wautersia eutropha* is capable of using vegetable oils as carbon source to produce PHAs. Because it has been reported that the structure of the carbon skeletons of the hydroxyalkanoic acids that incorporate to the PHAs during fermentation is related to the substrata used as precursors during the synthesis of PHAs[1], the composition and thermal characteristics of the PHAs obtained by means of the method of the invention were determined by different techniques.

- Extraction and purification of the PHAs obtained.

To extract the PHAs from the cells biomass obtained by the methods of the present invention, the cells of *Wautersia eutropha* previously dried were submitted to boiling in chloroform during 10 min. The chloroformic extract containing PHAs dissolved was filtered to remove the cell biomass. The latter step was repeated twice. Subsequently the PHA was precipitated with cold hexane, filtered and the residual solvent eliminated by evaporation.

- Magnetic nuclear resonance (MNR).

The purified PHAs obtained by means of the method(s) of the invention were analyzed by MNR to elucidate the structure of the biopolymer by means of the resonance of carbons and protons present in the structure of the monomeric units.

The identification of the monomeric units present in the produced PHAs was obtained analyzing the ^{13}C and ^{1}H spectra, using the bacterial biopolymer poly(p-hydroxybutyrate) (PHB of Goodfellow Cambridge Limited) as point of comparison.

The MNR was carried out in a Broker equipment (DM X 500 MHz) using deuterated chloroform as solvent. The ^{13}C and ^{1}H spectra were obtained at 500 and 125 MHz, respectively. The spectrums corresponding to ^{1}H were analyzed using the Spinkworks program version 2.5.5.

As it is observed in figure 10, the ^{13}C and ^{1}H spectrums of pure PHB showed the expected signals in accordance with the reported in literature[19,20], whereas the signals of the primary chain of the PHA produced by means of the method of the invention (see figure 11) correspond to the expected structures, coinciding with the reported structures[17].

Figure 11 shows the chemical displacements inside the intervals earlier mentioned corresponding to the carbons of the principal chain of the produced PHA. There are also observed signals corresponding to lateral chain carbons, indicating the presence of four different monomeric units in the synthesized polymer. Matsusaki[21] reports the resonance of a methyl group with a chemical displacement of 14 ppm corresponding to a terminal methyl of hydroxyoctanoate (C8) and hydroxydodecanoate (C12). They also report the hydroxyoctanoate and hydroxydodecanoate methylenes with a chemical displacement between 22.66 - 34 ppm. These displacements are coincident with those obtained in the ^{13}C spectrum of the PHAs produced with the method of the invention, either using only canola oil or using canola oil and fructose; besides presenting the signals that correspond to short chain hydroxyalkanoates as PHB and polihydroxyvalerate (C4 and C5, respectively).

The ^{1}H spectrum (figure 12) of the PHAs produced by the method of the invention shows the chemical displacements expected in an interval of 2.45 - 2.6 ppm, that correspond to the methylenes protons of the principal chain. The chemical displacement at 0.87 ppm indicated the presence of terminal methyl protons of medium chain, a signal that has been reported as a methyl group joined to a chain of 4 methylenes for the case of hydroxyoctanoate and 8 methylenes for hydroxydodekanoate. Besides a chemical displacement can be identified at 0.97 ppm that identifies as a methyl group corresponding to CH_3 of a monomeric chain of PHV (polihydroxyvalerate)[22,23].

The corresponding ^{1}H spectra analyzed with Spinworks program allowed the determination of the number of protons to which every signal integrates in order to obtain the relative percentage of medium chain units with respect to PHB, demonstrating that the percentage of medium chain monomeric units in the obtained polymer using canola oil and fructose as sources of carbon by means of the method of the invention was about 10%, whereas the polymer obtained with the use of canola oil only as carbon source contain about 5% of medium chain monomeric units. Therefore, the content of medium chain PHA chain is bigger for the polymer synthesized with canola oil and fructose than the obtained with canola oil only.

On the other hand the chemical displacement for the PHB is the signal obtained at 1.29 ppm as Gao[24] and Fukui[19] reported. The theoretical and experimental information of the chemical displacements of ^{13}C and ^{1}H spectrums of the PHAs synthesized with the method of the invention using only canola oil and, canola oil and fructose are resumed in table 6.

Table 6.

Chemical displacements in MNR of ^{13}C and ^{1}H for the PHAs synthesized in canola oil only and canola oil-fructose (both cases stage 3).

^{1}H		δ (ppm)	δ (ppm) theoretic displacement	^{13}C		δ (ppm)	δ (ppm) theoretic displacement
CH_3	8-HO	0.87	0.89	(10,12)CH_2	HDD	22.68	20-24
	12-HDD	0.87					
	3-PB	1.29	1.28	(10,12)CH_3	HDD	14.12	13-15
	3-HV	0.97	0.9				
				(9)CH_2	HDD	29.59	28-29
CH	8-HO	5.25	5.25				
	3-PB	5.33	5.3	(8)CH_2	HDD	31.8	29
	3-HV	5.33	5.3	(8)CH_3	HO	14.12	13.5-14
CH_2	8-HO	2.47, 2.61	2.49-2.62	(7)CH_2	HDD	29.59	29
	3-PB	2.47, 2.61	2.49-2.62		HO	22.66	22.3-22.7
	3-HV	2.47, 2.61	2.49-2.62				
				(6)CH_2	HDD	29.59	29
					HO	31.8	31
				(5)CH_2	HDD	27.2	29-31
					HO	24.91	22-25
				(4)CH_2	HDD	34.32	34-34.5
					HO	34	34-34.5
					HV	31.8	30-38
				(3)CH	HDD	76.9	65.5-71
					HO	67.5	65.5-71
					PB	67.5	65.5-71
					HV	67.5	65.5-71
				(2)CH_2	HO	40.88	38-41
					HB	40.88	38-41
					HV	40.88	38-41
				(1)C=O	12HDD	169	169-169.5
					8HO	169	169-169.5
					PB	169	169-169.5
					HV	169	169-169.5

It can be reasonably concluded that the PHAs obtained by means of the method of the invention is a polymer formed of hydroxybutyrate (HB), hydroxyvalerate (HV), hydroxyoctanoate (HO) and hydroxydodekanoate (HDD).

- Infrared spectroscopy (FT-IR).

The studies of FT-IR were performed using Perkin Elmer equipment (mod 1600) in an exploration window of 400 to 4,000 cm^{-1}. For the obtaining of the spectra 32 sweeps were realized by sample, whereas for its normalization air was used as reference.

The FT-IR spectrums of PHB and of the PHAs obtained by the method of the invention in the presence of canola oil show the expected signals (see figure 13) in accordance with the reported in literature, which are associated with the functional groups present in the structure of the materials. This implies that the obtained spectrums show a superposition of the vibratory bands of the obtained PHAs and of the PHB. The principal bands were observed in the interval comprised 4,000 to 650 cm^{-1} and appear in table 7.

Table 7. Principal signals of FT-IR obtained in the synthesized PHAs starting from canola oil, compared with reported signs for PHB as reference.

Chemical group	Pertaining to	Absorbance
CH_2-CH_2	PHA	2935
-CH_2-	PHB	2935
-CH_2 –CH_3	PHA	2874
-CH_3	PHB, PHA	2978
-C=O	PHB, PHA	1739
O-C=O	PHB, PHA	1721
-C-O-C	PHB, PHA	1300-1000

The typical spectrum of methyls and methylenes in the obtained PHAs and the PHB can be observed in the region 3600-2600, where diverse authors have reported that the signals in the regions 3015-2960, 2945-2925, and 2855-2875 cm^{-1} correspond to the stretching of CH_3 asymmetric, CH_2 antisymmetrical and CH_3 symmetrical respectively.

In figure 14 one observes the symmetrical stretching of CH_3 in the region 2870 cm^1, in which the intensity of the band is related to the conformational disorder obtained in the process of crystallisation[24], which indicates that the PHAs obtained by means of the method of the present invention are less crystalline than the PHB obtained by other methods presently used in the art.

The band at 2935 cm^{-1} corresponds to the stretching of anti symmetrical chains of CH_2, showing that in the PHA obtained with canola oil and fructose presents a more intense band than the PHA obtained with only canola oil and the PHB, these functional groups correspond to the lateral chains of the monomeric units. In case of the PHA obtained

from canola oil, this signal indicates a major quantity of methyl asymmetric groups, contained in the monomeric chains of the PHA. The band that corresponds to asymmetric methyls also is observed in the spectrum at 2978 cm^{-1}. This band is more intense for the PHB, and different authors indicate that this signal increases gradually with the presence of crystalline structures in material[25]. Also, the presence of CH_3 asymmetric stretchings has been recently reported near the region of 3000 cm^{-1} indicating the possible union of C-H---O, that is, of hydrogen bridges[24]. For this reason the stretching in the region 2978 cm^{-1} allows to suppose the presence of CH_3 interacting with C=O.

In the region 1850 to 1600 cm^{-1} appears the stretching of the C=O group. This one has been reported with two domineering bands, since the signal at 1739 cm^{-1} is associated with amorphous regions from the PHA, and the signal most defined at 1721 cm^{-1} the C=O is associated with crystalline groups[22,25]. In figure 15 one observes these two bands, nevertheless the signal at 1921 cm^{-1} in the obtained PHA obtained with canola oil and fructose is more intense than that of the two remaining materials. The percentage of crystallinity was estimated in 30% for both PHAs obtained with methods of the invention whereas 58% was obtained for the commercial PHB.

- Differentiated sweep calorimetry (DSC).

The DSC studies were done in a differential scanning calorimeter (Mettler Toledo DSC823). In order to erase the thermal history, the samples (4 mg approx.) were cooled at -20ºC to be warmed later at 180ºC. They were subsequently cooled down rapidly up to -20ºC being maintained 5 minutes at this temperature, to later carry out the thermal analysis warming them slowly up to 180ºC. The warming ramp was of 5ºC min^{-1}.

The percentage of crystallinity was estimated from the melting enthalpies determined in the calorimeter. The melting enthalpies were used in equation 1[26], taking as basis the melting enthalpy of the polymer 100% crystalline, presuming an enthalpy of 146 J/g for PHB[27].

Equation 1 $$Xp = \Delta H_m / Wp \times \Delta H^\circ_m$$

Where:

Xp = polymer crystallinity (%)

ΔH_m = melting enthalpy of the polymer (J/g)

Wp = proportion of the polymer when dealing with a polymeric mixture

ΔH°_m = melting enthalpy of the 100% crystalline polymer.

The DSC results show the signals that comprise the melting temperatures of the obtained PHAs. The obtained thermograms indicated a PHB melting temperature (Tm) of 170ºC with a ΔH_m of 84.74 Jg^{-1} (see figure 16), very similar to the reported in literature[21].

The melting temperature (Tm) which present the samples of the obtained PHAs with the method of the invention from canola oil and fructose and only canola oil, were 132ºC and 150ºC respectively with a ΔH_m 44.24 for both materials. In table 8 and figure 16 it is possible to observe that with the incorporation of small percentages of monomeric units of medium chain, the melting temperature of the PHAs is notably affected.

Table 8. Comparison between melting and enthalpy temperatures for different PHAs.

Sample	Melting temperature (ºC)	ΔHm (Jg^{-1})	Reference
PHB	170	84.74	Present invention
PHB	173.2	91	Matsusaki[21]
PHA (canola oil)	134	45.24	Present invention
PHA (canola oil, duplicate)	132	37.87	Present invention
PHA (canola oil and fructose)	150	44	Present invention
PHA (canola oil and fructose, duplicate)	150	48	Present invention
PHA (6% 6C-12C)	136	39	Matsusaki[21]
PHA (5% 6C)	125	47	Loo[29]
PHA (5% 6C)	155	38	Loo[29]

When the PHAs obtained according to the method of the invention in the presence of canola oil reach their melting temperature, double signals can be observed, which has been related to a recrystallization, that is, the first signal corresponds to the melting of

the PHA and the second one to a recrystallization. On this basis it is possible that small crystals of PHB remain dispersed during the cooling process and they might be places of nucleation for the longer chain monomers, of which the PHAs obtained according to the invention are formed.

In accordance with aforementioned, the chemical characterization demonstrated that the incorporation of co-monomers in the PHAs obtained by means of the method of the invention depends on the type of substrate used. When producing PHAs using canola oil as the only carbon source, one obtains 5% of monomeric units of medium chain incorporated in the PHAs of the invention, whereas when combining two sources of carbon (fructose and canola oil) it is possible to incorporate 10% of medium chain units in the PHAs of the invention.

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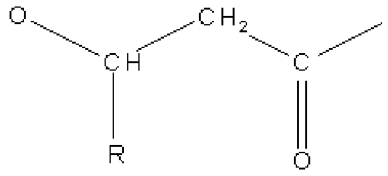
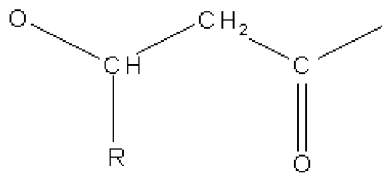
Claims

1. A poli-3-hydroxyalkanoate with repeated monomeric units of formula



characterized because comprises 4 to 10% of monomeric units where R is an alkyl group of 3 to 9 carbons and 90 to 95% of monomeric units where R is an alkyl group of 1 to 2 carbons.

2. The poli-3-hydroxyalkanoate of claim 1 characterized because comprises 4 to 10% of monomeric units where R is an alkyl group of 5 to 9 carbons and 90 to 95% monomeric units where R is an alkyl group of 1 to 2 carbons.
3. The poli-3-hydroxyalkanoate of claim 2 characterized because comprises 4 to 10% of monomeric units where R is penthyl or nonyl and 90 to 95% of monomeric units where R is methyl or ethyl.
4. The poli-3-hydroxyalkanoate of claim 3 characterized because comprises 4 to 10% of monomeric units of 3-hydroxyoctanoate and 3-hydroxydecanoate and 90 to 95% of monomeric units of 3-hydroxybutyrate and 3-hydroxyvalerate.
5. The poli-3-hydroxyalkanoate of claim 1 characterized because has a melting point of 130 to 150ºC.
6. A method for obtaining poli-3-hydroxialkanoates which comprise repeated monomeric units in its structure of formula



where R is an alkyl group of 3 to 9 carbons, characterized because comprises the phases of:

a) Cultivating a microorganism capable to produce poli-3-hydroxyalkanoates in a culture medium containing a saccharide and a nitrogen source, until the initial concentration of the saccharide is reduced by at least 80%,

b) Adding continuously a saccharide and a nitrogen source to the culture medium in such concentrations as to allow an increase of the total biomass at least 130% with respect to the total biomass generated in phase a),

c) Adding vegetable oil as carbon source to the culture medium under nitrogen or phosphorus limiting conditions and incubate up to produce a poly-3-hydroxyalkanoate rich biomass having at least 90% of poli-3-hydroxyalkanoate with respect to dry biomass , and

d) Recovering the poli-3-hydroxyalkanoate from the culture medium.

7. The method according to claim 6, characterized because the producer microorganism of poli-3-hydroxyalkanoates is selected from the group comprising *Wautersia*, *Klebsiella*, *Zoogloea*, *Bacillus*, *Aeromona*, *Azotobacter*, *Clostridum*, *Nocardia*, *Halobacterium*, *Burkholderia*, *Sphaerotilus* and *Pseudomona*.

8. The method according to claim 7, characterized because the producer microorganism of poli-3-hydroxyalkanoates is *Wautersia*.

9. The method according to claim 6, characterized because the saccharide is selected from the group comprising arabinose, glucose, mannose, fructose, galactose, sorbitol, manitol and inositol.

10. The method according to claim 9, characterized because the saccharide is fructose.

11. The method of claim 9, characterized because the saccharide is present in a concentration of 2 to 30 g/L in phase a).

12. The method according to claim 6, characterized because the nitrogen source is selected from the group comprising ammonia, ammoniac salt, ammoniac sulphate, urea, casein, peptone, yeast extract and flesh extract.

13. The method according to claim 12, characterized because the nitrogen source is ammonium sulphate.

14. The method according to claim 12, characterized because the nitrogen source is present in a concentration of 1 to 20 g/L in phase a).

15. The method according to claim 6, characterized because the saccharide is added to a final concentration of 2 to 40 g/L and the nitrogen source to a final concentration of 0.2 to 0.25 g/L in phase b).

16. The method according to claim 6, characterized because the vegetal oil is selected from the group comprising canola, soy, olive, corn, safflower, sunflower oil and mixtures of them.

17. The method according to claim 16, characterized because the vegetal oil is canola oil.

18. The method according to claim 6, characterized because the vegetal oil is added to a final concentration of 40 to 80 g/L in phase c).

19. The method according to claim 6, characterized because the poli-3-hydroxyalkanoate is recovered by a process comprising the steps of separating the cells from the culture medium, break the cells and extract the PHA with chloroform in phase d).

20. The method according to claim 6, characterized because the carbon and nitrogen sources are supplied continuously at 0.8 to 5 $g_{carbon\ source}$ /h per $g_{residual\ biomass}$ and 0.2 to 5 $g_{nitrogen\ source}$ /h per $g_{residual\ biomass}$ respectively.

21. The method according to claim 6, characterized because the C/N ratio in the culture medium is maintained at C/N=14 during phase a), whereas in phase c) it is maintained at C/N=120.

22. The method according to claim 15, characterized because the saccharide is added to a final concentration of 2 g/L and the nitrogen source to a final concentration of 0.25 g/L.

23. The method according to claim 22, characterized because the poli-3-hydroxyalkanoate obtained comprises until 5% of monomeric units where R is an alkyl group of 3 to 9 carbons and 95% of monomeric units where R is an alkyl group of 1 to 2 carbons.

24. The method according to claim 15, characterized because the saccharide is added to a final concentration of 30 to 50 g/L and the nitrogen source to a final concentration of 0.2 g/L.

25. The method according to claim 24, characterized because the nitrogen source is added in phase c) to a final minimum concentration of 0.4 g/L.

26. The method according to claim 25, characterized because the poli-3-hydroxykanoate obtained comprises until 10% of monomeric units where R is an alkyl group of 3 to 9 carbons and 90% of monomeric units where R is an alkyl group of 1 to 2 carbons.

FIGURE 1

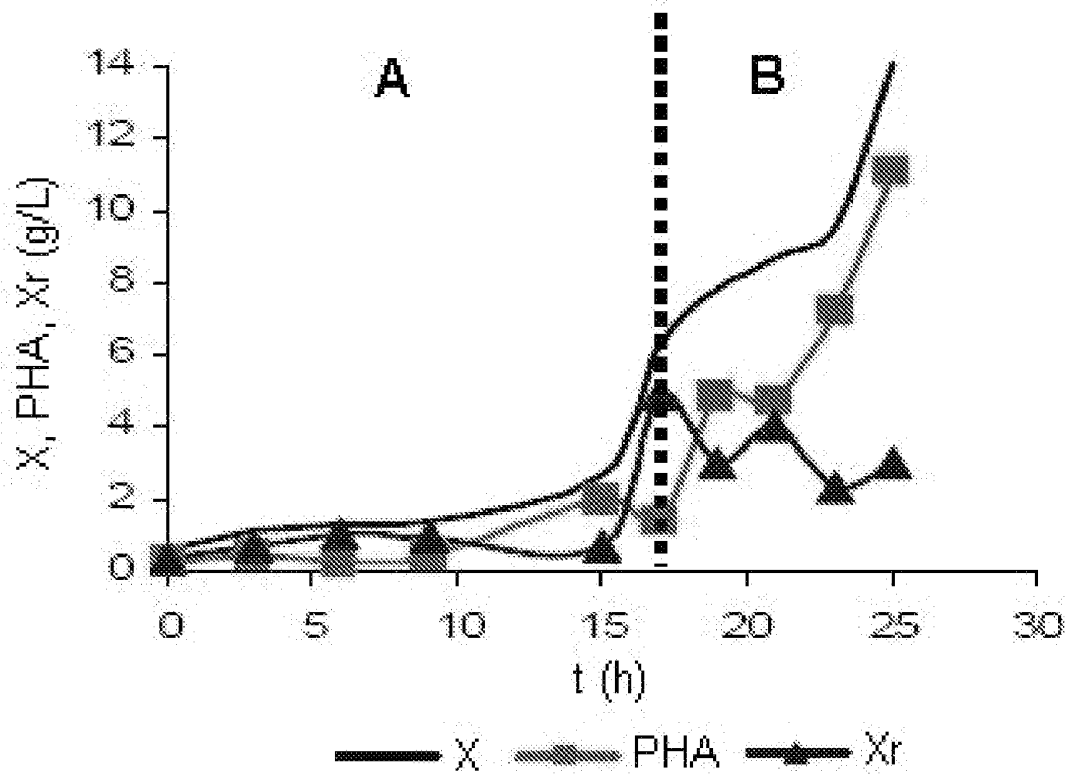


FIGURE 2

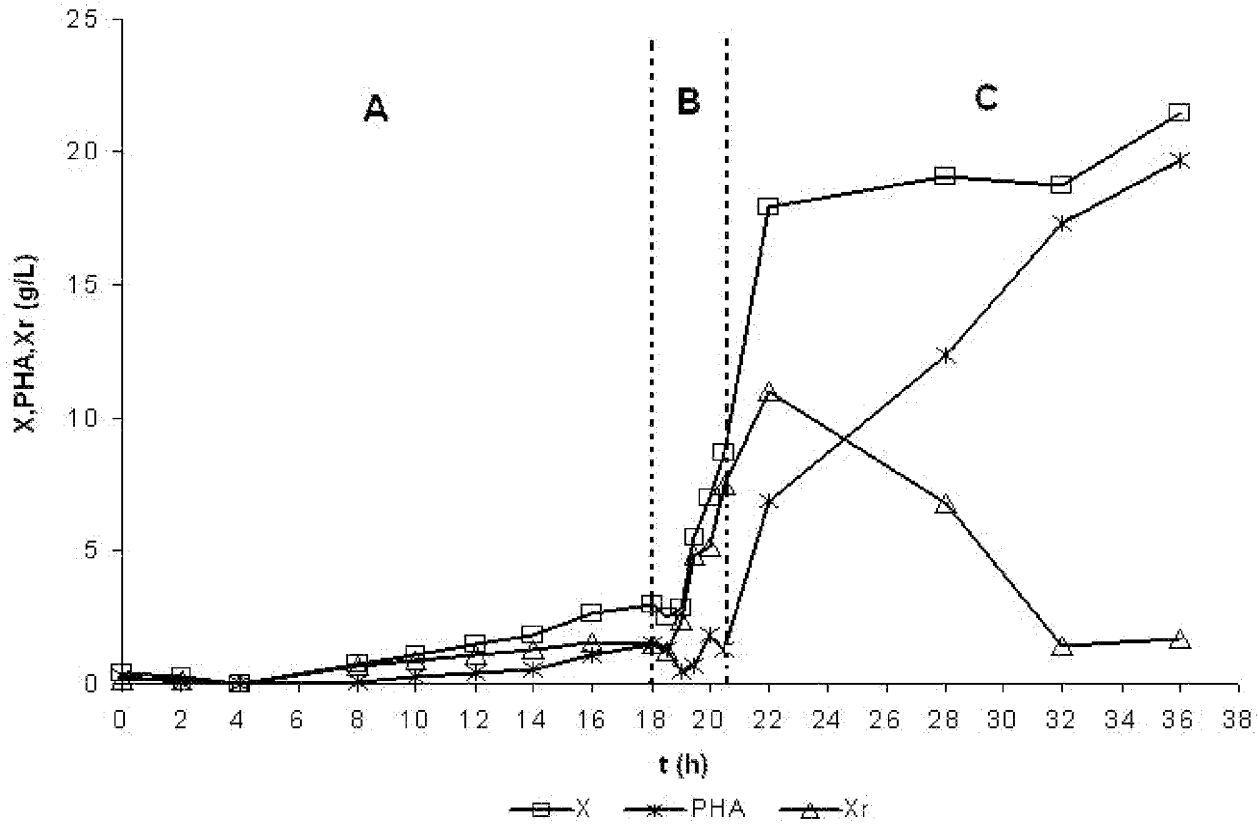


FIGURE 3

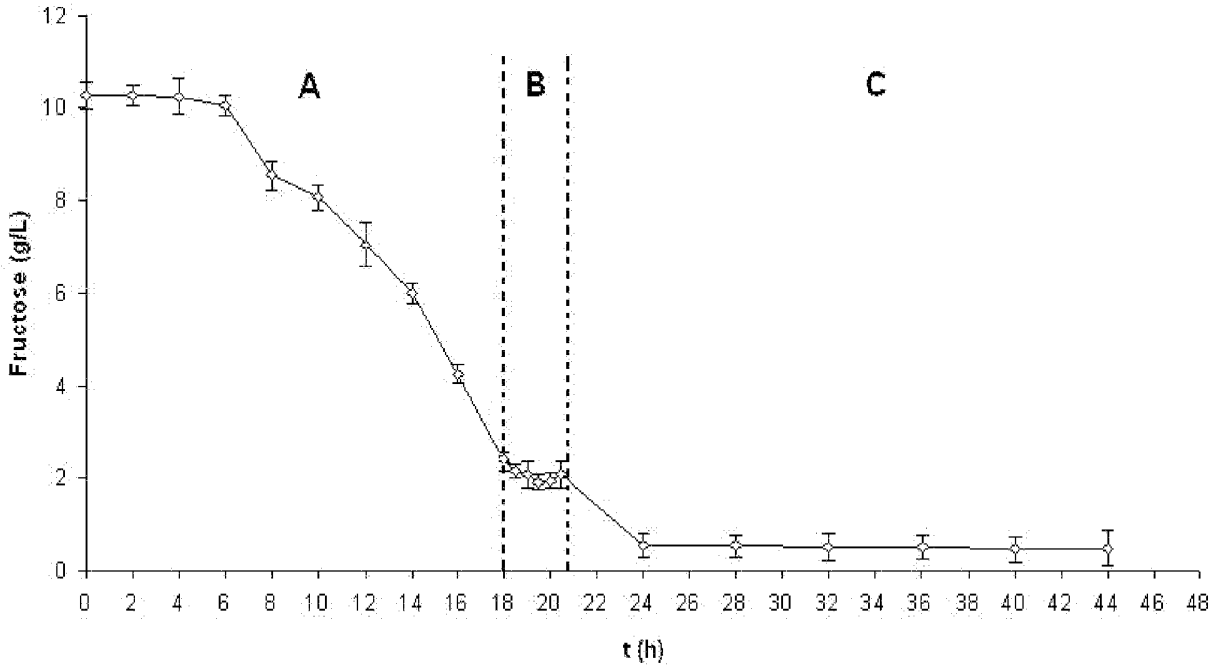


FIGURE 4

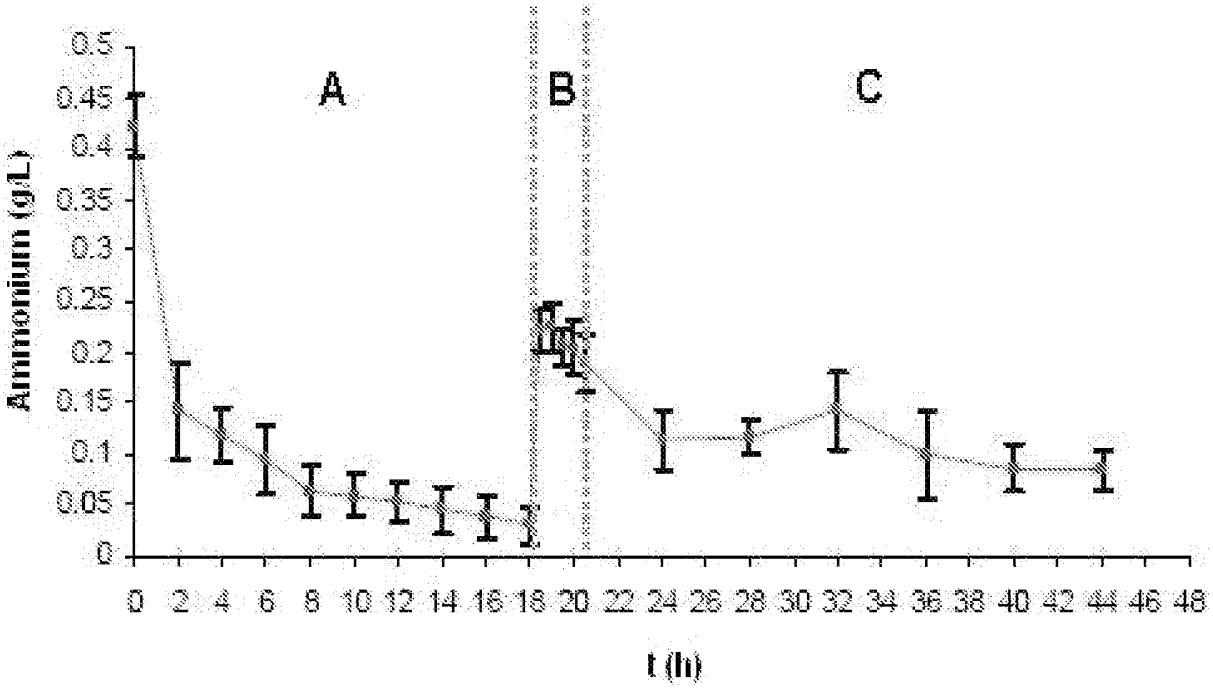


FIGURE 5

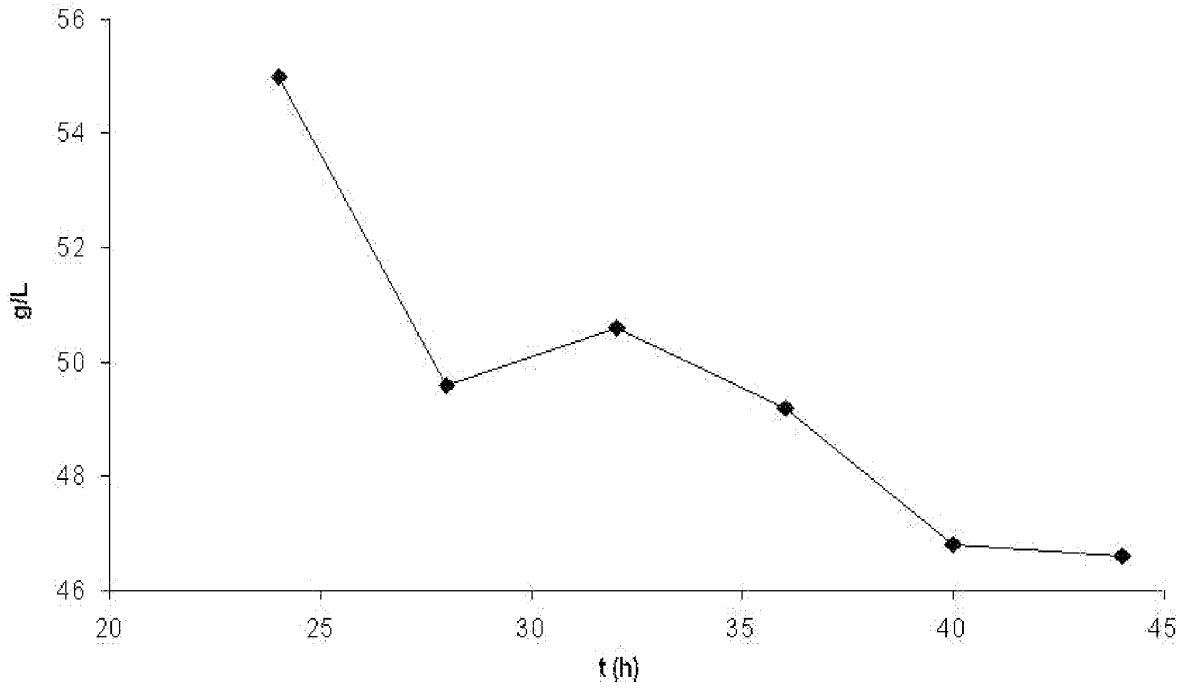


FIGURE 6

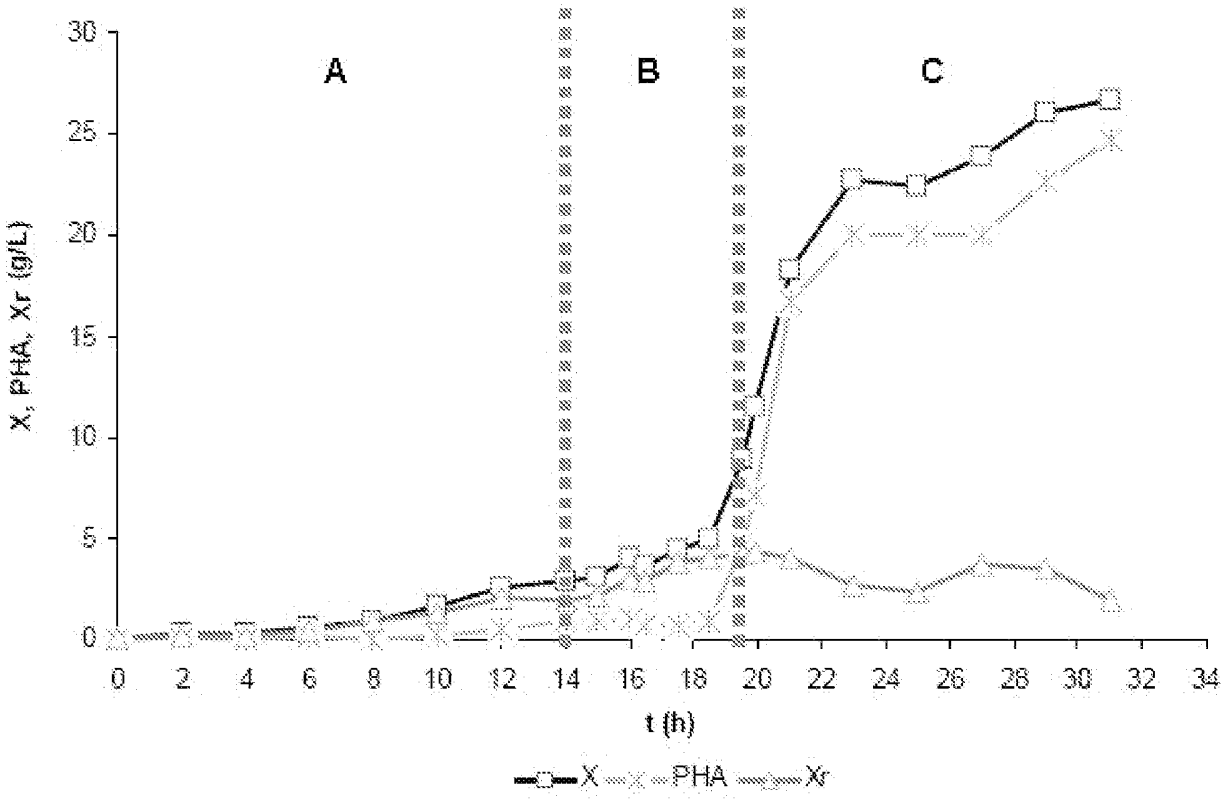


FIGURE 7

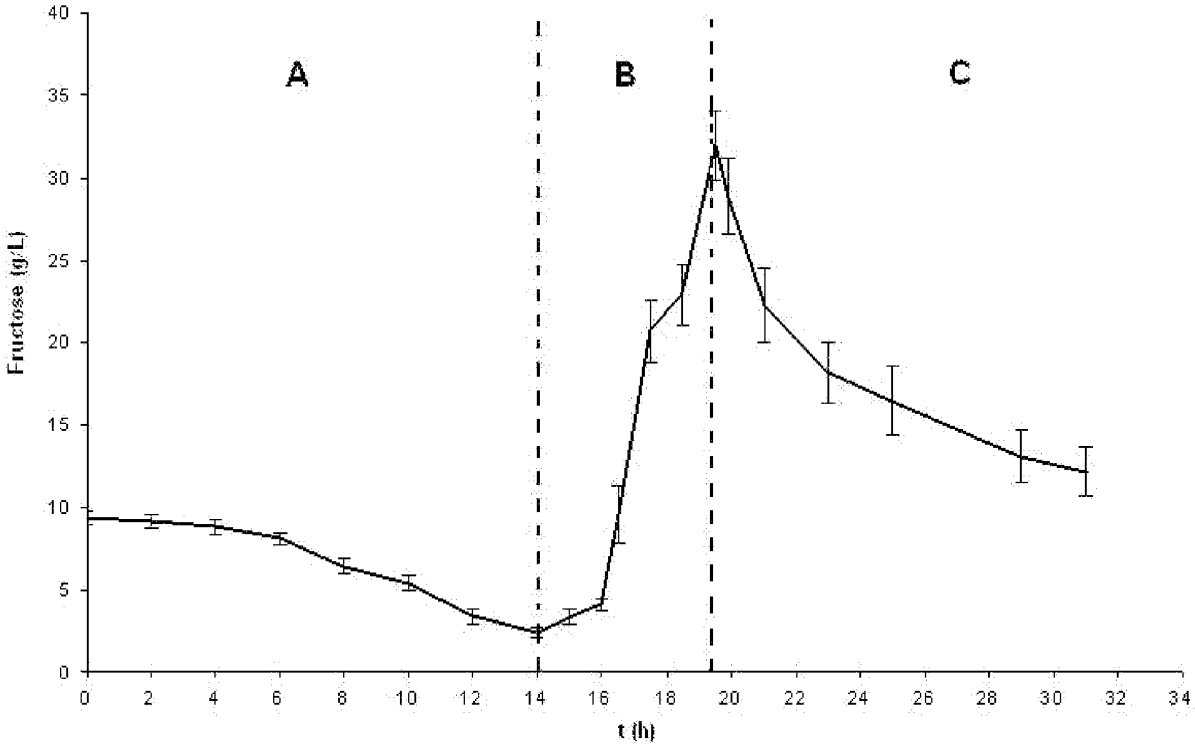


FIGURE 8

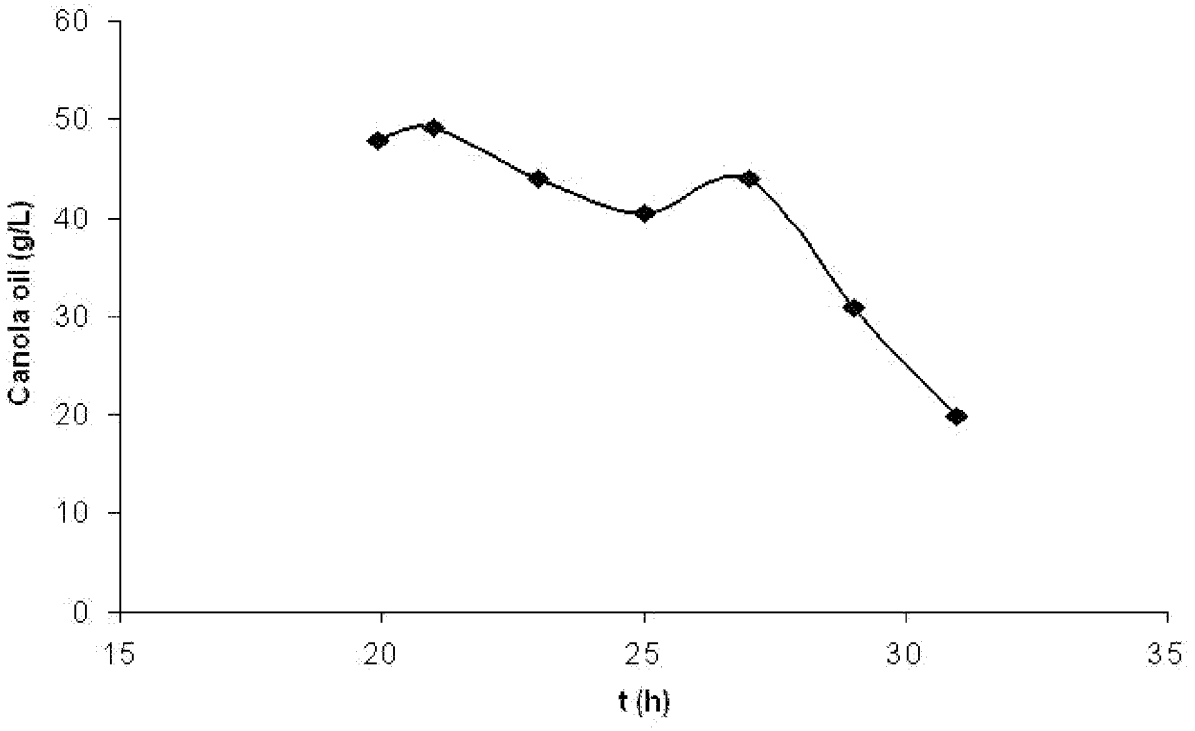


FIGURE 9

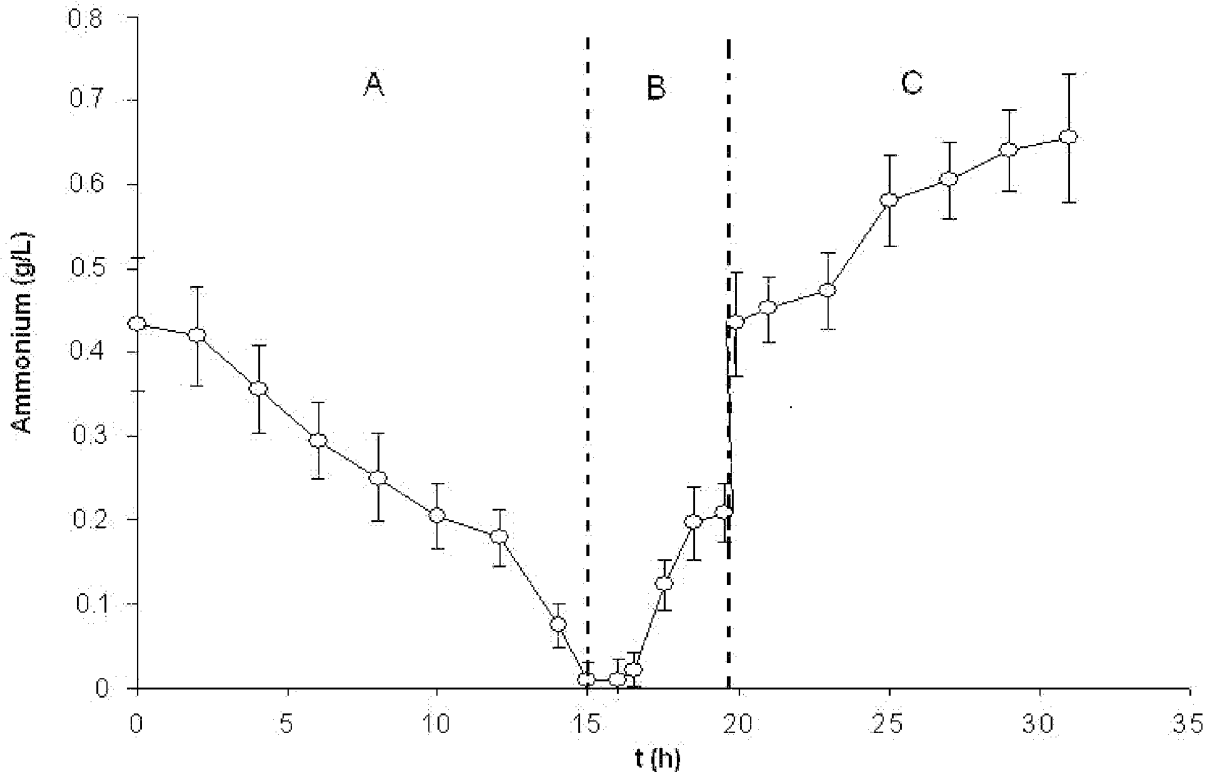


FIGURE 10a

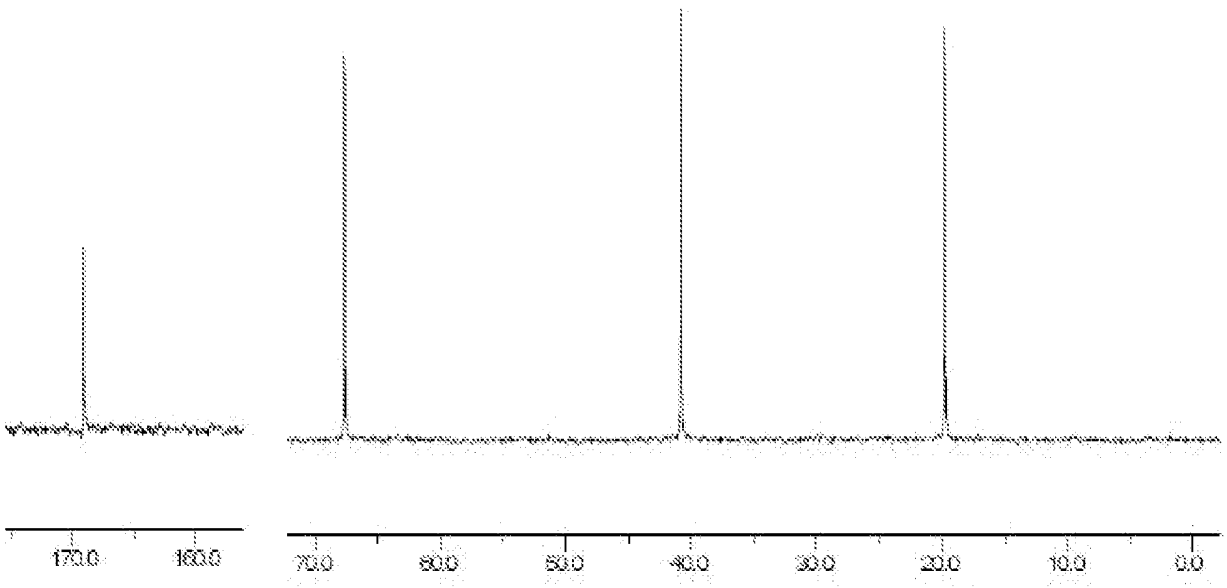


FIGURE 10b

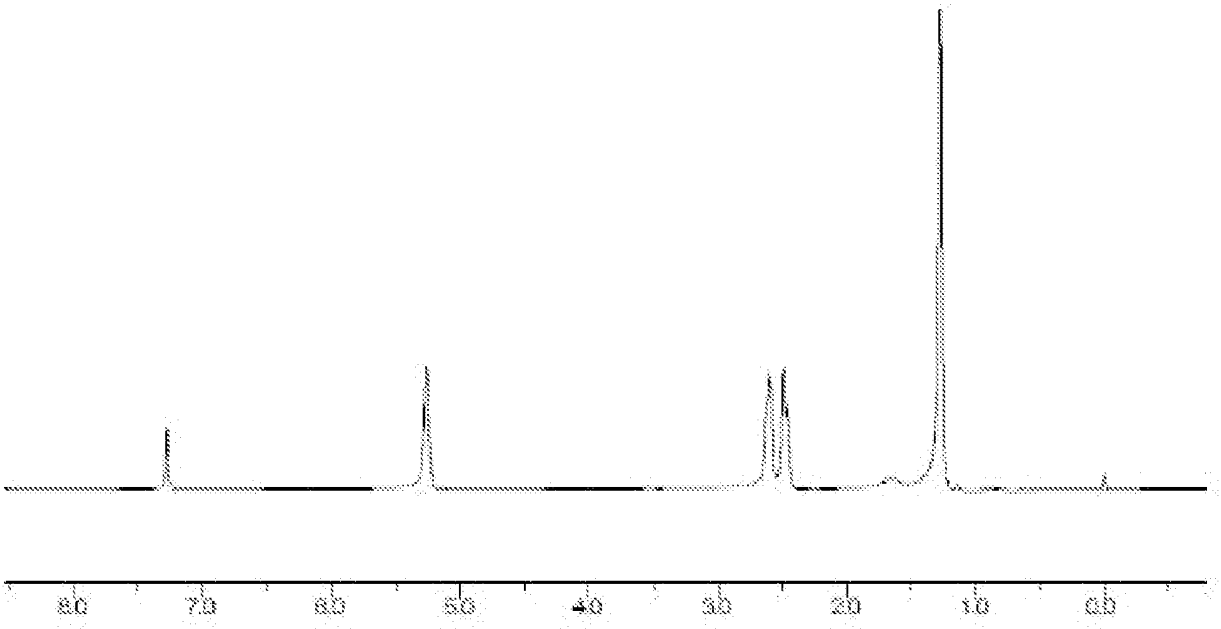


FIGURE 16

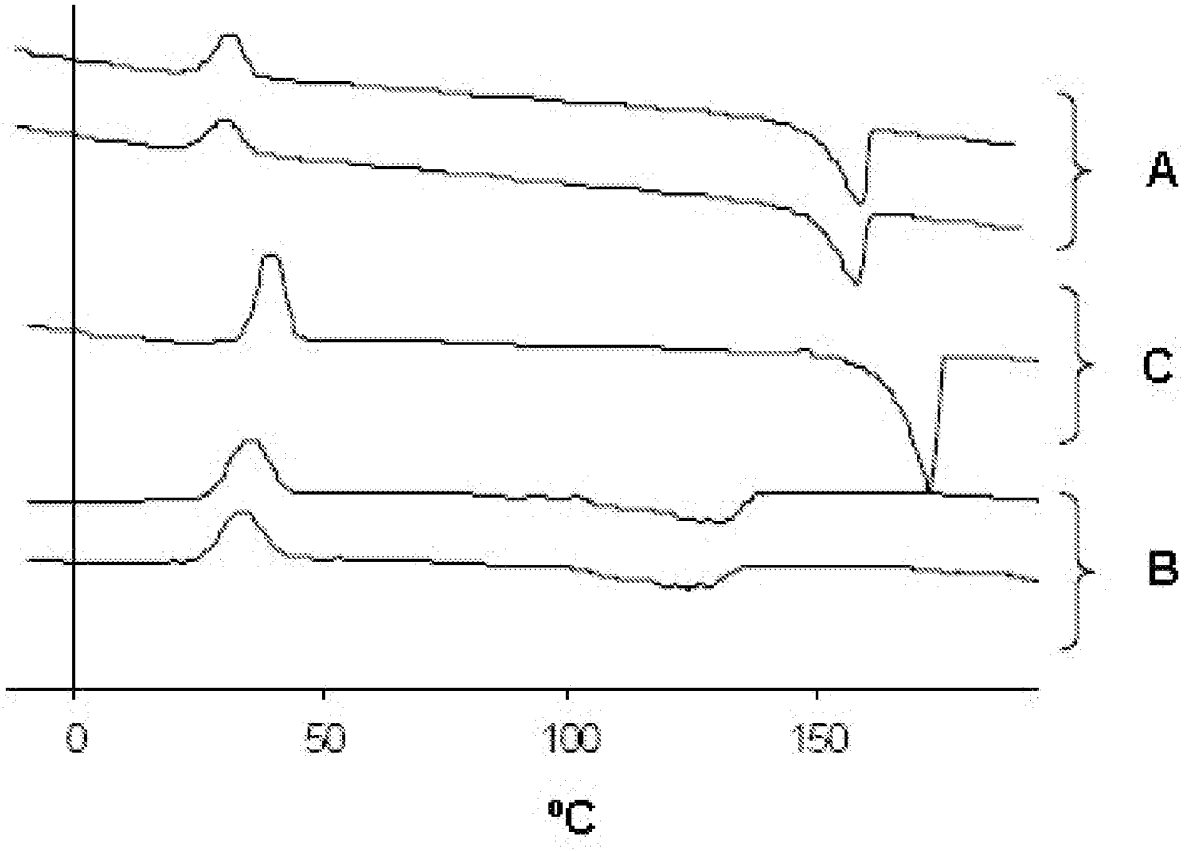
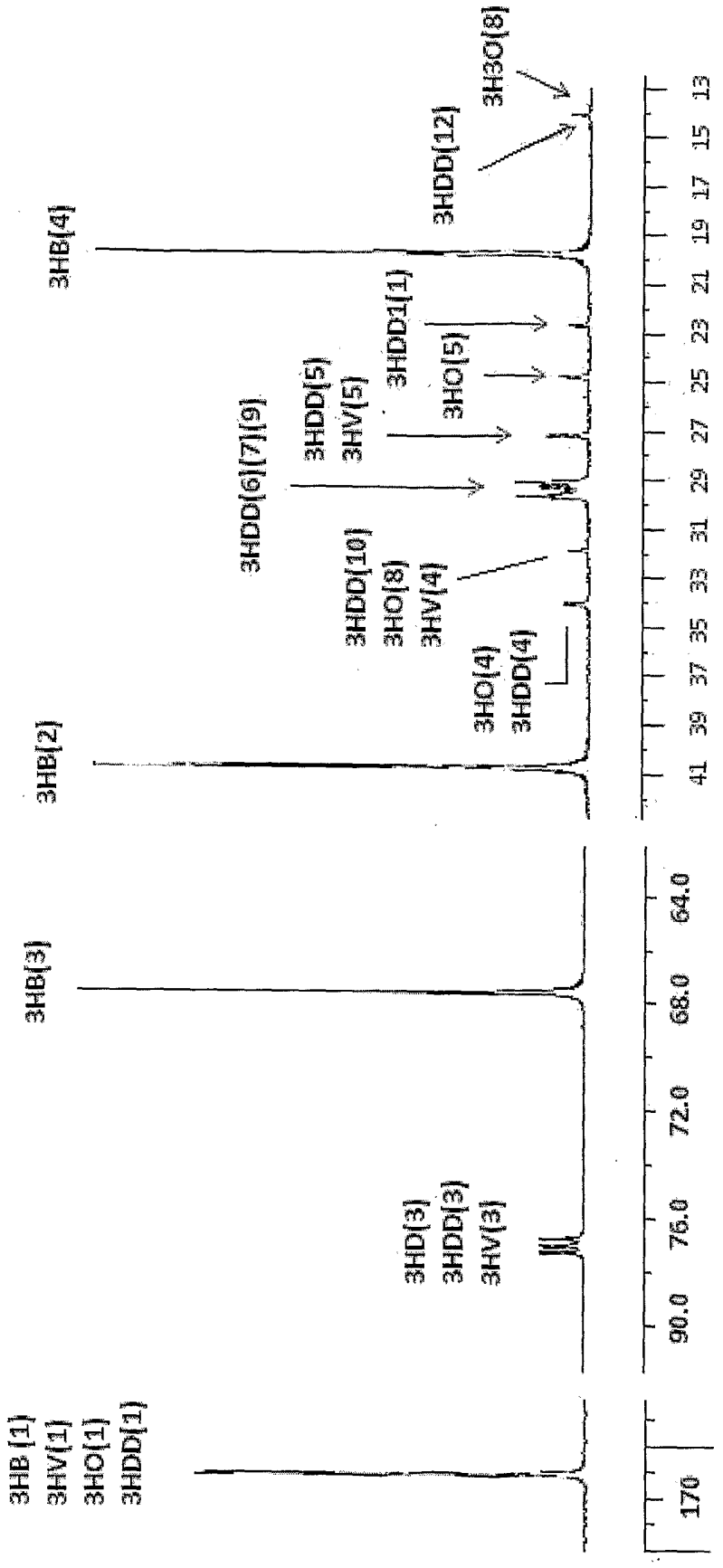


FIGURE 11a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3HB[3]
3HB [1]
3HV[1]
3HO[1]
3HDD[1]
3HDD[6][7][9]
3HDD[5]
3HV[5]
3HDD1[1]
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3HDD[12]
3H3O[8]
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3HO[4]
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3HDD[3]
3HV[3]
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64.0
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168
172
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FIGURE 11b

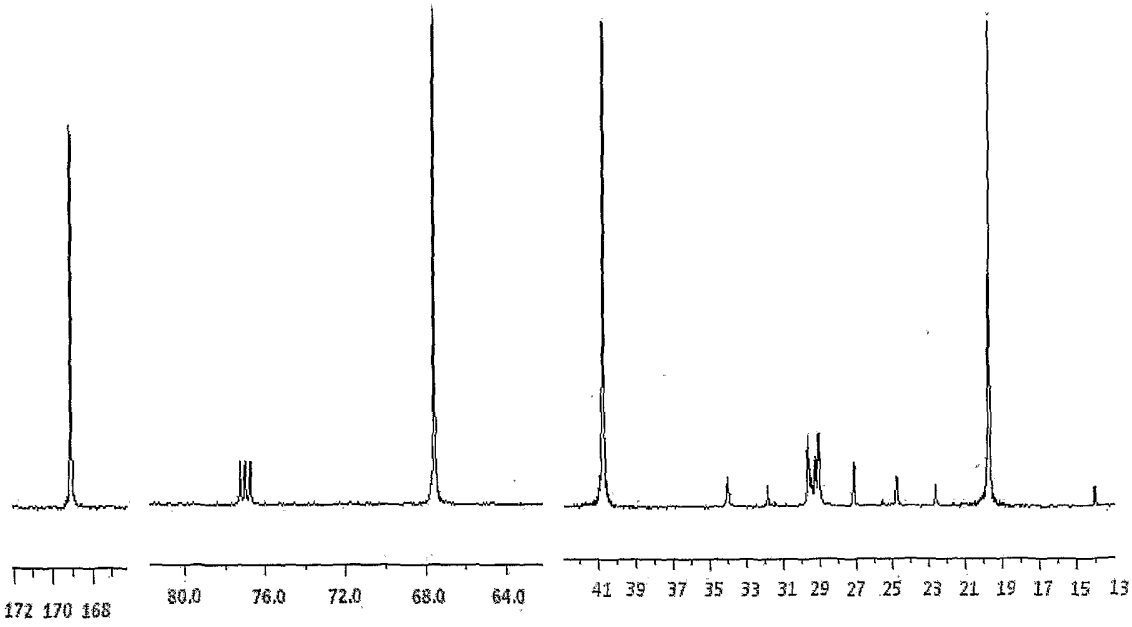


FIGURE 12

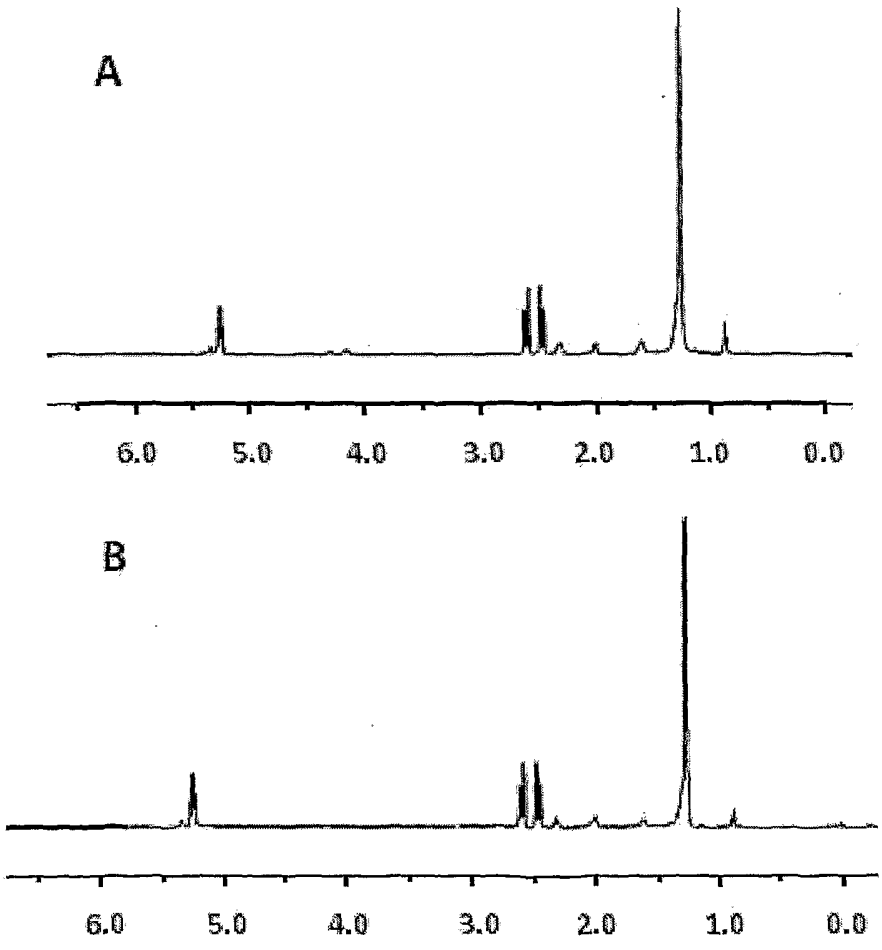
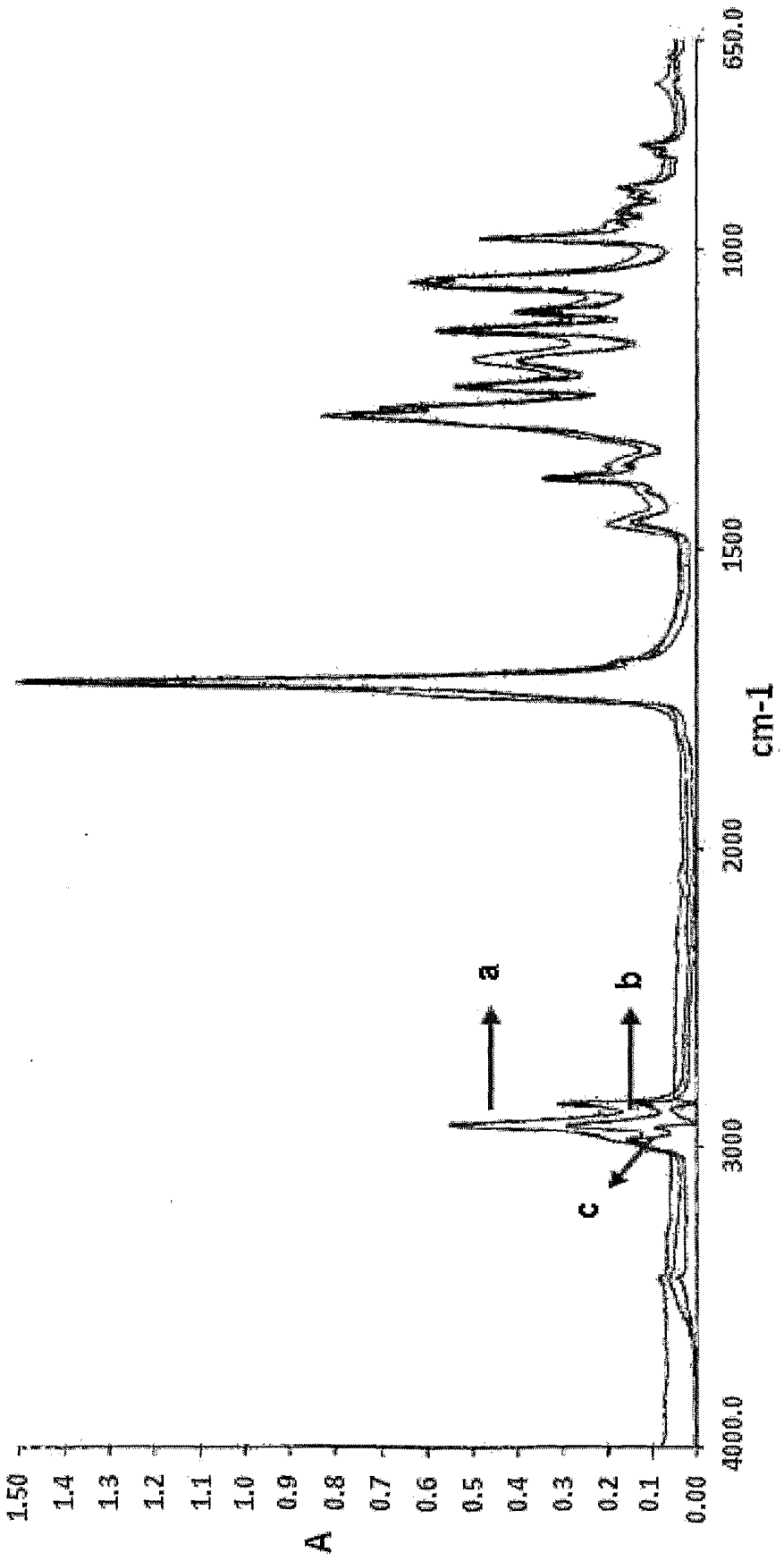


FIGURE 13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2000
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4000.0
cm-1
a
b
c
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1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
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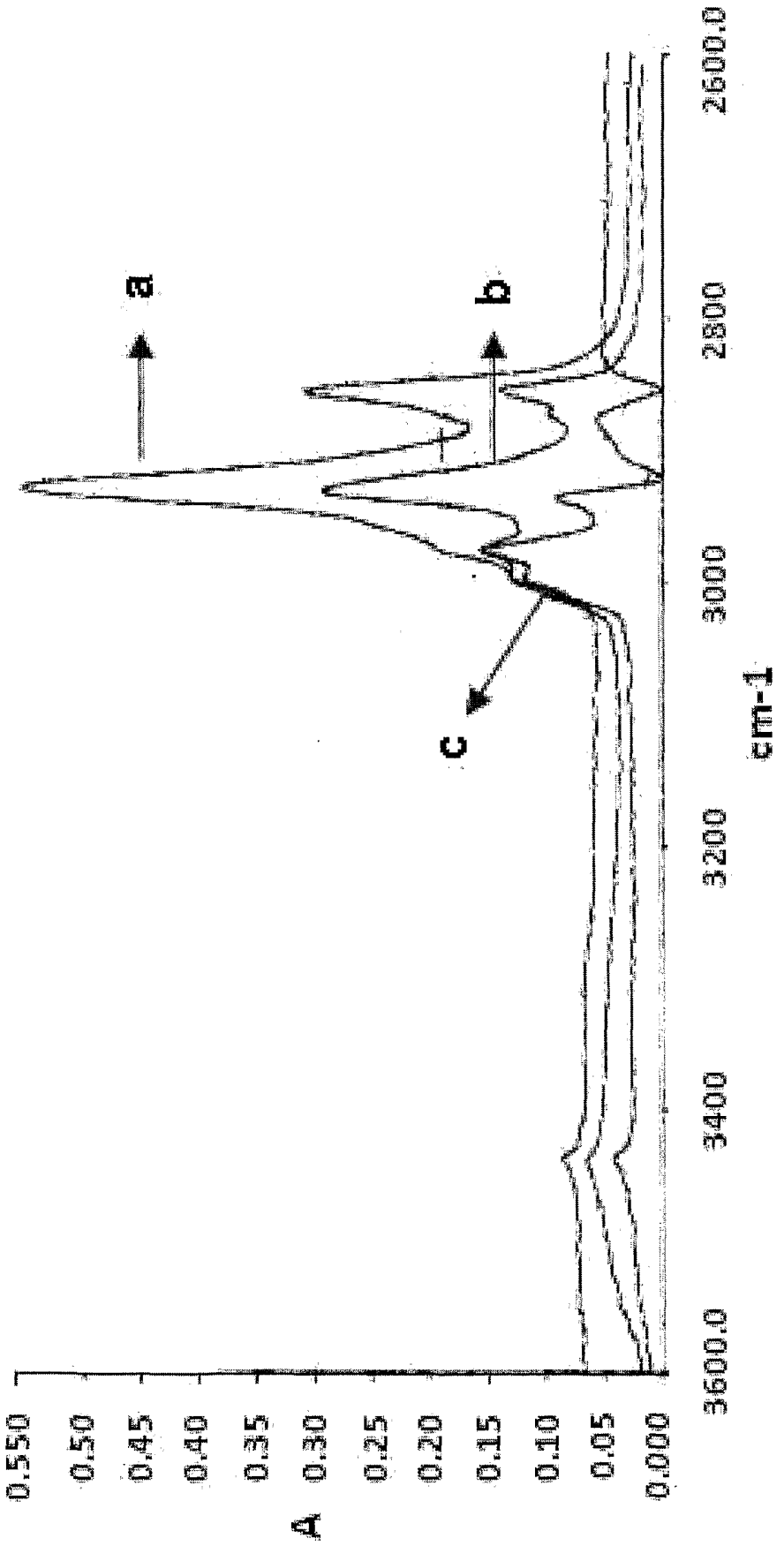
0.5
0.4
0.3
0.2
0.1
0.00

FIGURE 14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b
c
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cm-1
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0.350
0.300
0.250
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0.100
0.050
0.000
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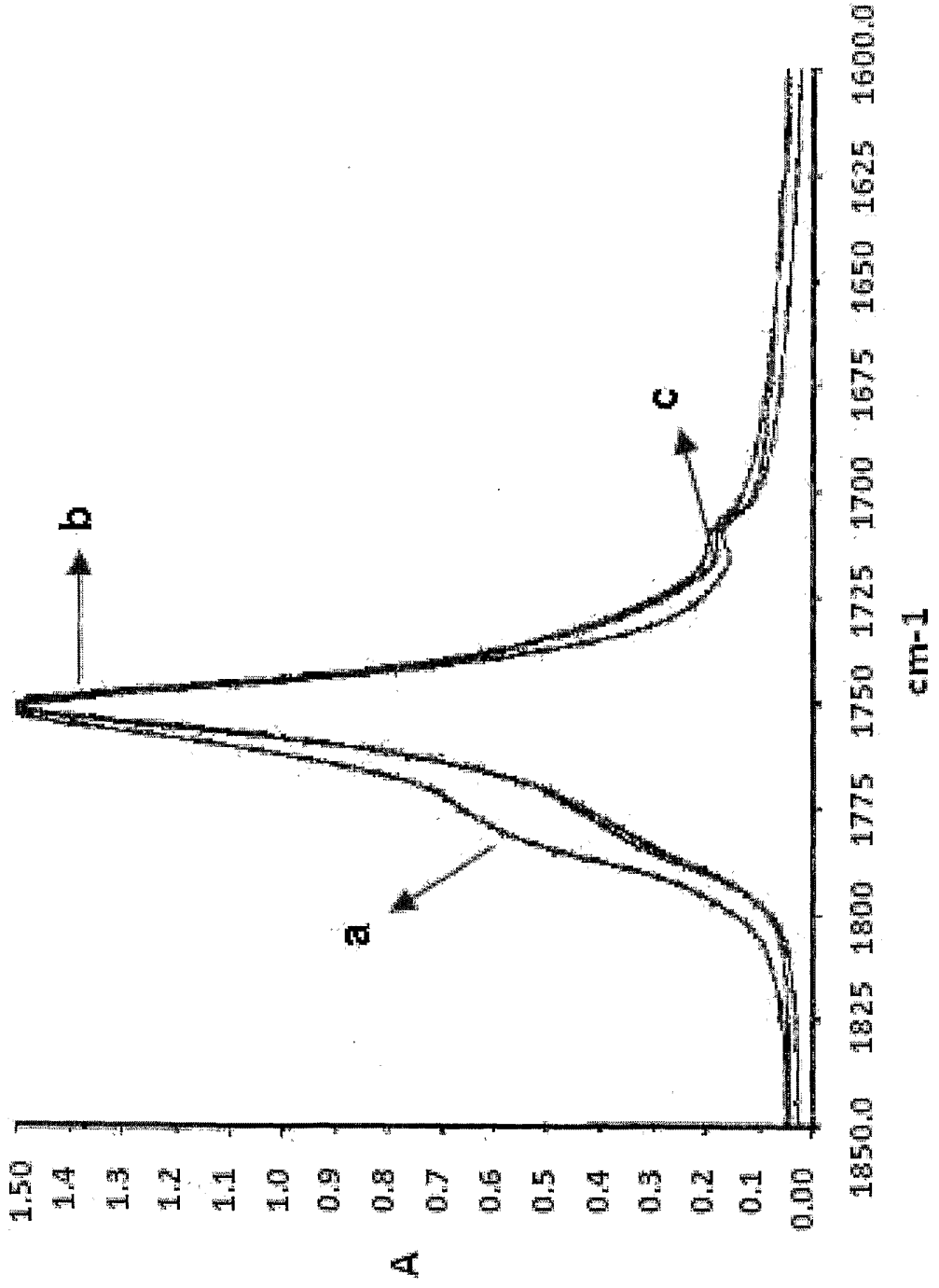
3400
3200
3000
2800
2600.0

FIGURE 15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