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(54) Title: METHOD OF PRODUCING POLYHYDROXYALKANOATES (PHA) FROM OIL SUBSTRATE

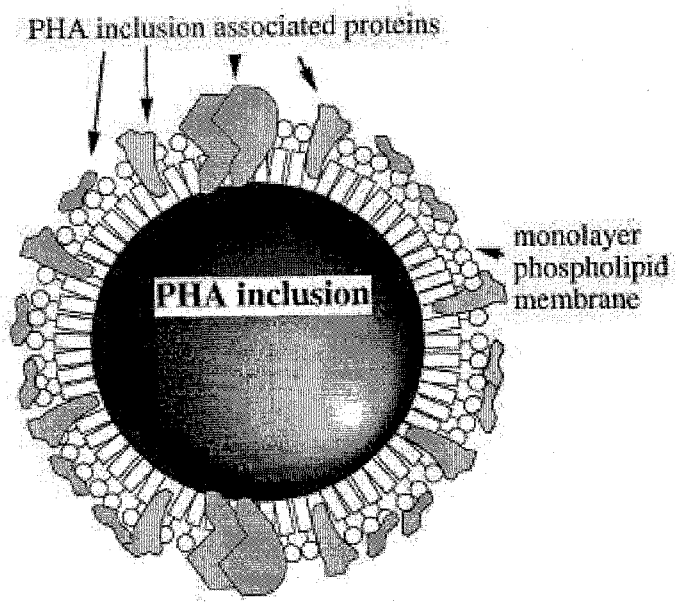

Fig. 4

(57) Abstract: In the method of producing polyhydroxyalkanoates (PHAs) on an oil substrate according to the invention, whereby the oil substrate comprises vegetable oil and/or edible oil and/or waste edible oil, preferably frying oil, the bacterial strain Cupriavidus necator H16 is grown, converting oil into PHAs and at the same time producing its own extracellular lipolytic enzymes, which are at least partially isolated from the culture medium during the fermentation process before PHA production and isolation has completed. Lipolytic enzymes isolated from C.necator during the cultivation are used for the treatment of the oil substrate in such a manner that they are added to the production medium either before the beginning of the cultivation or at the beginning of the cultivation together with inoculum and in both cases also during the cultivation together with an additional dose of oil. In the isolation of PHAs produced by the method according to the invention, after fermentation has completed, the content of the production reactor is warmed up to the temperature of 80°C for at least 30 minutes, subsequently it is cooled to the temperature of about 60°C and lytic agents containing a mixture of a detergent, such as SDS, and alkase (proteolytic enzyme) are added, by which means raw homogenate, or lysate, is obtained. Another option is isolation of PHAs by means of organic solvents - dimethyl sulfoxide or acetone.

WO 2014/032633 A1

METHOD OF PRODUCING POLYHYDROXYALKANOATES (PHA) FROM OIL SUBSTRATE

Technical field

The invention relates to a method of producing polyhydroxyalkanoates (PHAs) on an oil substrate comprising vegetable oil and/or edible oil and/or waste edible oil, preferably frying oil, on which the bacterial strain *Cupriavidus necator* H16 is grown, converting oil into PHA and at the same time producing extracellular lipolytic enzymes, which are at least partially isolated from the culture medium during the fermentation process before finishing production and isolation of PHA.

Backgound art

One of the most effective ways of producing biomaterials is making use of natural processes that take place in microorganisms. Some bacteria create reserve polymer PHA from substances present in the environment, which they utilize as food. The bacteria deposit this polymer against time to come and then, during periods of starvation, they can use it as inner food.

Polyhydroxyalkanoates (PHAs) are a group of optically active polyesters synthesized by some bacterial strains as energy storage or reserves. Monomers of PHA are (R)–3–hydroxyalkanoic acids. All monomeric building blocks are in (R) configuration due to stereo specificity of the enzyme, which is responsible for the synthesis PHA – PHA synthase. Only in a few cases a small amount of (S) monomer has been found in polyester. The molecular weight of PHAs ranges from 200,000 Da to 3,000,000 Da depending upon the microorganism and growth conditions. PHA is found in the cell in cytoplasm in the form of granules which vary in size from 0.2 to 0.5 μm [1].

PHAs may constitute up to 90 % of the cell weight. Unlike other biopolymers, such as polysaccharides, proteins or DNA, PHAs are thermoplastic. Due to their mechanical and technological properties PHA polymers are very interesting with respect to their applicability and may be an alternative to plastics produced synthetically from oil [2].

According to the number of atoms in a monomeric building block we distinguish short-chain-length PHAs (SCL) with 3 to 5 carbon atoms per monomer and medium-chain-length PHAs (MCL) with 6 to 14 carbon atoms per monomer. The structure of PHAs is illustrated in Fig.1, whereby for short-chain-length PHAs it applies that R = CH_3– C_5H_{11} and for medium-chain-length PHAs R = C_6H_{13}– $C_{14}H_{29}$.

It was as early as in the year 1926 when Lemoigne managed to isolate polyhydroxybutyrate (PHB) from the *Bacillus megaterium* bacterium. In the late 50´s of the twentieth century the presence of PHB as a form of energy and carbon reserve was confirmed in a number of Gram-negative bacterial species. Aside from the PHB, also copolymers containing 3-hydroxyvalerate and 3-hydroxyhexanoate were isolated in 1974. Since then, there have been identified many microorganisms capable of synthesis of polyhydroxyalkanoates, including industrial strains. These are both Gram-positive and Gram-negative bacteria (autotrophic, heterotrophic, phototrophic; aerobic as well as anaerobic – see the patent **WO2009156950A2**), but they also include some strains of archaebacteria. Microorganisms synthetize PHAs as a reserve form of energy and carbon under the condition of sufficient supply of carbon sources and lack of other nutrients. After exhaustion of the carbon source PHAs are then utilized as energy and carbon source. PHAs serve as an ideal reserve form of both carbon and energy, which is given by their low solubility and high molecular weight. Due to these properties they do not participate substantially in osmotic pressure in the cell [1].

In 1976 the British company Imperial Chemical Industries (ICI)-recognized the potential of hydroxybutyrate to replace synthetic polymers - mainly petrochemical polymers. Even though the bacterial production of PHB was expensive, it was anticipated that the soaring prices of oil would make the bacterial production of PHB cost-effective. As the expected increase in oil prices never happened, the polyhydroxyalkanoates find their use above all as a special biodegradable and biocompatible material. Due to high price, however, the market penetration of the polyhydroxybutyrate and P(HB-co-HV) (copolymer of hydroxybutyrate and hydroxyvalerate), which are known under the “Biopol” trademark, is rather scarce. In 1990 the German company Wella

used Biopol-made flasks for a new shampoo. In 1996 the Biopol material was bought from ZENECA BioProducts, a subsidiary company of ICI, by the American company Monsanto. Even today patents are presented to produce Biopol by using transgenic plants, which should reduce production costs (e.g. **US 2003/0017576 A1, US 2004/0101865 A1**).

The biochemical aspect of PHB biosynthesis is widely studied. With most bacteria, for example *Alcaligenes eutrophus* (also *Ralstonia eutropha;* now *Cupriavidus necator*) PHB is synthesized in a three-step reaction. First, two molecules of acetyl-CoA are coupled to form acetoacetyl-CoA in a reaction catalysed by 3-ketothiolase. The acetoacetyl-CoA is subsequently and stereospecifically reduced to (R)-3-hydroxybutyryl-CoA in a reaction catalysed by NADPH-dependent acetoacetyl-CoA reductase. Finally, PHB is synthesized by polymerization of (R)-3-hydroxybutyryl-CoA molecules by the enzyme PHB synthase [1].

The diagram of the PHB biosynthesis is shown in Fig. 2.

PHB biosynthesis happens when a carbon souce is available in sufficient amounts and under limitations of for example nitrogen, iron, phosphorus, sulphur, potassium or oxygen. PHB synthesis is regulated at the enzymatic level. From the point of view of the regulation of PHB synthesis, intracellular concentration of acetyl-CoA and of free HSCoA is essential. Under the balanced growth conditions acetyl-CoA is oxidized in the Krebs´ cycle. During the oxidation NADH, which is further used for biosynthetic purposes, is produced. After cessation of culture growth, the concentration of NADH increases, while the activities of citrate synthase and isocitrate dehydrogenase decrease. Acetyl-CoA then cannot be oxidized in the Krebs´ cycle and enters the PHB biosynthetic pathway. 3-ketothiolase is inhibited by free HSCoA, which is generated by the oxidation of acetyl-CoA in the Krebs´ cycle under normal growth conditions [4].

Some strains of bacteria generate copolymers PHA of various kinds. Copolymer PHB and PHV may be synthesized for example by the strain *Alcaligenes eutrophus* or by other types of microorganisms, namely when grown on substrates comprising glucose and propionic acid, or directly by 3-hydroxyvalerate (3HV) precursors. If propionic acid is used, the synthesis is

similar to PHB synthesis, except that acetyl-CoA condensates with propionyl-CoA to form 3-ketovaleryl-CoA, which leads to the incorporation of 3-hydroxyvalerate into the polymer structure. If an acid with an odd number of carbon atoms is used as carbon source, 3-hydroxyvalerate arises directly from the β-oxidation of fatty acids. In the case of *Alcaligenes eutrophus* it is also the low dissolved oxygen levels in the medium that increase the incorporation of 3-hydroxyvalerate into the polymer structure [1]. The diagram of the structure of PHB and P(HB-co-HV) is shown in Fig. 3.

Some microorganisms are capable of synthesizing P(HB-co-HV) when grown on a medium which does not include precursors of 3-hydroxyvalerate, such as some mutants of *Alcaligenes eutrophus.* The copolymer of 3-hydroxybutyrate and 4-hydroxybutyrate may be synthetized in *Alcaligenes eutrophus* from 4-hydroxybutyric acid, 1,4-butanediol, butyrolactone a 4-chloro butyrate [1].

PHAs are deposited intracellularly in the form of granules. The number and size of the granules depend on growth conditions, also varying within different bacterial cultures. The density of PHB granules ranges from about 1.18 to 1.24 $g.cm^{-3}$, the density of MCL PHA granules is approximately 1.05$g.cm^{-3}$. The granules contain polyesters, proteins and lipids. The granules have a polyester core, the surface being formed by a phospholipid monolayer, into which proteins fulfilling different functions are incorporated. Generally, PHAs have a hydrophobic character, therefore phospholipids and proteins constitute the interface between PHAs and the surrounding environment [1].

One of the proteins of PHA granules is the PHA synthase. There are probably three types of the PHA synthase, which differ in their substrate specificity and their primary structure. Their common feature is the active site containing cysteine. The first type of the PHA synthase catalyzes the SCL PHA synthesis (short-chain-length) from hydroxy acids consisting of 3-5 carbon atoms. The second type incorporates long-chain hydroxy acids (6-14 carbon atoms) into the structure of PHA polymers (MCL PHA). The third type differs from the first two types by its structure. Whereas the preceding types of PHA synthase consist of only one subunit having the size of 60-70 kDa, the third type of PHA synthases consists of two subunits: C-subunit (~ 40 kDa) and E-

subunit (~ 40 kDa). For the PHA synthesis by this type of enzyme both the subunits are necessary. The substrate specificity is not as strict a stipulation as in the case of the preceding PHA synthases, but, on the whole, it is the SCL PHA synthesis that is preferred [5].

The diagram of the PHA granule structure is shown in Fig. 4.

Another protein that can be found in the PHA granule is an intracellular PHA depolymerase. It is responsible for utilization PHA as a source of energy and carbon in case of limitation of carbon source from the environment. So far, research suggests that the process of PHA degradation by intracellular depolymerases is approximately 10 times slower than their synthesis. However, regulation of intracellular depolymerases has not been fully explained yet. In the structure of PHA granules there are also non-catalytic proteins, the so-called phasins. They are supposed to participate in the stabilization of hydrophobic PHAs in the aqueous environment of the cellular cytoplasm [6].

The PHB homopolymer is a polyester with all asymmetrical carbon atoms in the (R) configuration. It is relatively highly crystalline (approximately 50 to 80%), which makes it hard and brittle. The glass transition temperature is 5 to 9°C, the melting temperature is between 173 and 180°C. PHB decomposes at the temperature of 200°C, which is close to the melting point. In a chloroform solution it creates a dextrorotatory helical curve. The mechanical properties of PHB (for example Young's modulus of flexibility (3.5 GPa), elasticity of elongation (40 MPa)) is similar to that of polypropylene. Ductility, however, is only around 3%, which is considerably less than in the case of polypropylene [1].

Some properties of the PHA derivatives are shown in the following **Table 1**.

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

If 3-hydroxyvalerate is incorporated into the polymer structure, the mechanical properties improve considerably. Young´s modulus of flexibility drops below 0.7 GPa and elasticity of elongation decreases to 30 MPa. Material ductility grows with the growing proportion of 3-hydroxyvalerate. The melting point of copolymer falls to approximately 130°C, nevertheless also the decomposition temperature slightly decreases. Due to these properties copolymer may be melted without being decomposed. As stated above, poly-(HB-co-HV) comprising 0 to 30% of 3-hydroxyvalerate is commercially available under the trademark Biopol [1].

Copolymer of 3-hydroxybutyrate and 4-hydroxybutyrate does not form crystalline structures. The glass transition temperature drops from 5 to - 50°C, the melting temperature also decreases from 180 to 54°C with the growing content of 4-hydroxybutyrate (0-100%) in the polymer. For 94% 4-hydroxybutyrate Young´s modulus of flexibility is approximately 55 MPa, elasticity of elongation 39 MPa and ductility 500 % [1].

Despite the fact that there are numerous bacterial strains capable of producing PHAs, only a few of them can be used in industrial applications. Applicability of a bacterial strain is influenced by a number of factors. First of all, it is stability and safety, growth and accumulation capabilities, attainable amount of biomass and the amount of PHA. Next, it is the extractability rate of PHA, the molecular weight of PHA, the amount of usable substrates as well as financial demands on individual components of the medium [1].

The company ZENECA Bioproducts employed mutant strains of *Alcaligenes eutrophus* for the production of PHB and P(HB-co-HV). The process was realized as a two-phase fed-batch system. In the first step biomass was cultivated in a mineral medium comprising glucose as a source of carbon and energy and a precisely determined amount of phosphate. After the culture growth the phosphate was exhausted and in the second step phosphorus limitation occurred, which resulted in PHA accumulation. In the second step glucose was supplied to the culture, until a required amount of PHA was produced by biosynthesis. Each phase took approximately 48 hours and the final concentration of biomass dry weight was approximately 100 g/l. The copolymer P(HB-co-HV) was synthesized by adding a mixture of glucose and propionic acid in the second phase of the cultivation. The 3-hydroxyvalerate content in the polymer was controlled by the ratio of glucose to propionic acid [7].

Another process was developed by the Austrian company Biotechnologische Forschungsgesellschaft, which used the strain *Alcaligenes latus* DSM 1124 for the production of the PHB homopolymer, as it was capable of producing PHB in amounts up to 80% of the cellular dry weight. The process was realized as a one-step fed-batch and, as carbon source, sucrose was used [8].

Production costs could be reduced if for example methanol was used as a substrate, one of the cheapest carbon sources. The strain *Methylobacterium extorquens* produced PHB at discontinuous fed-batch cultivation on methanol. The optimum concentration of methanol was 1.7 g/l. 9-10 g/l of biomass concentration was achieved and the amount of PHB reached 30-33% of biomass. Nevertheless, even using the cheapest carbon source will not reduce operation costs, since a small amount of produced PHB makes the subsequent separation process more costly and more difficult.

There is great potential in the genetically modified bacterium *Escherichia coli,* which is capable of producing extremely large intracellular amounts of PHA (up to 80-90% of dry weight). Also, by means of methods of genetic engineering it is possible to develop strains producing copolymer P(HB-co-HV), or other copolymers. Synthesis of PHA with the aid of recombinant *E. **coli*** does not

require limitation, but it depends on the amount of available acetyl-CoA. Advantages include fast growth, ability to ultilize inexpensive carbon sources, high cell density of the grown culture and easy purification of the product (e.g. patents **KR20030070790 (A) — 2003-09-02**, **KR20030070789 (A) — 2003-09-02**, **KR20070097883 (A) — 2007-10-05**, **KR20070097884 (A) — 2007-10-05**, **US 2003/0004299 A1** etc.).

The main drawback of PHAs in comparison with synthetic petrochemical polymers is for the time being the problem of high production costs. Due to this, bacterially produced PHA is about 5-10 times more expensive than, for example, polypropylene or polyethylene. Our aim is to find an effective method of controlling PHA production in bacteria so that the bacteria would produce as much polymer as possible. The final price of polymer is affected especially by the cost of the incoming raw material for cultivation and by the demanding separation of polymer from the cells. Our strategy takes into account decreased input costs by using suitable waste substances as a food for bacteria, namely on the basis of waste oil. We also envisage optimization of the purification process, as well as using the by-product of fermentation – extracellular lipase - for the purpose of pretreatment of the substrate in a medium, or possibly as a separate co-product with numerous industrial applications.

Lipolytic enzymes belong to ester hydrolases, catalyzing in a two-phase system water – lipid, decomposition of mono-, di- a triacylglycerols to higher fatty acids, alcohol and glycerol through a complex mechanism dependent on many factors. Lipolytic enzymes are defined as carboxylesterases, which hydrolyze acylglycerols. Lipolytic enzymes, which hydrolyze acylglycerols with fatty acids with short chain lengths up to 10 carbon atoms, are considered to be esterases or carboxylases (EC 3.1.1.1). The enzymes which hydrolyze acylglycerols, comprising fatty acids with the number of carbon atoms ≥10, are referred to as lipases or also as triacylglycerol acylhydrolases (EC 3.1.1.3). Esterases or carboxylases are active in aqueous solutions, whereas „genuine" lipases are more active at the water/lipid interface than in the water phase [20]. Lipolytic enzymes are divided into three groups:

- *The first group is non-specific.* Lipolytic enzymes of this group release fatty acids from all three positions of acylglycerol and completely hydrolyze triacylglycerols into fatty acids and glycerol.
- *The second group* of lipolytic enzymes is 1,3-specific. They release fatty acids from the outer positions of the triacylglycerol molecules to form 1,2-diacylglycerol, 2,3-diacylglycerol a 2-monoacylglycerol, releasing fatty acids. Long incubation of triacylglycerol with 1,3-specific lipases generally leads to complete hydrolysis of triacylglycerols into fatty acids and glycerol.
- *The third group* includes lipolytic enzymes which prefer only some fatty acids.

Most lipases belong to extracellular enzymes, which are released to the environment during late exponential and early stationary phases of growth. The production of lipases is influenced by a number of different factors, such as temperature, pH, source of nitrogen, carbon and lipids, stress, and concentrations of dissolved oxygen and inorganic salts. The optimum pH for lipase activity is usually in the range of 6-9. However, lipases from *A. niger* and *Rhizopus* sp. are active even under acidic conditions at pH 4. In contrast, an alkaline lipase, which is active at pH 11, has been isolated from *P. nitroreducens*. Also optimum temperature and thermal stability vary. In mesophilic microorganisms the maximum lipase activity is at the temperature of 30-35 °C. In thermophilic MO it is mostly at 60°C. In psychrophilic MO lipases are produced already at low temperatures around 5°C. Most microorganisms can also produce more than one type of extracellular lipases with different specificities [21].

The mechanism of lipase activity is shown in Fig. 5.

Lipolytic enzymes belong to the so-called serine hydrolases. Three-dimensional 3D structure of these enzymes displays typical α/β-folding patterns – α-helices and β-sheets. Catalytic triad consists of three amino acid residues, namely serine, asparagine and histidine; in some lipases glutamine is found instead of asparagine. Lipolytic reaction takes place only at the lipid – water interface, therefore reaction rate is directly influenced by the substrate concentration at the phase interface. Thus in one phase there may be

molecules of the substrate in different states without directly influencing the reaction rate [22].

Esterase activity is a function of substrate concentration and undergoes Michaelis-Menten kinetics, the maximum reaction rate being achieved at a substrate concentration many times lower than saturation concentration. In contrast, lipases do not display any activity, as long as the substrate (lipid) is in the state of individual molecules in water. When the substrate concentration exceeds the solubility point, emulsion begins to form, the reaction rate increasing considerably. Lipase activity then depends directly on the presence of the phase interface. It has been confirmed by explaining the spatial structure of lipases that the active centre of an enzyme is protected by polypeptide chain, which blocks binding of the enzyme molecule itself to the enzyme and subsequent formation of the active complex. However, if lipase directly interacts with lipid phase, the starting conformational changes shift the protecting polypetide chain and thus the active centre of the enzyme becomes accessible for the lipid. As a result of hydrophobic interaction with the lipid phase the enzyme-substrate binding becomes stronger. This fact explains the phenomenon of lipase activation in the presence of the phase interface. If an enzyme acting on triacylglycerols does not show this type of activation, it should be regarded as esterase. Unlike esterases, the kinetics of the lipolytic reaction does not follow the Michaelis-Menten equation, a two-step mechanism is presumed, which includes the physical adsorption of lipase to the surface of the lipid phase, taking place concurrently with enzyme activation and shifting of the protecting polypeptide lid, and subsequent formation of the enzyme-substrate complex, which is afterwards hydrolyzed to a product and regenerated enzyme [20 - 22].

The mechanism for the hydrolysis of the ester binding is in principle identical both for lipase and for esterase and consists of four steps, as follows from Fig.6, which shows the diagram of microbial degradation of lipids. On the grounds of comparison of amino acid sequences to 3D lipase and esterase structure it was suggested that they should be distinguished on the basis of pH; the active site of lipases disposes of negative potential in the range of pH connected with the maximum lipase activity (typically at pH 8.0), whereas the

active site of esterase displays similar behaviour at pH 6.0, which is connected to the usual lower pH optimum of activity.

Most well-known microorganisms are capable of producing lipases, but only some kinds are used industrially. The reason for this is insufficient enzyme production, undesirable physical-chemical properties of lipases, limited possibilities of isolation form the cultivation medium, etc. The most common moulds that are commercially used include the species *Aspergillus, Penicillium, Mucor* and *Rhizopus.* The main producers of commercial lipases are *Aspergillus niger*, *Humicola lanuginosa*, *Mucor miehei*, *Rhizopus arrhizus, R. delemar, R. japonicus, R. niveus* and *R. oryzae* [20].

Lipolytic enzymes are currently attracting considerable attention because of their tremendous biotechnological potential. They constitute the most important group of biocatalysts for biotechnological applications, which are successfully used for the synthesis of biopolymers, bio-oil, for the production of agrochemicals and aromatic compounds. That is why the demand for industrial enzymes, particularly of microbial origin, is ever increasing. Enzymes are being exploited in various industries such as food, pharmaceutical, textile, and cosmetic industries, as well as in detergents. Lipases are used in brewing and wine making, cheese making and dietary supplements. They play an important role in pharmacy in transesterification and hydrolysis reactions and are essential for the production of special lipids. They are of great importance for modifications of monoglycerides, which are then used as emulsifiers. Some industrially important chemicals manufactured through a chemical process from fats and oils may be also produced by lipases whose specificity level is a lot greater and better. Moreover, lipases are used for the production of substitute for cocoa butter and for the production of esters which are employed in cosmetics industry. Lipases are used in the dairy industry for the hydrolysis of milk fat. Current applications contribute to intensifying the flavour of cheese, speeding up the aging process in the production of cheese, cheese making, as ingredients added to other products. Lipase from *Aspergillus oryzae* is exploited in detergents. Lipases are widely used in the processing of fats and oils, as cosmetic softeners, as well as industrial catalysts for the preparation of

prostaglandins, steroids, carboxylic nucleoside analogues and pharmaceutically important polyphenolic compounds [21].

PHAs can be used in many fields as well. It is presumed that the main use will be in the sphere of packaging industry, particularly for the production of feeding bottles and baby bottles, plastics for children´s products and ecological products (e.g. toys), packaging for cosmetics industry and the so-called intelligent packaging of food. Other interesting applications include production of containers (e.g. cups) designed for disposal after use, for instance for fast food restaurant chains, which in turn can provide waste oil as a substrate for the production of bioplastics.

Polymer PHA can be also used in other applications: nanofibres and nanoparticles can be prepared from it for targeting drug delivery system, and it is possible to use it for the production of biocompatible implants that can be employed in medicine as fibres, vascular substitutions, etc.

It should be noted that currently the production of bioplastics is relatively low, because their cost is for the time being higher than that of synthetic plastics, which discourages demand. In the near future, however, substantially stricter regulations for use of ecological plastics can be expected, which will presumably bring the expansion of bioplastic production.

Today the production of PHA from oil substrates by means of bacteria is well-known, but compared to classical production of plastics from crude oil it is too expensive, and so it is difficult to promote it.

The aim of the invention is to propose a method of PHA production from oil substrates which would be economical and would produce large quantities of PHA.

Principle of invention

The goal of the invention was achieved by a method of production of polyhydroxyalkanoates (PHAs) on an oil substrate comprising vegetable oil and/or edible oil and/or waste edible oil, preferably frying oil, on which the bacterial strain *Cupriavidus necator* H16 is grown, converting oil into PHA and at the same time producing extracellular lipolytic enzymes, which are at least

partially isolated from the culture medium during the fermentation process before finishing production and isolation of PHA, whereby the principle of the invention consists in that before starting the cultivation, extracellular lipolytic enzymes produced by *Cupriavidus necator* H16 are added to the oil substrate, thus accelerating the growth of the bacterial culture.

Concurrent production of PHA and extracellular lipolytic enzymes, during which, before the beginning of the cultivation, extracellular lipolytic enzymes produced by *Cupriavidus necator H16* are added to the oil substrate, represents an innovative method of PHA production, whereby extracellular lipolytic enzymes are induced by the presence of the oil substrate and bacteria *Cupriavidus necator* H16 produce an effective molecular form capable of effective accessing the oil substrate for utilization. It is of great advantage that enzymes are an extracellular product, whereas PHA is an intracellular product, which facilitates the separation of both products.

Owing to the fact that the production of extracellular lipolytic enzymes reaches its maximum several hours before PHA production reaches its maximum, a new technological method of isolation of extracellular lipolytic enzymes during PHA production with the aid of sterile or nonsterile ultrafiltration was devised.

Optimum conditions for bacterial growth are formed by maintaining pH values of the culture medium around 7, ranging in the interval +/- 0.3, by adding NaOH or H_2SO_4 and the dissolved oxygen concentration during the fermentation process is maintained in the range 10-50 % by controlled aeration.

By means of aeration the culture growth, PHA production and content of 3-hydroxyvalerate in the copolymer are regulated.

So as to support the bacteria growth on the oil substrate, the extracellular lipolytic enzymes, after being isolated, are at least partially added back to the culture medium together with an additional dose of oil/oil substrate.

Description of drawings

In enclosed drawings Fig. 1 represents the structure of PHA, Fig. 2 the diagram of biosynthesis PHB, Fig. 3 the diagram of PHB and P(HB-co-HV)

structure, Fig. 4 the diagram of PHA granule structure, Fig. 5 the mechanism of lipase activity, Fig. 6 the diagram of microbial degradation of lipids, Fig. 7 comparison of induction of extracellular lipase activity on different carbon substrates, Fig. 8 the yield of metabolites during a typical fermentation process, Fig. 9 characterization of the development of the centrifugation of a polymer product by the method of analytical centrifugation (4000 rpm, 2 hours, 5°C), Fig. 10 the effect of lipase addition on the process of growth of *Cupriavidus necator* H16 using oil as a carbon source, Fig. 11 an example of GC-FID chromatogram of PHA, Fig. 12 an example of GPC chromatogram of PHA, Fig. 13 TGA analysis of PHB, Fig. 14 DSC analysis of PHB, Fig. 15 pH optimum of extracellular lipase, Fig. 16 the effect of ionic strength on lipase activity a Fig. 17 record of protein separation by PAGE-SDS – silver dying.

Examples of embodiment

Production strain

For concurrent production of extracellular lipolytic enzymes and PHA the bacterial strain *Cupriavidus necator* H16 was used; Czech Collection of Microorganisms (CCM 3726). This strain is included in the Collection of Microorganisms and is approved for the production of PHAs intended for contact with food products, and it has not been genetically modified.

Bacteria growth on waste oil

Waste substrates are used for PHA production in a number of patents. One of the most general method of processing probably most types of waste is disclosed in the patent **US 2009/0317879 A1**, where, however, waste is mostly processed by methanotrophic bacteria to lower carboxylic acids (propionic, acetic) and to methane, by which means the waste is made accessible to the production strain. Another patent (**US 2010/0190221 A1**) even describes using substrates that may be toxic for microorganisms or the environment. By means of the enzyme methane-monooxygenase organic compounds are converted into utilizable substrates.

PHA production itself on waste oil of various kinds is also included in a number of various patents. The authors proposed using for example waste from landfill sites containing oil (**CN101255227 (A) — 2008-09-03**), waste edible oil for PHA production by means of hydrogen bacteria (**JP2004254668 (A) — 2004-09-16**), different types of vegetable oil – generally speaking (**WO 2009/156950 A2** – namely sunflowerseed oil, rapeseed oil, hempseed oil and other oils without further specifications; waste fritting frying? oil is not mentioned). Another possible substrate is the oil from *Brassica carinata* utilized by the bacterial species of the genus *Pseudomonas* (**WO 2010/0441180A1**). There are also several publications dealing with PHA production from oils and related substrates [9-19]. None of the available sources mentions the posibility of using waste fritting frying? oil pre-hydrolyzed by an enzyme induced during the growth process by the production strain itself.

Concurrent production of two industrially important metabolites (PHA and extracellular lipolytic enzymes), one of which (extracellular lipolytic enzyme) is induced by a sole type of substrate with a specific chemical composition (vegetable oil), and, furthermore, the substrate is preferably waste and specific (fritting oil frying?, which has no other uses), is degradable by the latter product (lipolytic enzymes) and at the same time provides the highest yields of the latter metabolite (on oil the highest yields have been achieved in the production strain – up to 96 % of biomass), has not yet been described in the case of PHA production either in technical literature or patent literature.

In some patents by-products are described (e.g. **WO 02/070659 A2**), in most cases, however, the process in question is at least a partly anaerobic process of the treatment of the substrate (in exceptional cases it is oil, but it must be utilized aerobically) and the products in question are mostly small molecules – products of typically fermentation processes (acetic acid, propionic acid, etc.), which may be further used. One published patent describes simultaneous production of intracellular PHA and extracellular polysaccharides, however, genetically modified strains are used (**WO 95/33838**).

The activity of extracellular lipolytic enzymes on different types of carbon substrate is given in **Table 2**

	Relative activity [%]	U/ml	U/mg
Oil	100.0 ± 1.1	34.37±0.37	72.23±0.77
Fructose	5.3±1.4	1.83±0.49	0.21±0.06
NB	38.7±7.1	13.29±2.46	0.26±0.05
Glycerol	38.1±4.1	13.11±1.40	10.15±1.09
Propionate	23.9±5.4	8.23±1.85	25.68±5.77
Palmitate	54.3±3.5	18.67±1.20	37.92±2.44

and comparison of induced activity of extracellular lipolytic enzymes on different types of carbon substrate is shown in Fig. 7.

Medium composition: inoculation; production; addition of precursors

The most common type of PHA so far, accumulated in the bacteria of the production strains, is homopolymer PHB. Due to low crystallinity and flexibility processes for the production of copolymers P(3HB-co-3HHx) were gradually developed, where especially copolymer with 3-hydroxyvaleric acid was produced, usually using the transgenic strain of *Ralstonia eutropha* or transgenic strains with genes of some kind of *Pseudomonas* sp. (Japanese patents **Kokai 57-150393, 59-220192**). Recently patent applications for the production of P(3HB-co-3-HHx) containing 3-hydroxyhexanoic acid (patents **Kokai 5-93049, 7-93049**) by means of soil bacteria *Aeromonas caviae* have been deposited. With 3-HH molar fraction high flexibility of polymer was achieved. Other filed patents include documents focusing on the production of copolymer with 3–hydroxyalkaonic acids with an additional methyl group, most frequently using recombinant strains. (e.g.**US 2011/0081692 A1**). Another presented patent deals with the PHA production by selected species of the genus *Pseudomonas* with controlled composition of copolymer regulated by means of the medium composition (C-source – fatty acids) and addition of suitable precursors (**US 2011/0166318 A1**), or a patent for the preparation of block copolymers by means of controlling enzymatic activities and medium composition (**WO 0006762 A1**) has been presented.

With regard to a wide range of uses and compliance of the copolymer P(3-HB-HV) structure with the European legislature concerning uses in food

industry (i.e. for potential applications), we focused on the production of copolymer P(3-HB-HV) with the 3HV content in the range 4-10%.

Medium composition

Medium for conservation and inoculum I

Beef extract	10 g
Pepton	10 g
NaCl	5 g
Distilled water	1000 ml

Medium for other inoculation steps and production medium:

Oil (waste, vegetable)	20 g
$(NH_4)_2SO_4$	3 g
Na_2PO_4	11.1 g
KH_2PO_4	1.02 g
Micro element solution*	1 ml
Distilled water	1000 ml*Micro element solution
$FeCl_3$	9.7 g
$CaCl_3$	7.8 g
$CuSO_4$	0.156 g
$CoCl_2$	0.119 g
$NiCl_2$	0.118 g
$CrCl_2$	0.062 g
Distilled water	1 000 ml

Cultivation of bacteria, production of biomass and its control

During two-step inoculation of bacterial culture inoculum I was prepared, having a total volume of 2000 ml (usually in Erlenmeyer´s flasks), after that second inoculum II was prepared in a fermentor having the volume of 25 l and after 23 hours of cultivation 200 l of the culture medium was inoculated with a second inoculum in the production tank.

Cultivation in the production medium was in progress performed for 32-38 hours. Between 8th and 12th hours 10 g/l of the precursor (sodium propionate), 3 g/l $(NH_4)_2SO_4$ and 20 g/l of oil (C-source addition) were added. In the period 8-21.5 hours the maximum activity of of extracellular lipolytic enzymes was usually achieved, part of the medium (10-50%) was therefore withdrawn, the biomass was separated out by means of ultrafiltration and subsequently (i) sterile returned to the process or (ii) added under non-sterile conditions to the reactor before the isolation of PHA. Permeate containing extracellular lipolytic enzymes was further concentrated by ultrafiltration (molecular weight exclusion limit, the so-called „cut-off" 10 kDa). Between 20th and 25th hours another dose of oil was added (20 g/l).

Metabolite yields during a typical fermentation process are represented in Fig. 8 and described in **Table 3 :**

Time	Biomass[g/l]	PHA [g/l]	PHA %	3HV [%]	Lipase[U/ml]
8	6.23 ± 0.04	4.08 ± 0.04	65.45 ± 0.72	0.00 ± 17.20	17.20 ± 2.70
14.5	11.52 ± 0.09	8.89 ± 0.25	77.15 ± 2.17	0.51 ± 18.68	18.68 ± 0.91
21.5	16.14 ± 0.49	10.85 ± 0.41	67.23 ± 2.54	1.32 ± 16.40	16.40 ± 1.27
24.5	37.65 ± 0.74	31.23 ± 0.20	82.95 ± 0.53	2.43 ± 5.22	5.22 ± 0.72
29.5	43.79 ± 0.18	36.19 ± 1.89	82.65 ± 4.31	4.01 ± 7.04	7.04 ± 1.82
32	53.90 ± 0.70	49.94 ± 1.73	92.66 ± 3.21	6.37 ± 9.27	9.27 ± 1.38

The yield coefficient $Y_{PHA/S}$ = 0.7 (both oil and precursor were included in the calculation).

During the cultivation pH of the culture medium was maintained on the value 7 +/- 0.3 by adding 1 M NaOH or 0.5 M H_2SO_4, aeration was regulated so that the dissolved oxygen concentration was during the fermentation process in the range of 10-50 %. Aeration regulated the culture growth, PHA production and the content of 3-hydroxyvalerate in the copolymer.

Single-step method of ecological polymer isolation from the cells directly in an industrial reactor immediately after finishing cultivation

First, cells are usually separated from the medium by centrifugation or filtration. Next, they have to be disrupted in order to release polymer. The most common method is the extraction of polymer by means of a suitable solvent (chloroform, methylene chlorid propylene carbonate, dichlorethylen). Nevertheless, this process is very demanding as to the solvent consumption and due to this fact it is expensive. Another method exploits sodium hypochorite, which, however, causes a partial degradation of PHB and lowers its molecular weight. A number of patents have been published disclosing solutions using nearly all sorts of available organic solvents – including those in which PHA does not provably dissolve. Methods of polymer PHA isolation with the aid of organic solvents can be found in numerous publications as well as patents, e.g. **RU 2199 587 C2**, **US 2002/0081646 A1** and others.

On the other hand, processes have been described (usually in general), which can be used for commercial production of PHB and P(HB-co-HV) as an alternative to solvent extraction. One of the options is lyophillization (freeze-drying; **WO 2006103699 A1**, **WO 2010/082810 A1**), furthermore, procedures using a mixture of enzymes and detergents have been described, without specification of the lytic mixture composition (e.g. **WO2010116681 (A1) — 2010-10-14**),

In the presented application cells are not separated first, polymer is isolated directly in the fermentation reactor immediately after the cultivation has completed (usually 32-38 hours). The cells in the culture medium are first exposed to warming-up, when the culture medium is warmed up to the temperature of 80°C (30 min) and, after subsequent cooling to the environment temperature, a mixture containing proteolytic enzyme is added (i.e. an enzyme hydrolyzing proteins, e.g. alcalase) and a detergent (e.g. sodium dodecyl sulfate) with optimized concentration (0,04 g SDS/1 g CDW; Alcojet – neutral industrial detergent). Most cell components are hydrolyzed by the acting of these two agents, whereas polymer remains untouched. After that polymer is

separated by fractional membrane ultrafiltration, is washed with water and dried by lyophilization.

Centrifugation as a common technique for separating cells from the medium, or polymer from the rest of the cells proved to be difficult to use. Above all, centrifugation of the product is rather difficult, since the residual oil carries a relatively large part (about 1/4-1/3) of the centrifuged sample to the surface, which represents a considerable loss, as follows from Fig. 9, illustrating the characterization of the process of centrifugation of the polymer product by a method of analytical centrifugation (4000 rpm, 2 hours, 5°C).

For this reason the presented patent application proposes optimization of fractional membrane ultrafiltration using filtration cassettes with a „cut-off" exclusion limit in the range of 200-400 kDa.

The final product can be also washed in order to increase its cleanness – purity?. The following table (**Table 5**) shows a list of possible purification conditions and their influence on the cleanness purity? of the PHA product.

Table 5

	PHA %
H_2O 25°C	92.39 ± 6.15
H_2O 40°C	93.79 ± 9.78
H_2O 60°C	101.64±0.86
H_2O 80°C	102.44±5.91
0.1 M NaOH	100.55±1.92
0.1 M HCl	91.73±1.42
3% H2O2	58.36±1.42
1% EtOH	91.34±1.43
control	91.72±2.49

Production, isolation and exploitation of extracellular lipolytic enzymes

If the bacteria grows on oil (and solely on oil), which may be vegetable oil and/or edible oil and/or waste edible oil, preferably frying oil, they produce extracellular lipolytic enzymes, which help to decompose the oil and utilize it. Extracellular lipolytic enzymes are industrially important enzymes and during

this cultivation process they are produced in large quantities, which is economically attractive. Therefore we propose an overall technological solution including the concurrent production of PHA (intracellular polymer; yield 93-96 %) and lipase (extracellular enzyme; activity approximately 100 U/ml)). In the concurrent production it is necessary to carry out the step consisting of the isolation of extracellular lipolytic enzymes from the culture medium prior to the final cell lysis and PHA isolation.

We have proposed an effective technological solution of isolating extracellular lipolytic enzymes during a fermentation process by a method of fractional membrane filtration. Reaching the maximum PHA production is preceded by achieving the maximum production of extracellular lipolytic enzymes by several hours, which means that during PHA production the culture medium is either withdrawn completely in a sterile manner, or part of the culture medium is withdrawn in a non-sterile manner together with the cells (it depends on the biotechnological equipment). And subsequently, there are two ways of isolating extracellular lipolytic enzymes that are technically feasible:

a) sterile ultrafiltration of the culture medium from the cells over a desirable period of time (usually after 24 hours of cultivation), adding a new culture medium with a substrate and a precursor to the growing cells. The precursor is added to the culture medium together with the additional dose of oil (addition) and with extracellular lipolytic enzymes.

b) non-sterile withdrawal of part of the culture (e.g. 1/3 of the cells in the culture medium), isolating the cells and subsequent purification of the extracellular lipolytic enzymes by ultrafiltration; afterwards, re-entry of the cells having the polymer content of about 70% to the reactor just before the final isolation of PHAs.

Extracellular lipolytic enzymes can be further utilized in the subsequent cultivation as a factor enhancing biomass production and consequently accelerating the whole process. If extracellular lipolytic enzymes, isolated according to the procedure described above in the amount of 0,5 – 3 U per ml of growth medium (i.e. about 2%) are added to a medium containing oil and subsequently the medium is inoculated by the culture *Cupriavidus necator*, the bacterial culture growth increases by approximately 20-30%. Extracellular

lipolytic enzymes produced by *Cupriavidus necator* seem to be more suitable for this purpose than for example commercially available lipase produced by the microorganism *Rhizopus oryzae.* The effect of the added extracellular lipolytic enzymes on the process of cultivation of *Cupriavidus necator H16* using oil as carbon source is shown in Fig. 10.

Digestion of biomass and extraction of PHA with organic solvents

In principle, when isolating PHB from bacterial biomass it is possible to apply two approaches - one consists in extracting PHB from bacterial biomass by means of a solvent which dissolves PHB - most often by means of chlorinated organic solvents, such as chloroform. The second method consists in disrupting and "dissolving" (digestion) of biomass other than PHB (proteins, cell wall, lipids, etc.), for which purpose it is possible to use chemical agents, such as sodium hydroxide (NaOH), detergents, sodium hypochlorite, hydrolytic enzymes, or , as the case may be, their combinations. [Jacquel N.et al., 2008].

For digestion of biomass other than PHB, organic solvents, which under the conditions of isolation do not significantly dissolve PHB, were used – DMSO and acetone. This strategy applied to biomass with the content of PHB 79.2 % increased the cleanness of the product to the value of 92.0 % (aceton) and 91.1 % (DMSO). As far as we know, this strategy has not been published by anybody yet. In the only publication, in which acetone is exploited for isolating PHB from biomass, the first strategy is used, when at a very high temperature and pressure PHB is dissolved in acetone [Koller M. et al., 2013]. The technology that we are proposing provides a procedure under less dramatic conditions (energetic profitability), when PHB does not dissolve. Moreover, in our case it is not necessary to isolate PHB from the dried biomass. The fact that the cells before being isolated do not have to be dried is also energetically favourable and within the process it is also logistically advantageous.

	Cleanness of PHB %
Before isolation	79.2± 4.2
Acetone	92.0 ± 3.8
DMSO	91.1± 2.1

Procedure: After the cultivation has completed, the biomass is separated from the culture medium by centrifugation and subsequently an organic solvent from the group dimethyl sulfoxide (DMSO) or acetone in the amount corresponding to 20-80 g of wet biomass per 1 litre of solvent is applied on the wet biomass. Incubation process takes between 1 and 5 hours at the temperature in the range from 30 to 80°C. Subsequently the organic solvent is removed for example by centrifugation and the solid fraction containing PHB having cleanness higher than 90% may be further purified using any of the methods mentioned within the patent application, or any of the generally known methods. The used organic solvent may be regenerated for example by distillation.

Characterization of PHA polymer

Determination of content and structure of produced PHA

The concentration and structure of PHA is most often determined by a method of gas chromatography (GC) with detection by FID (flame ionization detector). Before the actual analysis polymer is subjected to acid hydrolysis and subsequent methylation to form volatile methyl esters of 3-hydroxy acids. These esters are afterwards separated and detected by means of GC. Fig. 11 shows a typical chromatogram of polymer composed of 3-hydroxybutyrate and 3-hydroxyvalerate. By peak integration and by a method of calibration curve (Table 6) it is possible to determine the content of each component and express their ratio, in other words, the content of 3-HV in copolymer PHB-HV.

Table 6 *Ratio between peak area and 3HB (3HV) concentration*

3HB ($mg \cdot ml^{-1}$)	3HB area (-)	3HV ($mg \cdot ml^{-1}$)	3HV are (-)
2.0592	16133.8	0.2808	3076.1
4.1272	31629.3	0.5628	6159.2
6.1952	44894,3	0.8448	8801.3
8.2632	65493.6	1.1268	12837.5
10.296	77344.3	1.4040	15265.1

Determination of molecular weight of produced PHA

The molecular weight of PHA is a very important parameter, especially with respect to its applications. Its value is important not only for processing of PHA for example to bioplastics, nanofibres or particles, but also for numerous pharmaceutical applications, particularly in connection with biodegradability of the used materials. Therefore it is essential to characterize the produced microbial polymer in the greatest detail possible, or generalize the conditions for the production of polymer having a defined molecular weight.

The molecular weight of the produced PHA was determined by a method of gel permeation chromatography (GPC). An example of GPC chromatogram of PHA is shown in Fig. 12. During a number of testing experiments it was found out that it was possible to influence the molecular weight of the final polymer by mere modification of growth conditions (e.g. nutrition stress) and by selection of the cultivation medium. For this purpose some authors used enzyme preparative, chemical substances or radiation (see e.g. **US 2006/0183205 A1**).

In our case the molecular weight of the produced polymer achieved values in the range 1.85-2.41 E+05, whereby the values achieved on a saccharide substrate were more than twice as high.

The molecular weight of selected samples is shown in **Table 7**

	Mn	**Mw**	**D**
Biomer PHB (commercially available material)	$1.75 \cdot 10^5$	$5.15 \cdot 10^5$	2.94
Sigma P(HB-co-HV); 5 % HV (commercially available material)	$1.84 \cdot 10^5$	$5.51 \cdot 10^5$	2.99
Sigma P(HB-co-HV); 12 % HV (commercially available material)	$1.36 \cdot 10^5$	$2.95 \cdot 10^5$	2.17
PHB, cultivation *C. necator* H16, substrate fructose	$6.94 \cdot 10^5$	$1.81 \cdot 10^6$	2.61
PHB, cultivation *C. necator* H16, substrate oil	$3.84 \cdot 10^5$	$1.15 \cdot 10^6$	3.00
P(HB-co-HV), 6 % HV; cultivation *C. necator* H16, substrate oil, precursor sodium propionate	$2.41 \cdot 10^5$	$6.60 \cdot 10^5$	2.74

Thermal stability

The thermal properties of PHA depend on the content of individual monomers (see Tab. 1). Specific parameters, such as the temperature of

material degradation, the melting point etc. are routinely determined by means of TGA (thermogravimetric analysis), which is represented in Fig. 13, and DSC (differential scanning calorimetry), which is shown in Fig. 14.

Temperature of the melting point and the degradation point of selected samples of PHAs is given in **Table 8**.

	Temperature degradation [°C]	**Temperature melting [°C]**
Biomer PHB (commercially available material)	264	168.52
Sigma P(HB-co-HV); 5 % HV (commercially available material)	264	144.87
Sigma P(HB-co-HV); 12 % HV (commercially available material)	278	146.36
PHB, cultivation *C. necator* H16, substrate fructose	281	172.36
PHB, cultivation *C. necator* H16, substrate oil	281	171.05
P(HB-co-HV), 6 % HV; cultivation *C. necator* H16, substrate oil, precursor sodium propionate	256	166.40

Solubility of PHA

PHA is not soluble in polar implicit solvents or in non-polar implicit solvents. Solvents from the centre of an eluotropic series (medium Rf values) have the ability to partly dissolve a polymer or create gel. Solubility of PHA in selected organic solvents is shown in **Table 9:**

	Hexane HX	**Dichlormethane DCM**	**Xylene XYL**	**Toluene TOL**
Solubility	nr.	mr.	mr. + gel	gel
Liquid colour	light yellow	light yellow	light yellow	light yellow
	Dioxane DOX	**Chloroform CHL**	**Dimethyl sulfoxide DMSO**	**Butyl acetate BuAc**
Solubility	mr.	gel	gel	mr. + gel
Liquid colour	light yellow	light yellow	light yellow	light yellow
	Ethyl acetate EthAc	**Acetone ACE**	**2-propyl alcohol 2PrOH**	**Ethanol EtOH**
Solubility	nr.	nr.	nr.	nr.
Liquid colour	light yellow	light yellow	light yellow	light yellow

R – highly soluble; r – soluble; mr - slightly soluble; nr – insoluble; gel – creates gel

Characterization of extracellular lipolytic enzymes

Basic molecular characteristics include pH optimum, whose values are summarized in Fig. 15 and shown in **Table 10**, which indicates changes of extracellular lipolytic enzyme activity at varying pH assessed in % compared to the maximum value of 100 %.

pH	Activity (%)
3.0	7.42 ± 2.22
4.0	6.52 ± 1.96
4.8	9.59 ± 0.31
5.8	1.8 0± 1.13
6.6	27.49 ± 1.48
7.0	37.21 ± 2.36
7.4	76.60± 9.89
8.0	79.28 ± 5.69
8.3	54.6 ± 1.78
9.0	10.1 ± 2.66
9.4	16.88 ± 2.49
9.8	68.54 ± 3.49
1.6	100 ± 17.47
1.0	60.23 ± 8.70
1.5	2.05 ± 0.96
1.0	6.14 ± 5.04
13.0	11.13 ± 6.93

The development of the pH optimum displays two maximums, which corresponds to two molecular forms of extracellular lipolytic enzymes. In microorganisms a similar type of secretion of lipolytic enzymes usual; with respect to the value of pH optimum it can be assumed that both forms decompose acylglycerols and they are probably enzymes with different preference for the esteric bond in triacylglycerols.

The effect of ionic stregth (NaCl, 0 – 5 mol/l) on the activity of extracellular lipolytic enzymes is illustrated in Fig. 16. From the course of the dependence it is obvious that the activity of extracellular lipolytic enzymes decreases with growing ionic strength, which is significant with regard to the cultivation medium composition.

Partial purification of extracellular lipolytic enzymes

After the separation of the cells the culture medium contains, apart from extracellular lipolytic enzymes, also a number of other proteins and other

substances. Furthermore, all these substances are considerably attenuated, including the targeted enzyme. For the purpose of condensation and more detailed characterization of extracellular enzymes the following experiments were carried out in order to verify cheap and effective isolation and concurrent enzyme purification.

As a result of condensation by aceton, substantial enzyme activity was lost, the yield represented about 1%. At the condensation by ammonium sulphate within the range of concentration between 60 and 80%, the saturation was more successful, nevertheless the subsequent dialysis practically resulted in complete loss of activity, the yields reaching around 3%. Another method used for the purification of extracellular lipolytic enzymes was fractional ultrafiltration. In the first phase the cells were removed (on a filter with cut-off 1 mil.[A1] kDa) and afterwards extracellular lipolytic enzymes were isolated on an ultrafilter with „cut-off" exclusion limit 10 kDa. Consequently, the concentration increased 8 times and the yield was approximately 91 U/ml of the culture, see **Table 11**. Ultrafiltration was therefore proposed as an optimum part of the technology.

Table 11

	Activity ($U \cdot ml^{-1}$)	Protein concentration ($mg \cdot ml^{-1}$)	Activity ($U \cdot mg^{-1}$)	Volume(ml)	Yield (-)
Supernatant	29.92 ± 3.24	0.30± 0.01	99.74 ± 10.83	400	1.00
Ultrafiltration	90.68± 1.47	0.44 ± 0.01	206.11 ± 3.36	50	0.38

Molecular weight of extracellular proteins a purity of preparates of extracellular lipolytic enzymes determined by PAGE/SDS

In order to achieve characterization of protein composition and thus also the purity of a condensed preparate of extracellular lipolytic enzymes, a method of polyacrylamide gel electrophoresis (PAGE) in the presence of dodecyl sodium sulphate (SDS) was applied, wherein proteins are separated in accordance with mobility in the unidirectional electric field. The mobility is determined by the ratio of molecular weight and the particle charge. As all

proteins have under these conditions the same charge and shape, separation in principle takes place on the basis of differences in molecular weight. The record of separation (silver dyeing) is in Fig. 17. Explanatory note for Fig. 17 is in the following **Table 12**

line	Sample – purification step
1	Standard
2	Dialysis
3	Dialysis
4	Ultrafiltration
5	Acetone precipitation
6	$(NH_4)_2SO_4$ precipitation 60%
7	$(NH_4)_2SO_4$ precipitation 70%
8	$(NH_4)_2SO_4$ precipitation 80%

and **Table 13**, which describes protein fractions of electrophoresis

Zone	relative molecular weight ($Da \cdot 10^3$)
Protein fraction1	15.95
Protein fraction2	18.80
Protein fraction3	24.34
Protein fraction4	47.54
Protein fraction5	64.52

Extracellular fraction of *C.necator* H16 comprises 5 main protein fractions having molecular weights of 15.95 kDa; 18.80 kDa; 24.34 kDa; 47.54 kDa and 64.52 kDa. The first four of them were visible even after the dialysis. After the application of ultrafiltration (membrane filter with 10 kDa „cut-off" exclusion limit) the majority fractions were proteins with membrane filter 18.80 kDa and 24.34 kDa, the fraction having 15.95 kDa was only slightly visible. In addition, it was confirmed by the method PAGE/SDS that altough the preparative of extracellular lipolytic enzymes obtained by fractional ultrafiltration has a relatively low concentration, it contains a minimum of impurities and can be used for a direct application – either as addition to the culture medium for industrial production of PHAs, or for the pre-treatment of the oil substrate, or for other industrial uses.

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22. Bornscheuer U.T.: Microbial carboxylesterases: classification, properties and application in biocatalysis; *FEMS MicrobiologyReviews 26* (2002) 73-81.
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PATENT CLAIMS

1. A method of producing polyhydroxyalkanoates (PHAs) on an oil substrate comprising vegetable oil and/or edible oil and/or waste edible oil, preferably frying oil,on which the bacterial strain *Cupriavidus necator* H16 is grown, converting oil into PHA and at the same time producing extracellular lipolytic enzymes, which are at least partially isolated from the culture medium during the fermentation process before PHA production and isolation has completed, **characterized in that** before starting the cultivation, extracellular lipolytic enzymes produced by *Cupriavidus necator* H16 are added to the oil substrate, thus accelerating the growth of the bacterial culture.

2. A method of producing PHAs according to Claim 1, **characterized in that** extracellular lipolytic enzymes are isolated from the culture medium by sterile ultrafiltration of the culture medium, in which the cells of the bacteria *Cupriavidus necator* H16 are separated and return to the production device.

3. A method of producing PHAs according to Claim 1, **characterized in that** extracellular lipolytic enzymes are separated from the culture medium by non-sterile withdrawal of part of the fermented growth medium, where upon from the withdrawn part of the growth medium are separated the bacterial cells, which return to the culture medium before the isolation of PHA, and from the rest of the medium extracellular lipolytic enzymes are isolated.

4. A method of producing PHAs according to Claim 1 or 2, **characterized in that** after the isolation of extracellular lipolytic enzymes these return to the culture medium at least partially together with an additional dose of oil substrate to support the growth of bacteria *Cupriavidus necator* H16.

5. A method of producing PHAs according to any of the preceding claims, **characterized in that** during the cultivation pH values of the culture medium are maintained in the range 7 +/- 0.3 by adding NaOH nebo H_2SO_4 and the dissolved oxygen concentration during the fermentation process is maintained in the range 10 - 50 % by controlled aeration, whereby the culture growth, PHA

production and content of 3-hydroxyvalerate in copolymer are regulated by aeration.

6. A method of producing PHAs according to any of the preceding claims, **characterized in that** the oil substrate contains an additional substance from the group sodium propionate, n-propanol, levulinic acid.

7. A method of producing PHAs according to any of the preceding claims, **characterized in that** after finishing fermentation the content of the production reactor is warmed up to the temperature of 80°C for at least 30 minutes, subsequently it is cooled to the temperature of 20 to 60°C and after that lytic agents containing a mixture of a detergent, for example SDS, and proteolytic enzyme are added, by which means raw homogenate, or lysate, is obtained.

8. A method of producing PHAs according to Claim 7, **characterized in that** the raw homogenate, or lysate is cleaned by fractional membrane filtration and washed with water at the temperature of 20 to 60°C, whereby the resulting yield of PHA is up to 96 % from the original cell biomass.

9. A method of producing PHAs according to Claim 2 or 3, **characterized in that** extracellular lipolytic enzymes are condensed by ultrafiltration, which results in their cleanness ranging from 100 to 200 U/mg.

10. A method of producing PHAs according to any of the Claims 1 to 6, **characterized in that** after the fermentation has completed the biomass is separated from the culture medium by centrifugation and subsequently directly on the undried biomass a nontoxic organic solvent is applied from the group dimethyl sulfoxide (DMSO) or acetone in an amount of 1 l of solvent per 20-80 g of damp biomass and is left there to act for 1-5 hours at the temperature ranging from 30 to 80°C, whereupon the organic solvent is removed .

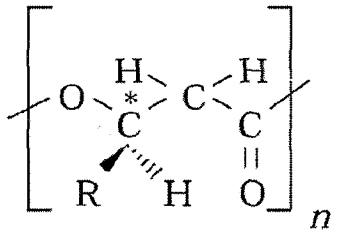
H
O
C
C
H
C
C
R
H
O
n

Fig. 1

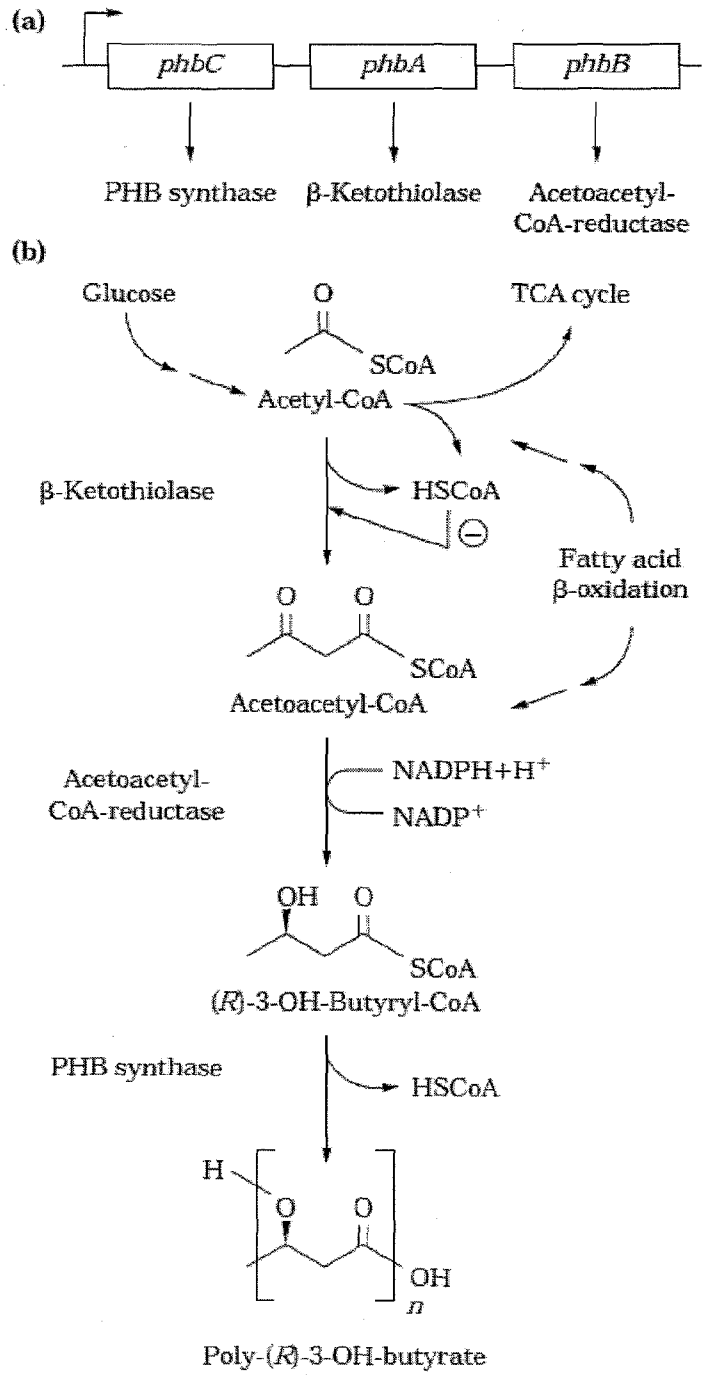
(a)
phbC
phbA
phbB
PHB synthase
β-Ketothiolase
Acetoacetyl-CoA-reductase
(b)
Glucose
TCA cycle
SCoA
Acetyl-CoA
β-Ketothiolase
HSCoA
Fatty acid β-oxidation
SCoA
Acetoacetyl-CoA
Acetoacetyl-CoA-reductase
NADPH+H+
NADP+
OH
SCoA
(R)-3-OH-Butyryl-CoA
PHB synthase
HSCoA
H
OH
n
Poly-(R)-3-OH-butyrate

Fig. 2

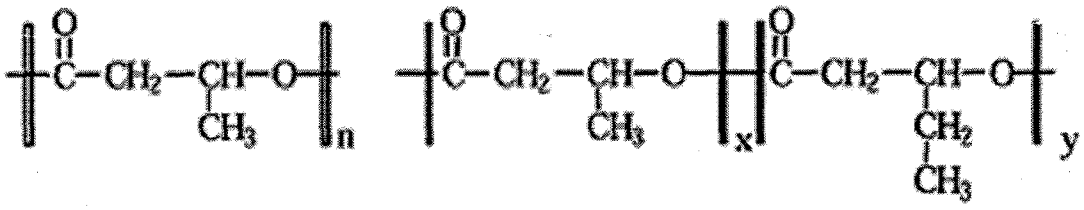
Poly(3HB)
Poly(3HB-co-3HV)

Fig. 3

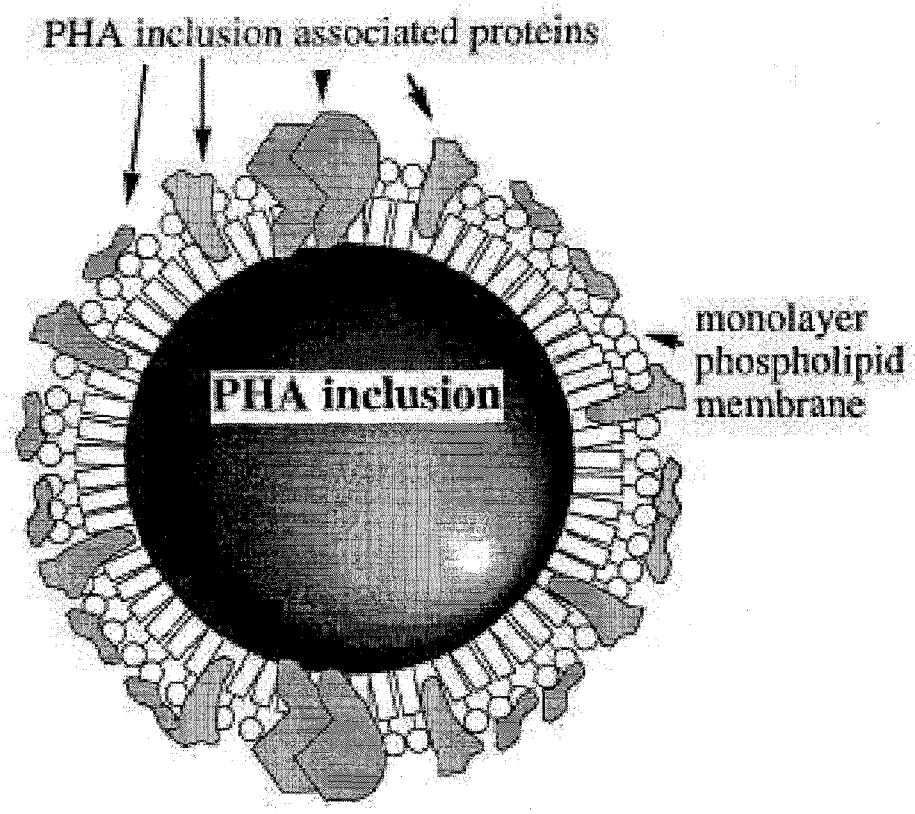
PHA inclusion associated proteins
PHA inclusion
monolayer
phospholipid
membrane

Fig. 4

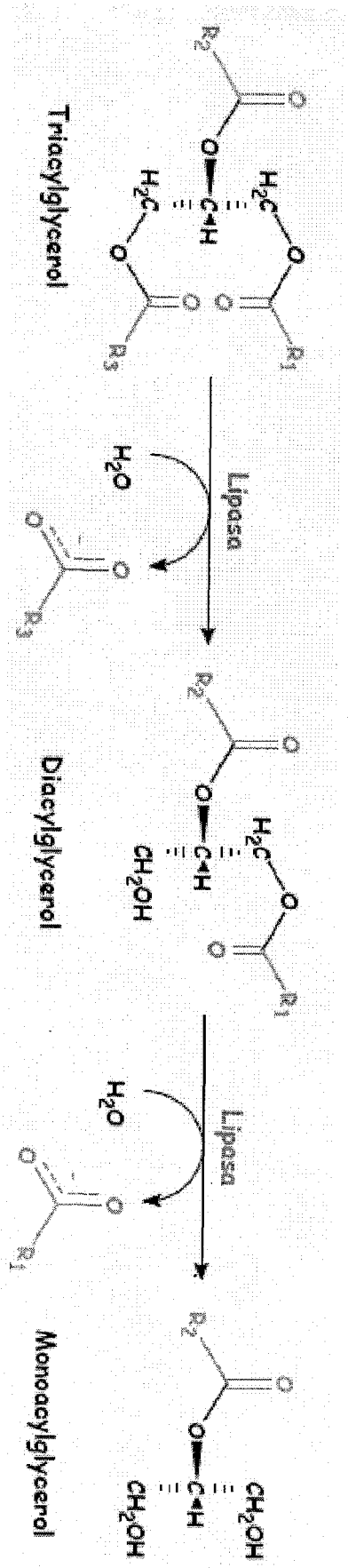
Triacylglycerol
Lipasa
Diacylglycerol
Lipasa
Monoacylglycerol

Fig.5

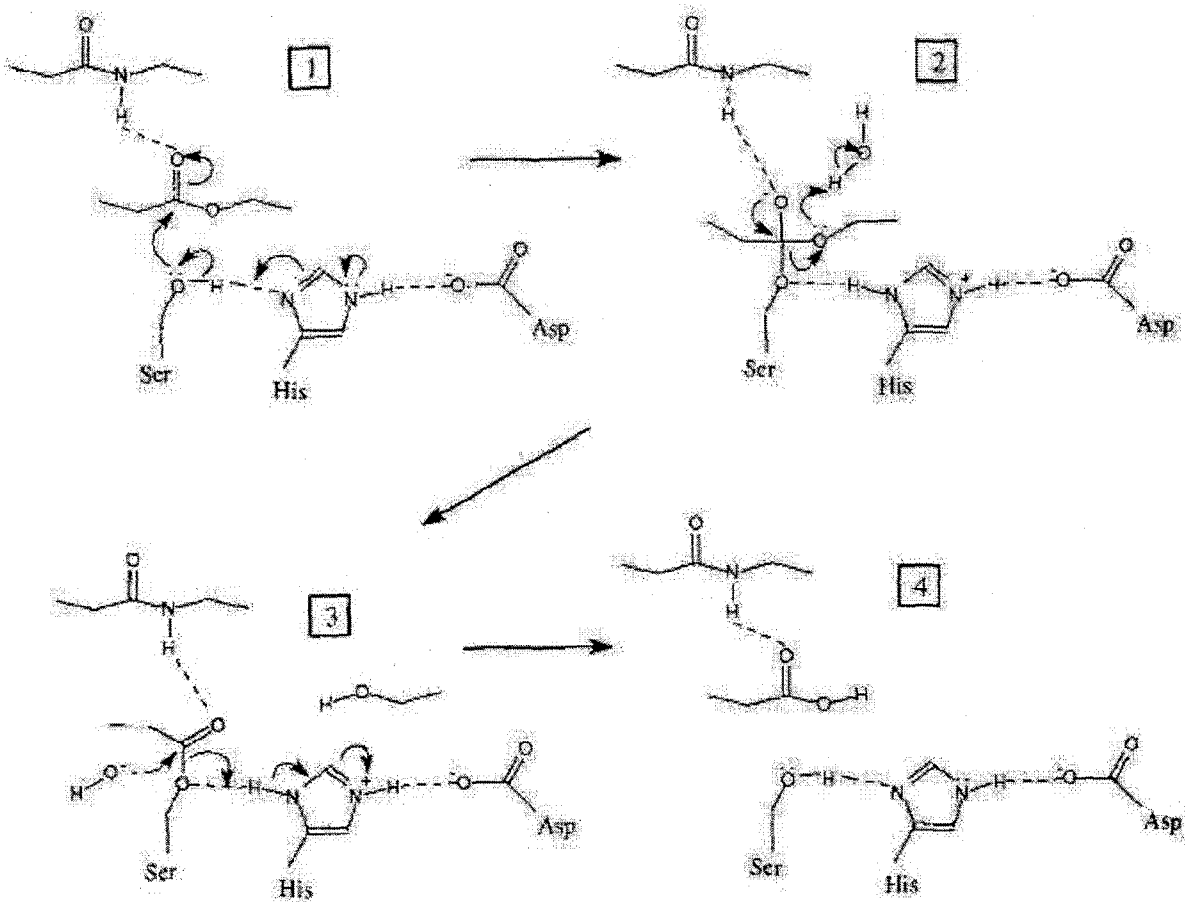
1
2
3
4
Ser
His
Asp

Fig. 6

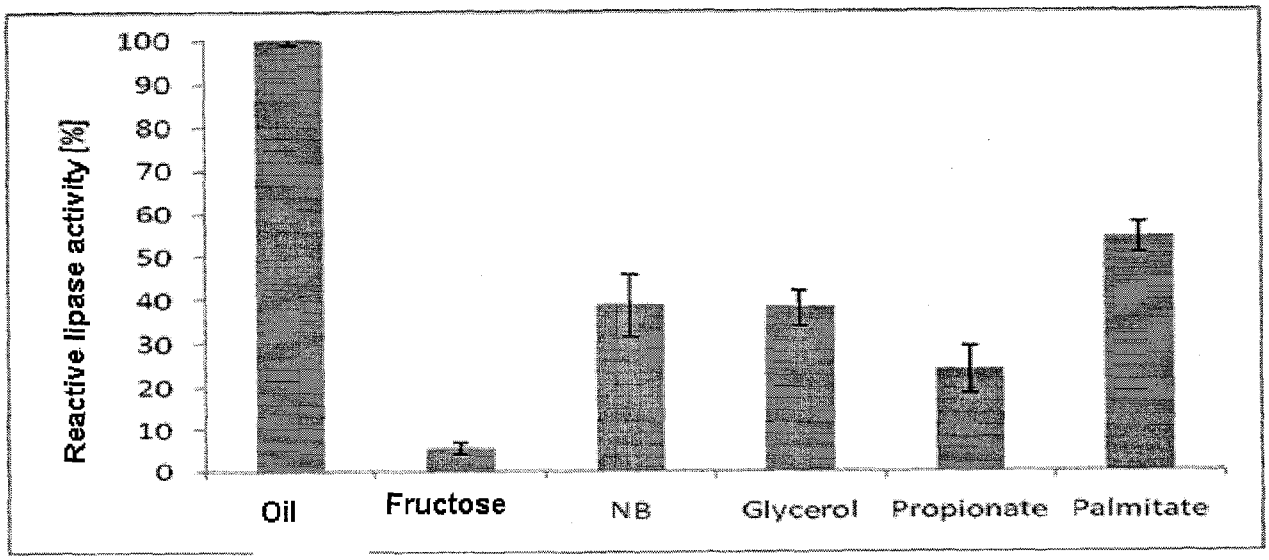
Reactive lipase activity [%]
100
90
80
70
60
50
40
30
20
10
0
Oil
Fructose
NB
Glycerol
Propionate
Palmitate

Fig. 7

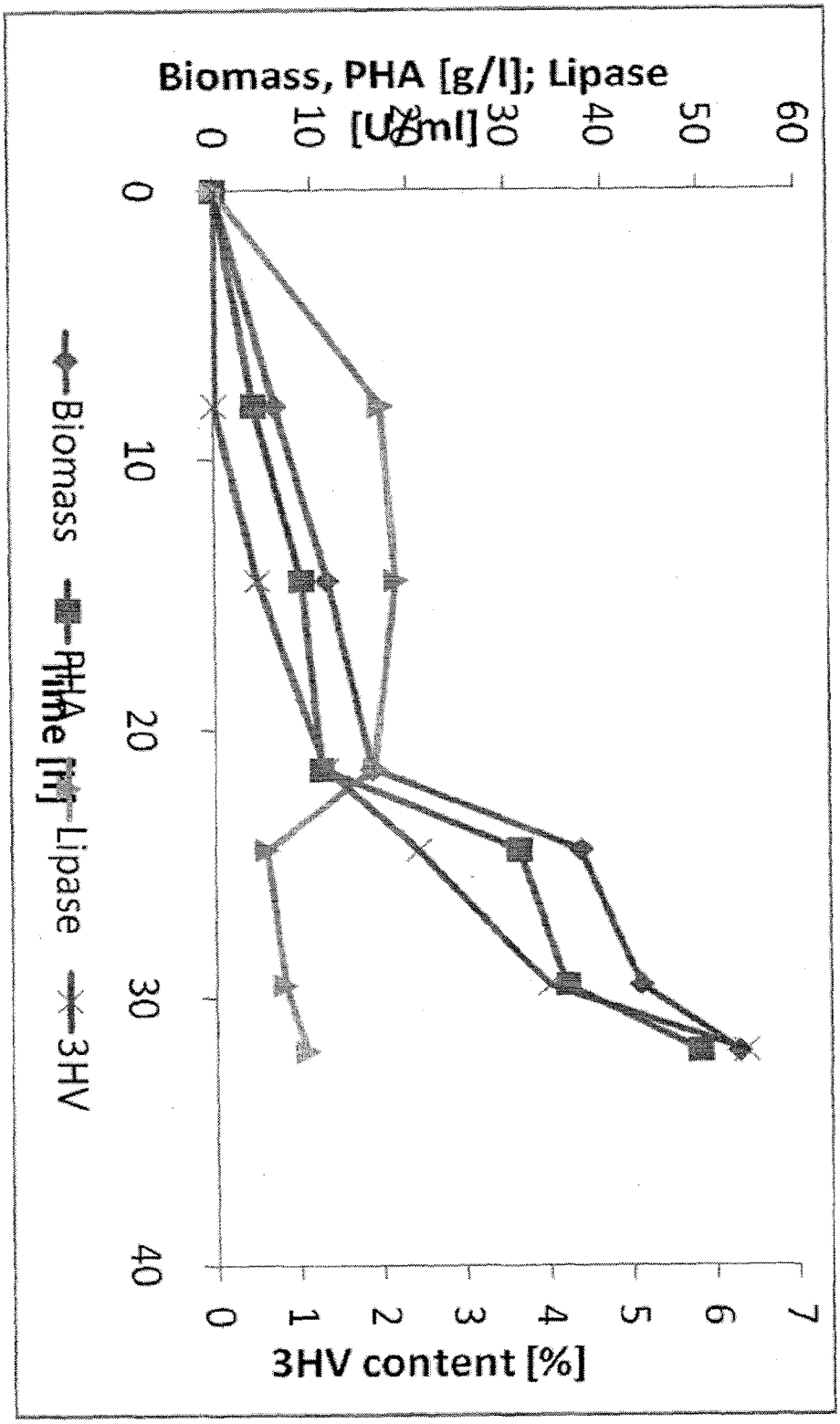
Biomass, PHA [g/l]; Lipase [U/ml]
0
10
20
30
40
50
60
0
10
20
30
40
Biomass
PHA
Lipase
3HV
Time [h]
0
1
2
3
4
5
6
7
3HV content [%]

Fig. 8

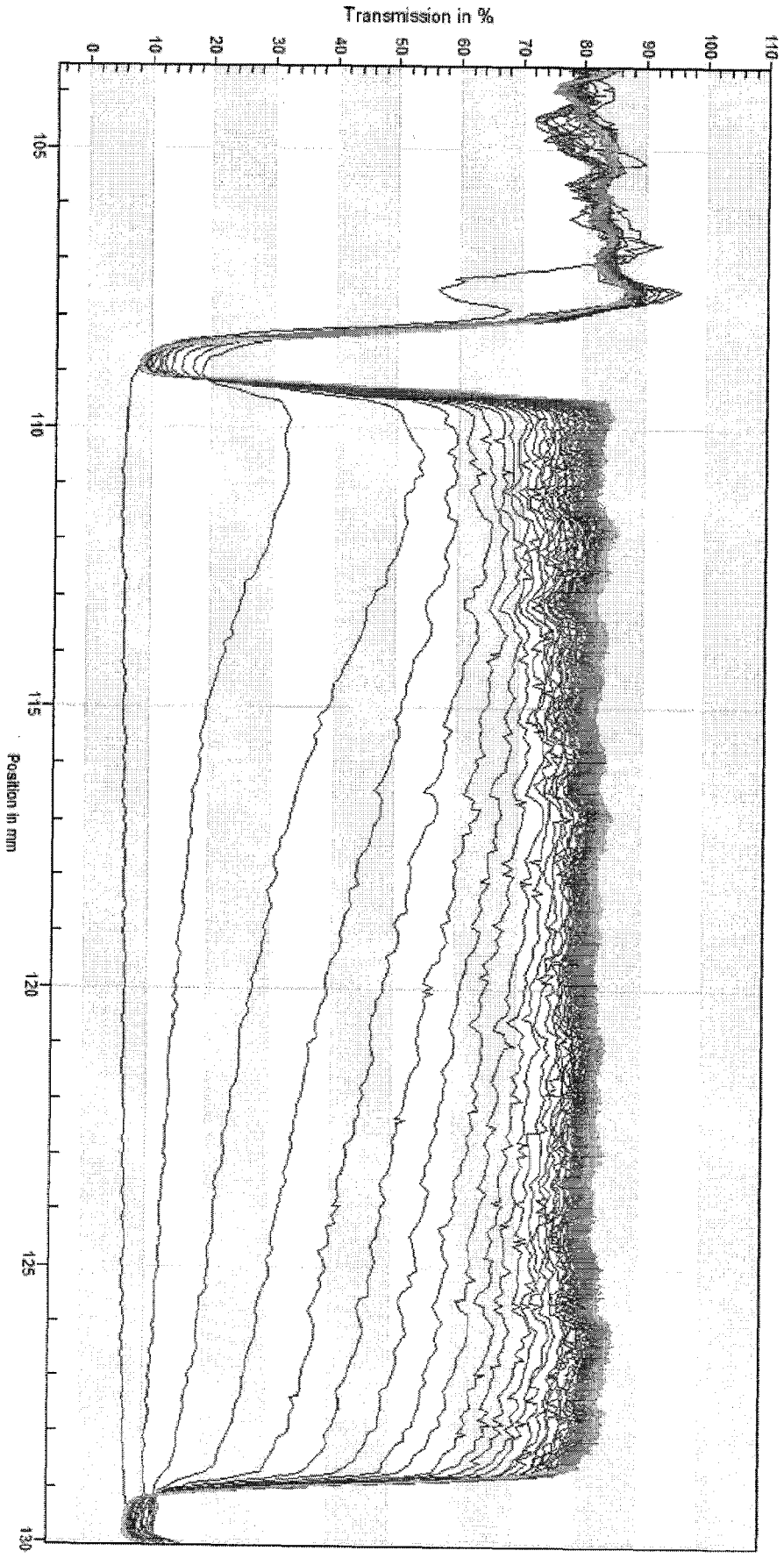
Transmission in %
0
10
20
30
40
50
60
70
80
90
100
110
Position in mm
105
110
115
120
125
130

Fig. 9

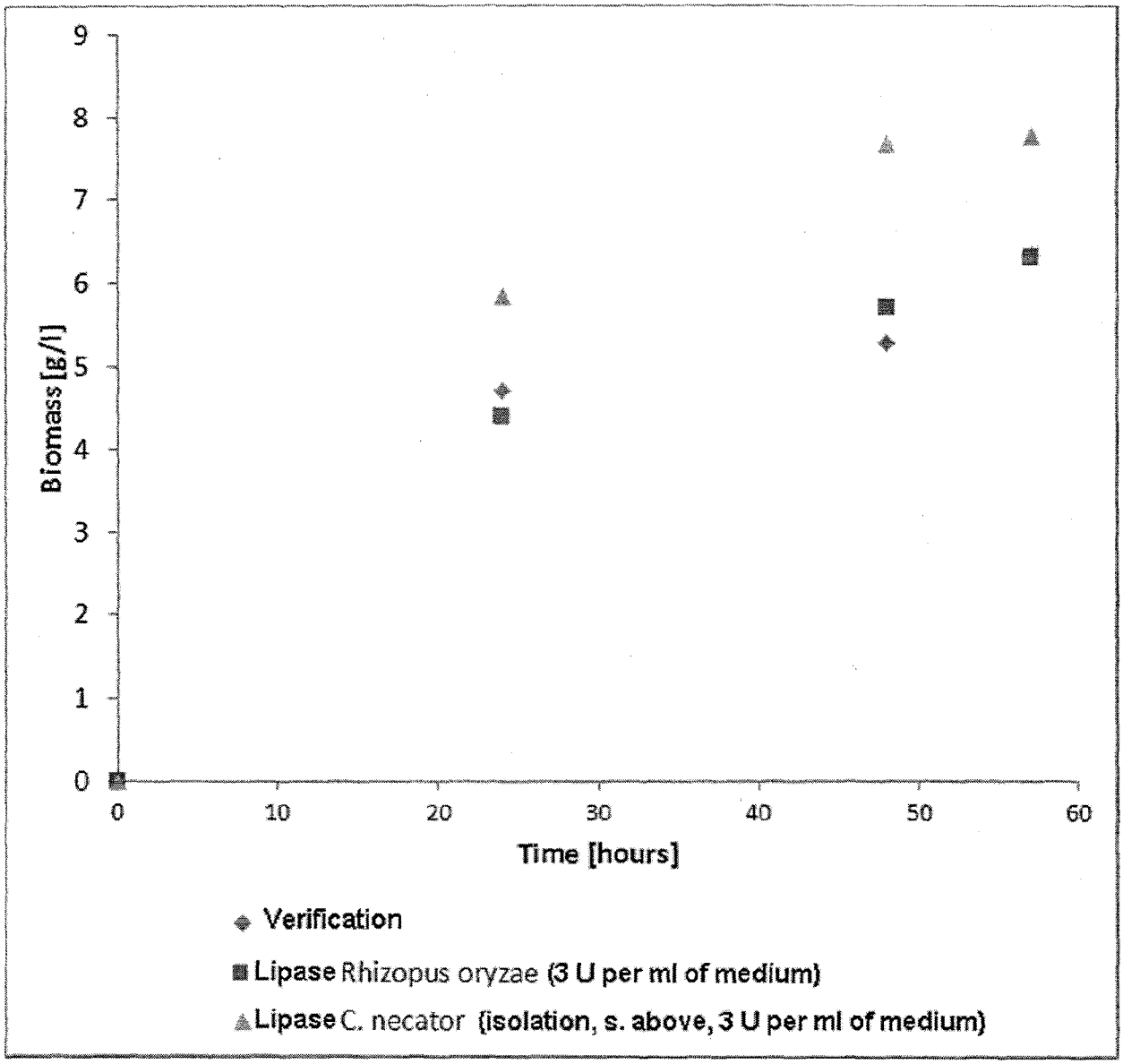
Biomass [g/l]
0
1
2
3
4
5
6
7
8
9
0
10
20
30
40
50
60
Time [hours]
Verification
Lipase Rhizopus oryzae (3 U per ml of medium)
Lipase C. necator (isolation, s. above, 3 U per ml of medium)

Fig. 10

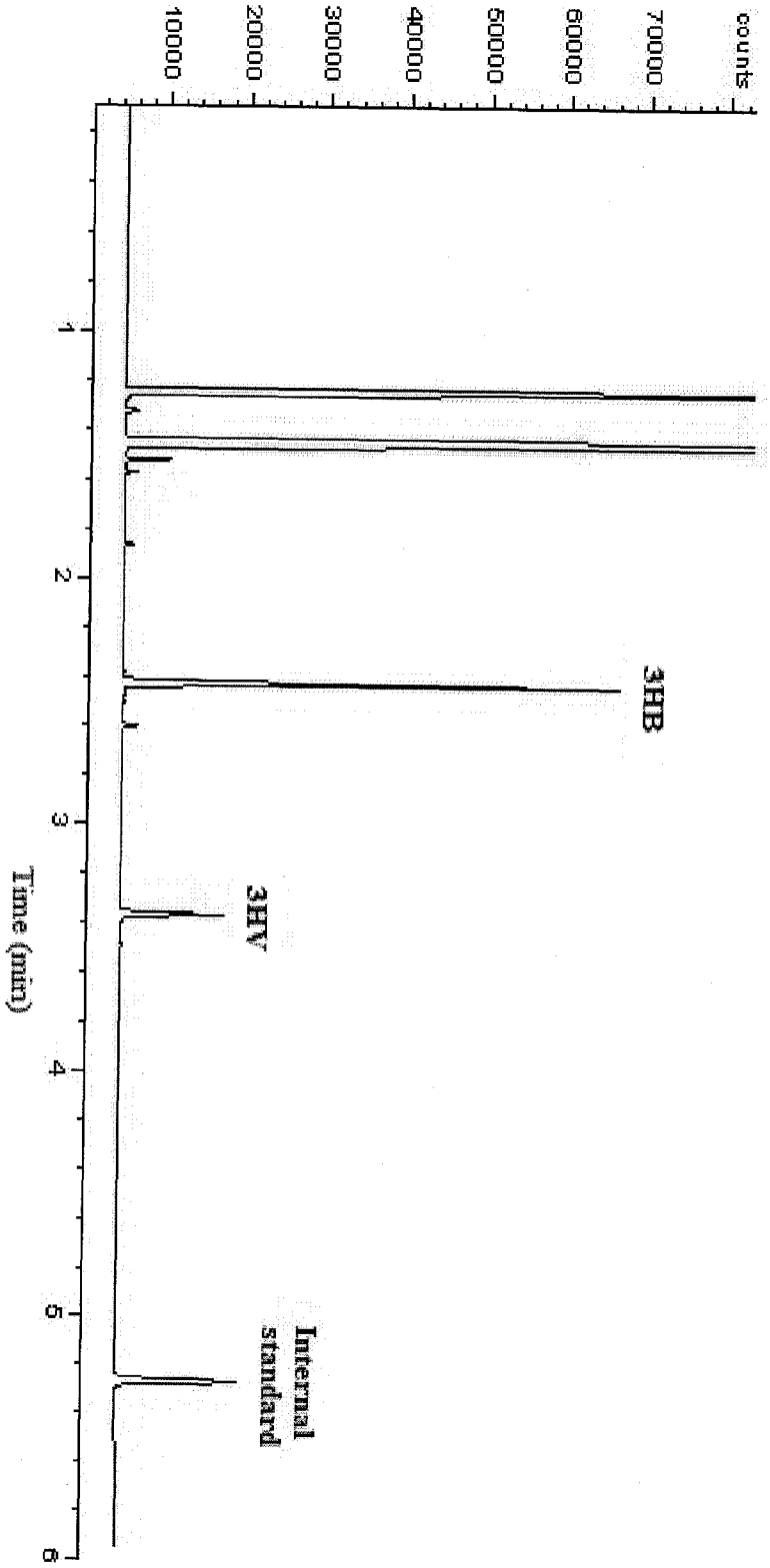
counts
10000
20000
30000
40000
50000
60000
70000
1
2
3
4
5
6
Time (min)
3HB
3HV
Internal standard

Fig. 11

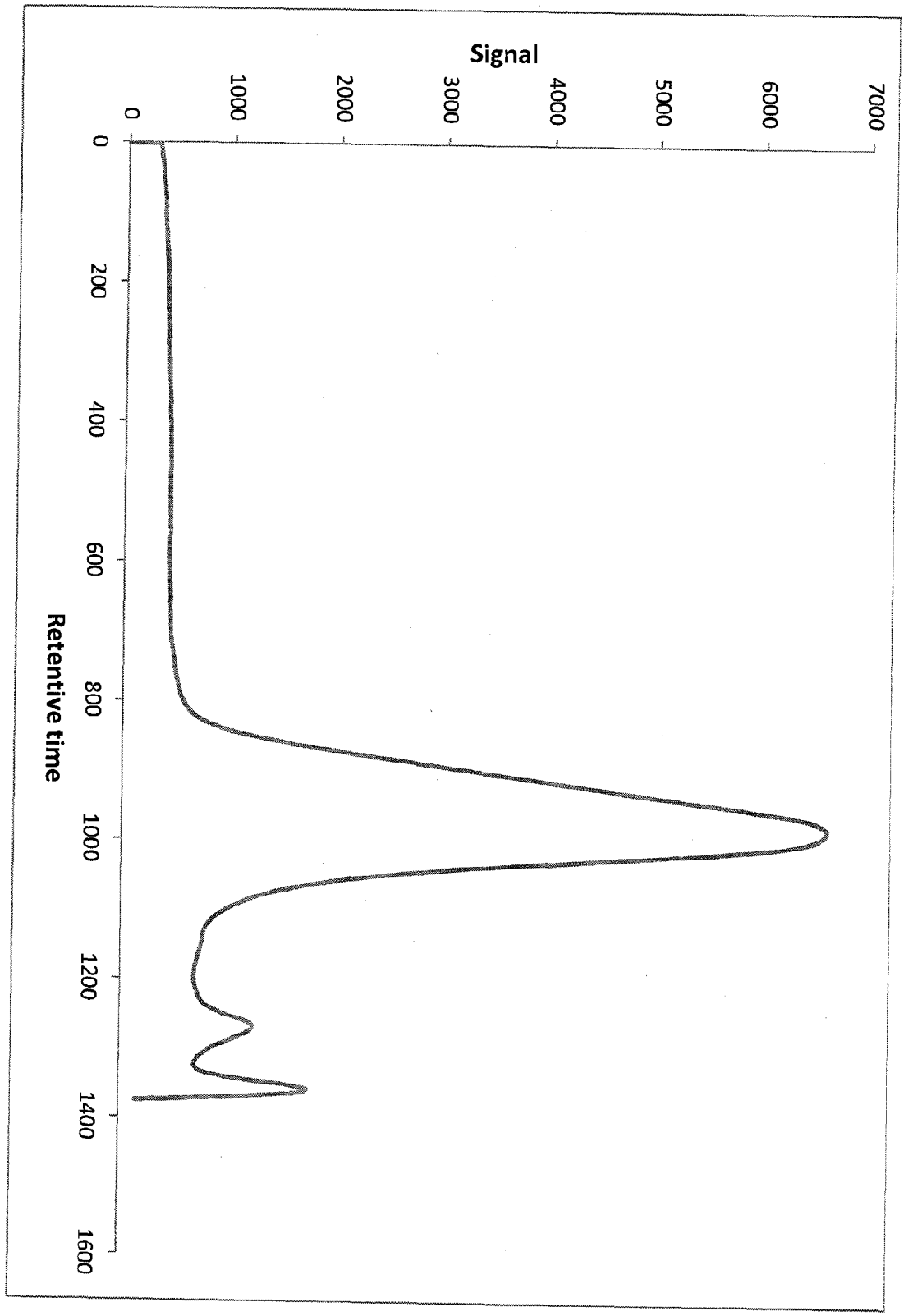
Signal
0
1000
2000
3000
4000
5000
6000
7000
Retentive time
0
200
400
600
800
1000
1200
1400
1600

Fig. 12

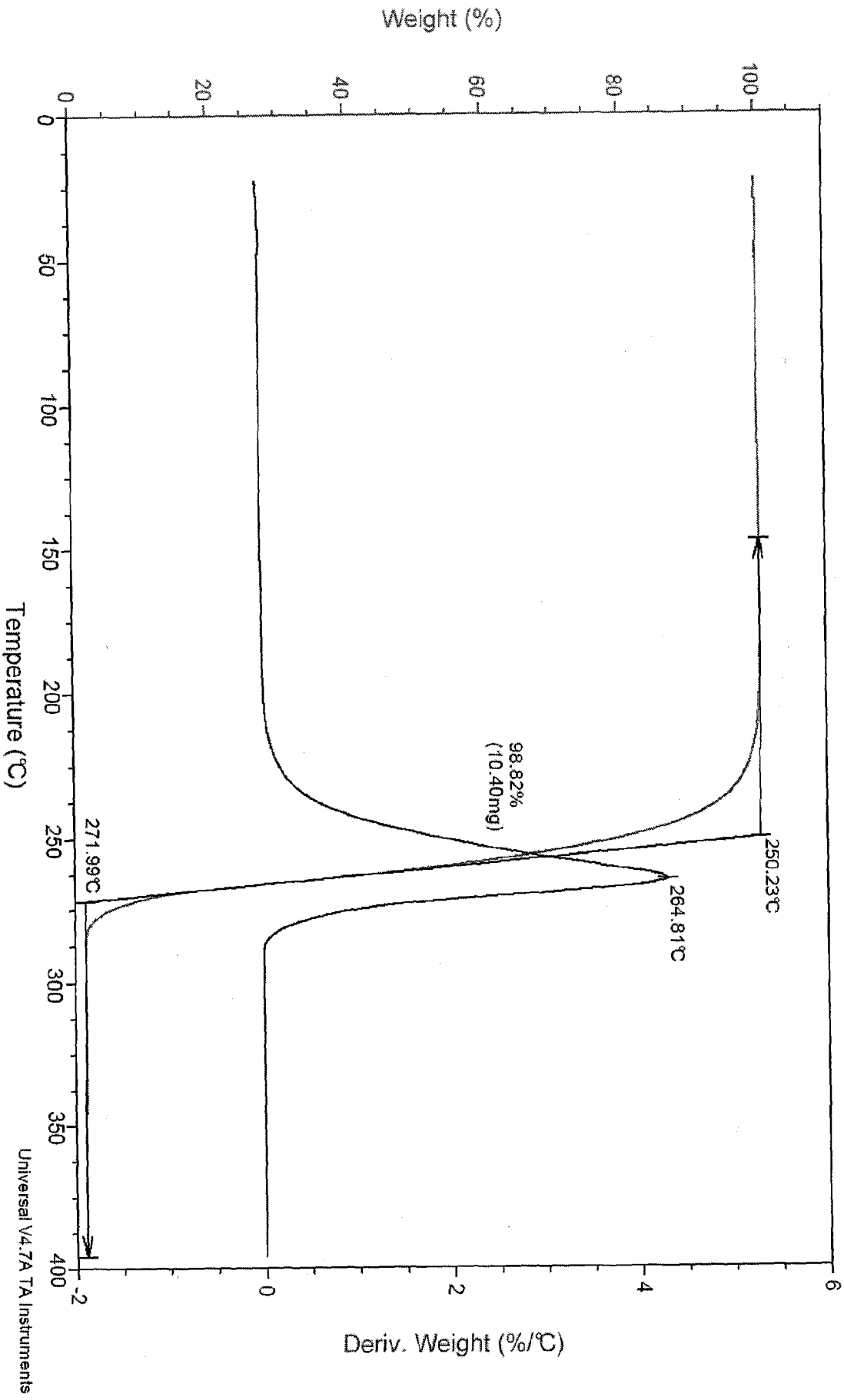
Weight (%)
0
20
40
60
80
100
Temperature (°C)
0
50
100
150
200
250
300
350
400
98.82%
(10.40mg)
271.99°C
250.23°C
264.81°C
Deriv. Weight (%/°C)
-2
0
2
4
6
Universal V4.7A TA Instruments

Fig. 13

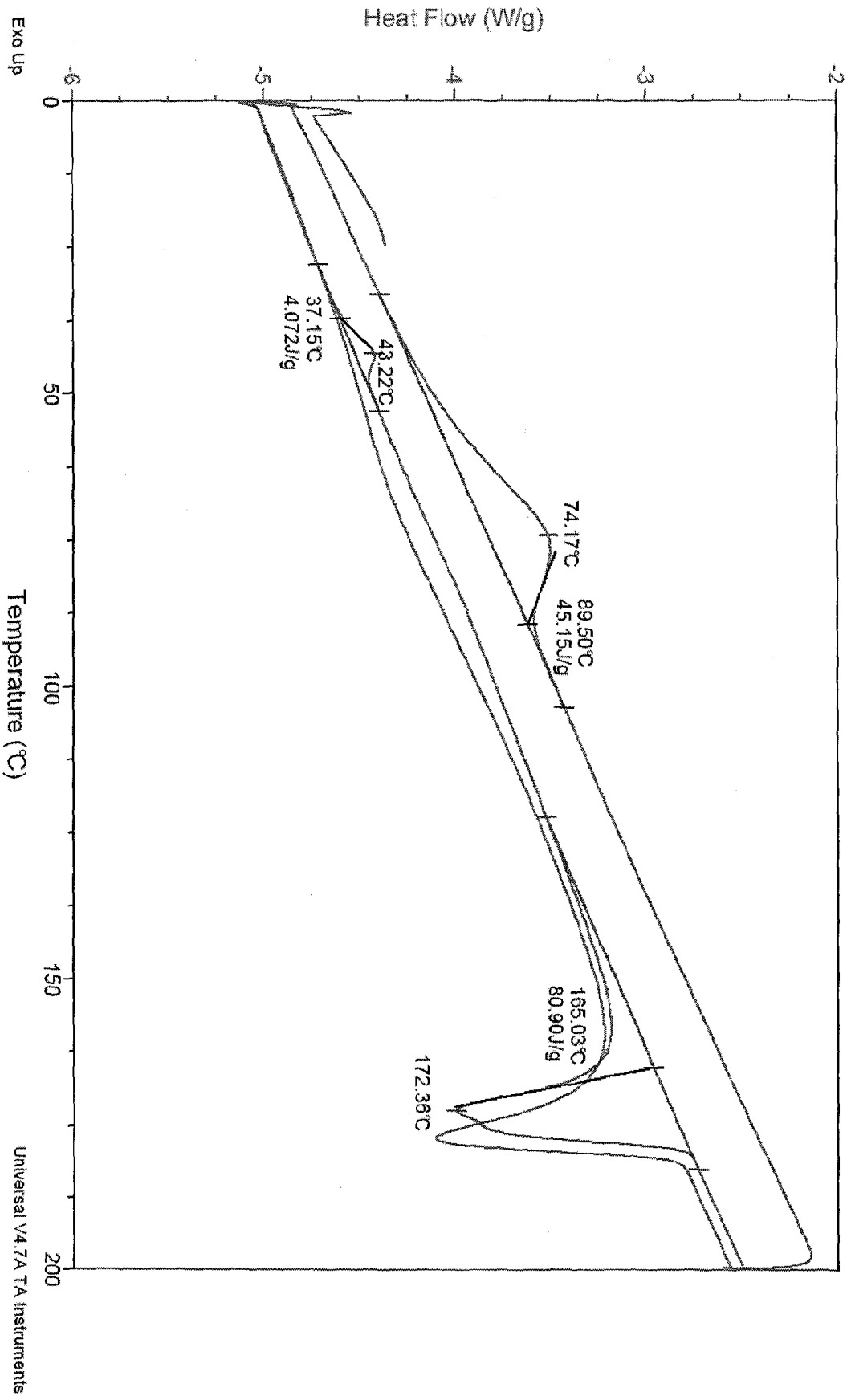
Heat Flow (W/g)
Exo Up
-2
-3
-4
-5
-6
0
50
100
150
200
Temperature (°C)
37.15°C
4.072J/g
43.22°C
74.17°C
89.50°C
45.15J/g
165.03°C
80.90J/g
172.36°C
Universal V4.7A TA Instruments

Fig. 14

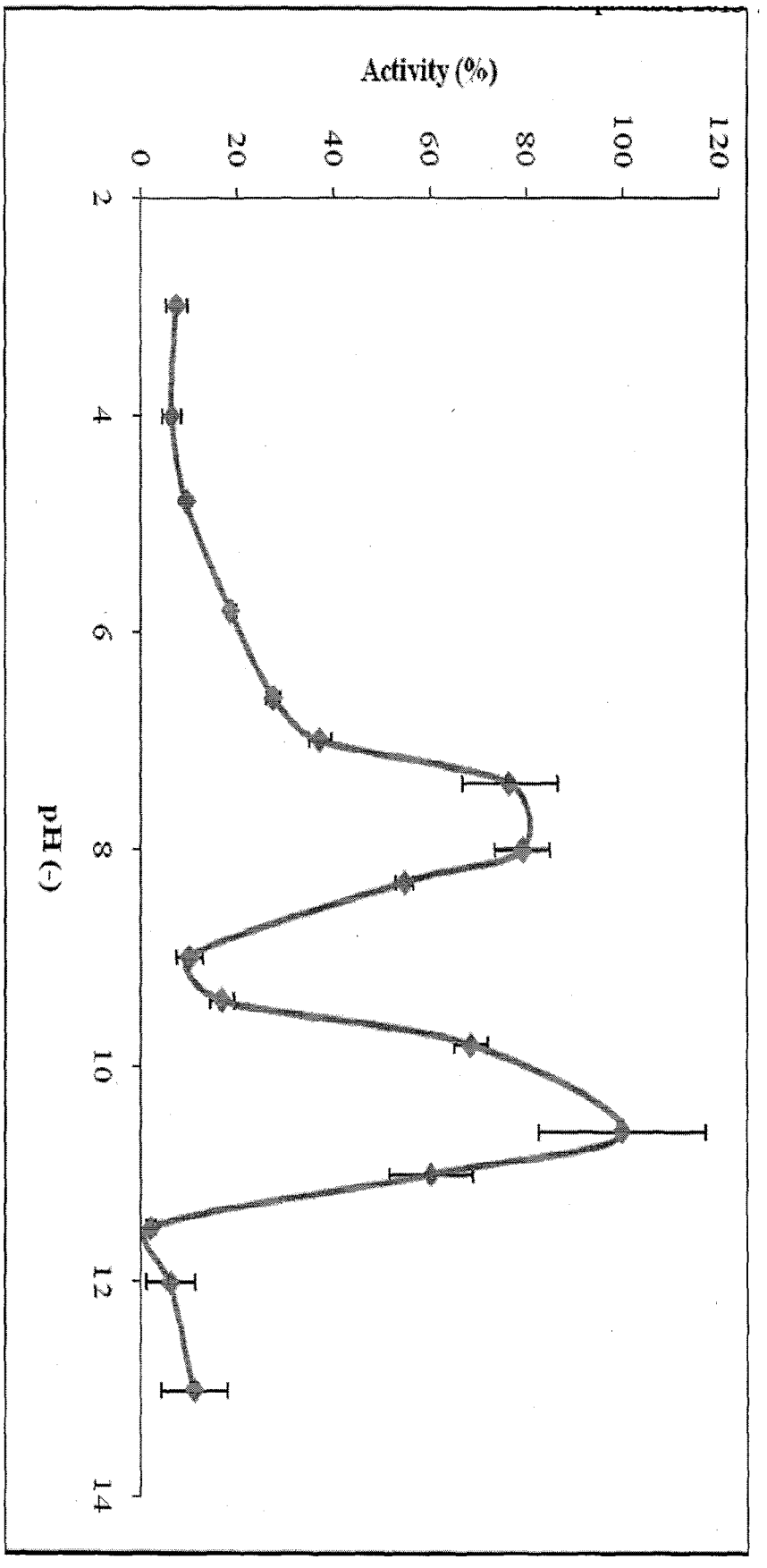

Fig. 15

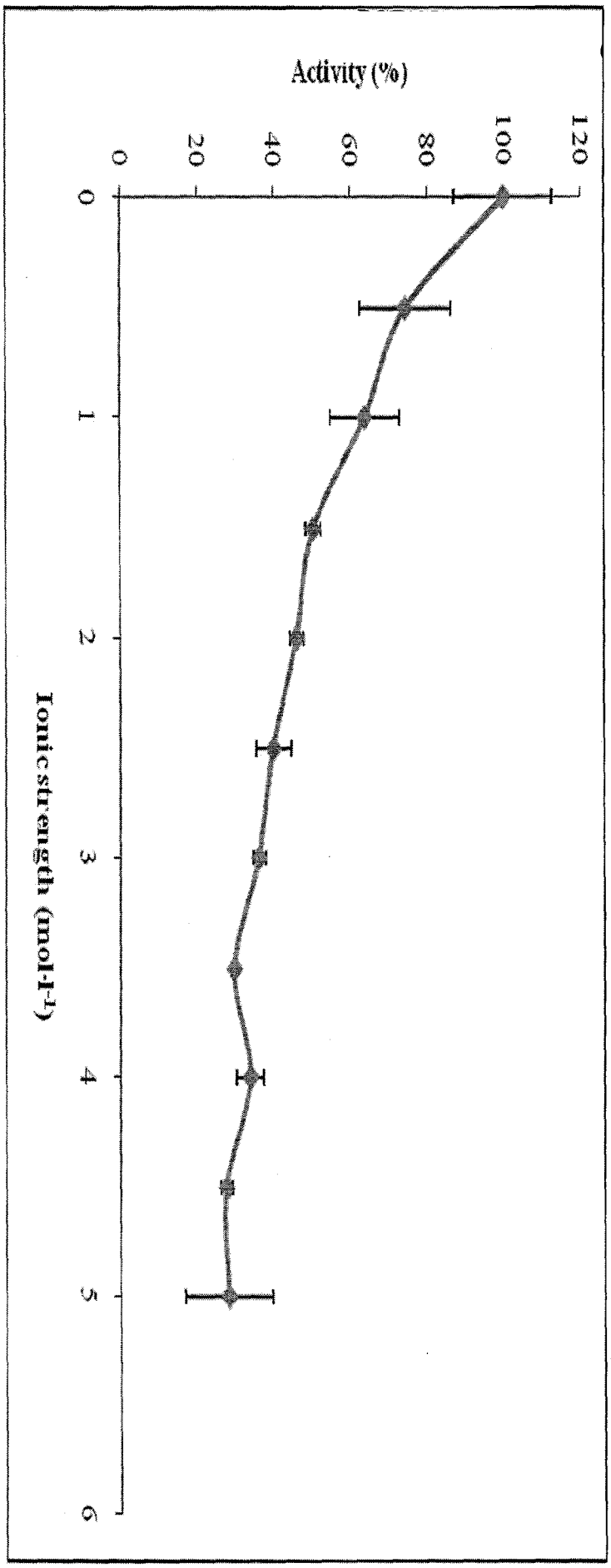

Fig. 16

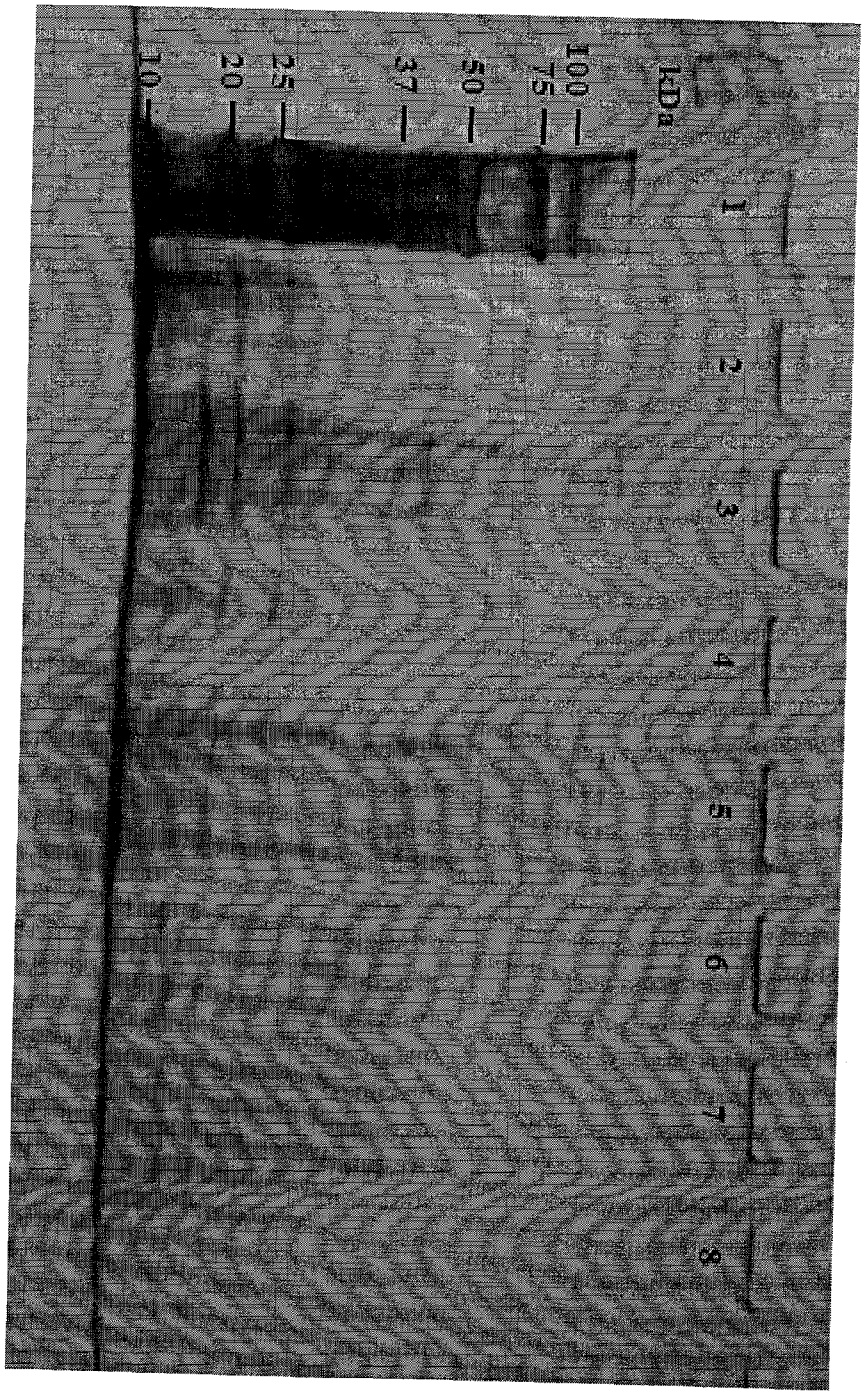

Fig. 17

INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2013/000100

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P1/04 C12P7/02 C12P7/42
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NG K S ET AL: "Evaluation of jatropha oil to produce poly(3-hydroxybutyrate) by Cupriavidus necator H16", POLYMER DEGRADATION AND STABILITY, BARKING, GB, vol. 95, no. 8, 1 August 2010 (2010-08-01) , pages 1365-1369, XP027122906, ISSN: 0141-3910 [retrieved on 2010-01-28] the whole document ----- -/--	1

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 January 2014	31/01/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Boeker, Ruth

INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2013/000100

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	STANISLAV OBRUCA ET AL: "Production of poly(3-hydroxybutyrate--3-hydroxyvalerate) byfrom waste rapeseed oil using propanol as a precursor of 3-hydroxyvalerate", BIOTECHNOLOGY LETTERS, SPRINGER NETHERLANDS, DORDRECHT, vol. 32, no. 12, 12 August 2010 (2010-08-12), pages 1925-1932, XP019859040, ISSN: 1573-6776, DOI: 10.1007/S10529-010-0376-8 the whole document	1-10
A	RAO U ET AL: "Biosynthesis and biocompatibility of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) produced by Cupriavidus necator from spent palm oil", BIOCHEMICAL ENGINEERING JOURNAL, ELSEVIER, AMSTERDAM, NL, vol. 49, no. 1, 15 March 2010 (2010-03-15), pages 13-20, XP026889220, ISSN: 1369-703X, DOI: 10.1016/J.BEJ.2009.11.005 [retrieved on 2009-11-14] paragraphs [02.4], [03.2], [0004]; figure 2	1-10
A	SANG Y L: "Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria", TRENDS IN BIOTECHNOLOGY, ELSEVIER PUBLICATIONS, CAMBRIDGE, GB, vol. 14, no. 11, 1 November 1996 (1996-11-01), pages 431-438, XP004069641, ISSN: 0167-7799, DOI: 10.1016/0167-7799(96)10061-5 the whole document	1-10
A	WO 2011/031566 A1 (NEWLIGHT TECHNOLOGIES LLC [US]; HERREMA MARKUS [US]; KIMMEL KENTON [US) 17 March 2011 (2011-03-17) claims	1-10
A	Rob Aj Verlinden ET AL: "Production of polyhydroxyalkanoates from waste frying oil by Cupriavidus necator", AMB Express, 1 January 2011 (2011-01-01), pages 1-8, XP055097273, Retrieved from the Internet: URL:http://www.amb-express.com/content/1/1/11 [retrieved on 2014-01-20] the whole document	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/CZ2013/000100

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011031566 A1	17-03-2011	US 2012165500 A1	28-06-2012
		WO 2011031566 A1	17-03-2011