ELSEVIER Resources, Conservation and Recycling 27 (1999) 309–332

resources, conservation and recycling

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Biodegradation and bioconversion of cellulose wastes using bacterial and fungal cells immobilized in radiopolymerized hydrogels

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Accepted 21 May 1999

Abstract

Annually, great amounts of cellulose wastes, which could be measured in many billions of tons, are produced worldwide as residues from agricultural activities and industrial food processing. Consequently, the use of microorganisms in order to remove, reduce or ameliorate these potential polluting materials is a real environmental challenge, which could be solved by a focused research concerning efficient methods applied in biological degradation processes. In this respect, the scope of this chapter is to present the state of the art concerning the biodegradation of redundant cellulose wastes from agriculture and food processing by continuous enzymatic activities of immobilized bacterial and fungal cells as improved biotechnological tools and, also, to report on our recent research concerning cellulose wastes biocomposting to produce natural organic fertilizers and, respectively, cellulose bioconversion into useful products, such as: 'single-cell protein' (SCP) or 'protein-rich feed' (PRF). In addition, there are shown some new methods to immobilize microorganisms on polymeric hydrogels such as: poly-acrylamide (PAA), collagen-poly-acrylamide (CPAA), elastin-poly-acrylamide (EPAA), gelatin-poly-acrylamide (GPAA), and poly-hydroxy-ethyl-methacrylate (PHEMA), which were achieved by gamma polymerization techniques. Unlike many other biodegradation processes, these methods were performed to preserve the whole viability of fungal and bacterial cells during long term bioprocesses and their efficiency of metabolic activities. The immobilization methods of viable microorganisms were achieved by cellular adherence mechanisms inside hydrogels used as immobilization

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PII: S0921-3449(99)00028-2

matrices which control cellular growth by: reticulation size, porosity degree, hydration rate in different colloidal solutions, organic and inorganic compounds, etc. The preparative procedures applied to immobilize bacterial and fungal viable cells in or on radiopolymerized hydrogels and, also, their use in cellulose wastes biodegradation are discussed in detail. In all such performed experiments were used pure cell cultures of the following cellulolytic microorganisms: *Bacillus subtilis* and *Bacillus licheniformis* from bacteria, and *Pleurotus ostreatus*, *Pleurotus florida*, and *Trichoderma viride* from fungi. These species of microorganisms were isolated from natural habitats, then purified by microbiological methods, and finally, tested for their cellulolytic potential. The cellulose biodegradation, induced especially by fungal cultures, used as immobilized cells in continuous systems, was investigated by enzymatic assays and the bioconversion into protein-rich biomass was determined by mycelial protein content, during such long time processes. The specific changes in cellular development of immobilized bacterial and fungal cells in PAA hydrogels emphasize the importance of physical structure and chemical properties of such polymeric matrices used for efficient preservation of their metabolic activity, especially to perform in situ environmental applications involving cellulose biodegradation by using immobilized microorganisms as long-term viable biocatalysts.

Keywords: Bacteria and fungi; Cellulose biodegradation; Hydrogels; Immobilization; Radiopolymerization

1. Introduction

Biodegradation of cellulose wastes by fungal or bacterial enzymatic activities represents a large area of research experiments concerning the influence of different physical and biochemical factors, which interfere in cellular dynamics of such biotechnological processes [1]. The survey of these important factors could give much useful information about the optimal parameters of biodegradation in continuous-culture systems in order to maximize the efficiency of these biotechnological procedures and to achieve increasing rates of biocomposting and bioconversion into useful products, using immobilized bacteria and fungi [2].

There are many well-known methods to immobilize bacterial and fungal spores by entrapment inside various polymeric matrices prepared by chemical polymerization. The main disadvantage of these procedures is their inability to be used as immobilization technique for whole viable microorganism cells, by which it could be generated a new potential efficiency for many biotechnological continuous processes [3]. So far, most of the available immobilization methods have already been applied, especially to filamentous fungi and, in fact, several industrial bioprocesses have been developed using as much as possible the enzymatic potential of these microorganisms to convert useless cellulose materials into useful products [4–6].

The cellulose biodegradation using bacterial and fungal cells immobilized in polymerized hydrogels is essentially based on the complex interactions between biotic factors, in first place, including: cell wall composition, cell age, and morphogenetic specificity of microorganisms, and, on the other hand, the cellulose composition, especially its complexity with hemicellulose and lignin, as well as the abiotic

ones, such as: physico-chemical surface properties of polymerized hydrogels which will be used as immobilization matrices, their porosity, pH value, and ionic strength of nutritive solutions added to substrata. The immobilization of whole viable cells is achieved, in this way, by natural adherence and biofilm growth of bacterial and fungal cells inside of polymerized hydrogels during their cellular cycles. At the same time, the immobilization efficacy usually depends on the porosity degree of polymeric hydrogels used as optimal immobilization matrices and the surface contact of enzymatic compounds with cellulose particles by the adsorption effects [6–9]. At all events, the interest about the further role of immobilized cells in biotechnology, and the current level of research, are going to lead to much more extensive commercial applications being considered as potential substitutes for conventional fermentation systems [10–12].

2. Chemical and physical features of cellulosic materials and their susceptibility to enzymatic hydrolysis

Basically, the cellulose is the most widely distributed skeletal polysaccharide and represents about 50% of the cell wall material of plants. Beside hemicellulose and lignin, cellulose is a major component of agricultural wastes and municipal residues [12–14].

The cellulose and hemicellulose comprise the major part of all green plants and this is the main reason of using such terms as 'cellulosic wastes' or simply 'cellulosics' for those materials which are produce especially as agricultural crop residues, fruit and vegetable wastes from industrial processing, and other solid wastes from canned food and drinks industries [14,15]. The cellulose molecules are composed of longer slander bundles of long chains of β-D-glucopyranose residues linked by 1-4 glucosidic bonds, called 'elementary fibrils'. Within each elementary fibrils the cellulose molecules are laterally bound and the adjacent molecules run in opposite directions, but in parallel, with various degrees of orientation [3,6,15].

These molecules have two regions: one of this, which is called 'crystalline cellulose' is composed of highly-oriented molecules, and another one called 'amorphous cellulose', which comprises less-oriented molecules. Many of these elementary fibrils form together a microfibril and, furthermore, several microfibrils joined together form a macrofibril [3,6,12,14–17].

For the complete hydrolysis of cellulose to glucose the cellulase systems must contain the following enzymes: endoglucanase (1,4-β-glucan glucanohydrolase, EC 3.2.1.4), exoglucanase (1,4-β-glucan cellobiohydrolase, EC 3.2.1.91) and β-glucosidase (β-D-glucoside glucohydolase or cellobiase, EC 3.2.1.21)[15–19]. Only the synergy of these enzymes makes possible the cellulose hydrolysis to glucose [17–19]. In addition, C_1 activity (cotton hydrolyses activity) is necessary for splitting off the elementary fibrils from the crystalline cellulose [20–23].

As a result of its swelling and H-bond-breaking effects, C_1 activity from the cellulase system releases microfibrils as well as macrofibrils from the cell wall and continues to cut the open ends of the microfibrils as well as on macrofibrils by

penetrating deep inside the cellulose structure to liberate elementary fibrils and single cellulose molecules [6,12,21–23].

To penetrate into the crystalline cellulose region, nevertheless, C_1 activity must be augmented by endoglucanase to break the 1,4-β linkage in the cellulose molecules (glucose chains). As soon as the two broken ends of the cellulose molecules are lifted, the C_1 activity enters beneath the cellulose molecules to release the glucose chain from the rest of the cellulose by its swelling and H-bond-breaking effects. The newly released long glucose chain (cellulose molecule) is broken down by endoglucanase into shorter chains (oligosaccharides, cellotetraoses, cellotrioses) [21,23–25]. Direct physical contact between enzyme and surface of cellulose molecules is a preliminary requirement to hydrolysis [25–28]. Since the cellulose is an insoluble and structurally complex substrate, this contact can be achieved only by diffusion of the enzymes into the complex structural matrix of the cellulose [23,28–30].

The ability of cellulolytic microorganisms to degrade cellulose vary greatly with the physico-chemical characteristics of the substrate, such as: (a) the size and permeability of cellulolytic enzymes and other reagent molecules, which are involved in relation to the size and surface properties of the grown fibrils and the space between microfibrils and cellulose molecules from amorphous region; (b) the degree of crystallinity of cellulose; (c) the unit cell dimensions of cellulose; (d) the stereoscopic conformation and rigidity of the anhydroglucose units; (e) the degree of polymerization of cellulose molecules; (f) the nature of components with which cellulose is associated; (g) the nature, concentration and distribution of substituted groups [30–35]. The crystallinity degree of cellulose is one of the most important structural parameters which affects the rate of enzymatic degradation by hydrolysis. Therefore, the rate of degradation should be a function of the surface properties of cellulose which makes possible the access of enzymes to polymeric molecules [35–37].

3. Microorganisms used as biocatalysts in cellulose biodegradation

Many fungi from *Asco-* and *Basidiomycetes* can produce extracellular enzymes that enable them to break down polysaccharides such as celluloses and convert these polymeric compounds into sugars, and otherwise, there are several Gram-positive bacteria, such as those from *Bacillus* genus, which have the enzymatic potential to cut down proteins into amino-acids that can be assimilated easily by other organisms during the specific food chains existing in any ecosystems [38,39]. In this respect, there will be shown some short morpho-physiological characteristics of such microorganisms which were used as biocatalysts in our experiments concerning cellulose biodegradation by immobilized viable cells [39].

3.1. Bacillus subtilis and Bacillus licheniformis

The bacterial species from *Bacillus* genus (*Eubacteria*, Gram-positive endospore-bearing bacteria, *Bacillaceae*) are aerobic or facultatively anaerobic microorgan-

isms, with wide diversity of physiological ability with respect to heat, pH and salinity. *Bacillus* bacterial cells are rod-shaped and straight, and often are arranged in pairs or chains, with rounded or squared ends. Many species are normally present in soil and in decaying animal and vegetable matter [40]. *B. subtilis* is responsible for spoilage in fruit and vegetable products and it is used in industry to manufacture enzymes for biological washing products [41].

3.2. *Pleurotus ostreatus and Pleurotus florida (Fr.) P. Kumm.*

Pleurotus species (*Basidiomycota*, *Basidiomycetes*, *Holobasidiomycetidae*, *Poriales*, *Lentinaceae*) are characterized by hyphae with a dolipore septum and parenthesomes, with basidiospores forming directly a mycelium and basidium clavate, cylindrical, uniform, or furcate, lacking internal septation. These species are hymenophore lamellate and hyphal system monomitic or dimitic with skeletal or skeleto-ligative hyphae [42–45]. They are commonly cultivated in Europe (*P. ostreatus*) and in tropical countries (*Pleurotus pulmonarius*), and usually, their cultivation has long been a bioconversion process and simultaneously a useful approach to treating solid agricultural and industrial cellulose wastes [45].

3.3. *Trichoderma viride Pers.ex S.F. Gray aggr.*[1]

The species of *T. viride* (*Ascomycota*, *Hypocreales*, *Hypocreaceae*) may have either loosely-floccose or compactly-tufted colonies and there are numerous intermediate types between these two extremes, sometimes, both of them occurring on the same colony. Typically, colonies of *T. viride* always have green coloration, but in the other aggregate species, some isolates of this species may also have an entirely different coloration, which varies from yellow to yellowish or light green. In cultures, colonies grow rapidly, covering 9-cm Petri dishes after about 4 days at 20°C and form, at first, a smooth-surfaced mycelial net, which later may become hairy from the formation of loose aerial hyphae [46].

4. Immobilization matrices preparation by radiopolymerization procedure

Recently, many biotechnological processes using immobilized microorganisms on different matrices using the gels shape were described, e.g. agar–agar, alginate or calcium alginate, which all have almost the same characteristics concerning the preparative procedures [47–49]. The 'classical' poly-acrylamide (PAA) gel, prepared by chemical polymerization reaction, is actually the most widely used matrix for immobilizing bacterial or fungal spores [47].

The main disadvantage of this method, which involves the use of *bis*-acrylamide as chemical initiator of polymerization reaction, consists of denaturing effects of

[1] Aggregate species, an entity which can be defined as aggregations of morphologically very similar and often hardly-separable species [46].

viable microorganisms and the considerable loss of cellular activity, caused by the chemical toxicity of the redundant non-polymerized monomers [48–50].

We have developed a method to produce micropellets containing immobilized bacterial and fungal cells on radiopolymerized hydrogels, that overcomes many disadvantages of the older chemical methods [51].

The first step of PAA hydrogels preparative procedure consists of preparing such concentration of monomer solutions which make possible a controlled size of reticulation net pores to be hydrated by adding specific nutritive media [51–53]. Aqueous solutions of pure acrylamide monomers (10%, 15%, 20%) were prepared by mixing 20% acrylamide solutions with adequate solutions of fibrillar proteins such as: collagen, elastin, or gelatin; the following solutions were obtained: 1, 2, and 3% collagen acrylamide (CAA); 1, 3, and 5% elastin acrylamide (EAA); 3, 5, and 7% gelatin acrylamide (GAA). These mixed solutions of acrylamide monomers and fibrillar proteins were gamma irradiated by using a ^{60}Co radioactive source with a debit of 5–7 KGy [51–53].

During gamma polymerization a continuous air-flow of bubbles inside of the acrylamide monomer solutions was carried out which controlled the absorption dose of gamma rays by monomer solutions composition. After radiopolymerization colorless and gelatinous hydrogels were obtained, containing many air bubbles in polymerized hydrogels. The size of these air gaps was determined by the air-flow pressure and the reticulation of hydrogels was controlled at the same time by gamma-radiopolymerization dose and, also, by radioabsorption dose. This procedure was carried out on the corresponding types of hydrogels, respectively: 10, 15, and 20% poly-acrylamide (PAA); 1, 2, and 3% collagen-20% poly-acrylamide (CPAA); 1, 3, and 5% elastin-poly-acrylamide (EPAA); 3, 5, and 7% gelatin-poly-acrylamide (GPAA).

In the next stage of this procedure, the hydrogels are broken up into small pellets, dehydrated by vacuum, and sterilized by gamma irradiation at 25 KGy. Then, these PAA micropellets were rehydrated with specific liquid-nutritive media for each bacterial and fungal species. Using almost the same procedure as above, three variants of adequate concentrations of hydroxy-ethyl-methyl-methacrylate (HEMA), respectively, 10, 15, and 20% were prepared. All stages of this procedure were almost the same as in the first mentioned method, excepting the radiation treatment which was longer than previous PAA preparative procedure. These obtained poly-hydroxy-ethyl-methacrylate (PHEMA) were physically harder and less elastic, having a light yellow aspect and their rehydration power was three times lower than that one of PAA hydrogels [53].

5. Bacterial and fungal cells immobilization

So far, a multitude of definitions have been proposed to explain the concept of microorganisms immobilization and, obviously, there are many difficulties in order to accept the precise boundaries of this subject and identify them as universal paradigms which could be applied in any biotechnological process without any change [54].

In spite of these comprehensive problems, this concept could be designed to include such methods and systems, which determine the physical confinement or chemical-surface bonding of microorganisms inside or on the surface of a matrix. In this way, a long-term metabolic activity during continuous bioprocess and the economic re-use of such microorganism-matrix complexes will be achieved [55]. In this respect, the meaning of this definition might be sufficiently broad to cover by its extension both adsorption and entrapment procedures and, also, to include surface mycelial culture systems and fixed-bed reactors in which the immobilization process is achieved by natural adherence and biofilm growth [5,7,56]. To achieve a good immobilization of cells the inner structure of the matrix is an important factor. In most of cases the spherical shape of the matrix has the advantage of combining the fast production with control of particle diameter [57].

Most of the methods used for immobilization of intact microorganisms cells are essentially based on adsorption and entrapment techniques, being adaptations of those previously developed for enzyme immobilization. The adsorption method is currently achieved by linking cells directly to water-insoluble carriers and the adsorption effect is mainly a result of electrostatic interactions between microorganism cell surface and carrier material. Although this process is essentially mild and allows a good retention of cell viability, a certain desorption reaction can occur rapidly under certain circumstances [58–60].

Procedures much more extensively used than adsorption of whole-cell immobilization, involve the entrapment methods which are based on the use of particular polymeric hydrogels, such as: PAA and calcium alginate. These methods have been successfully applied to many filamentous fungal species from *Ascomycetes* [60–62]. These methods are based on the inclusion of cells within polymeric matrices, allowing diffusion of substrate and product, but, nota bene, preventing cellular activity loss by a higher protection against any hazardous shocks which could be produced any time by mechanical or chemical means, especially in environmental applications [62–64].

Ideally, the nature of matrix and the preparative procedure should minimize the loss of cellular activity in order to preserve the intact viable microorganisms and achieve the highest cell density per unit volume, as Coughlan and Kierstan reported [47]. In this respect, PAA produced by chemical polymerization has been widely used in numerous immobilization methods, and the general applied procedure involves the chemical polymerization of an aqueous solution of acrylamide monomers, in which the microorganisms are suspended, followed by the subdivision of prepared hydrogels into small pellets to be used in biotechnological processes [51–53,56]. However, this polymerization procedure requires the use of a chemical initiator, such as *bis*-acrylamide, which can cause denaturing effects which are translated in considerable loss of cellular activity [64–66].

5.1. Bacterial cells adsorption on hydrogel-coated zeolites

Many immobilized-cell reactors contain films of biomass growing on some type of support particles [67]. All these reactors share the problems associated with the

mass-transfer resistance in the biomass, by which the substrate must diffuse into the biofilm and, to the contrary, the product must diffuse out. Since both of these processes require a concentration gradient, the deep region inside the biomass can become a very low substratum compared with a high product environment where metabolic activity is severely inhibited [68].

Adsorption to a solid support such as PAA-hydrogel-coated zeolites offers a simple and effectiveness method to immobilize bacterial cells. As ordinary ceramic materials the zeolites are a well-known group of hydrated aluminosilicate minerals which are usually used as molecular sieves. This means that such natural materials have a porous inner structure which allows the PAA-hydrogel coating inside them during the preparative procedure of immobilization matrices [69–72].

The immobilization technique was achieved by hydrating the zeolites with standardized acrylamide monomer solutions until complete saturation. After the radiopolymerization reaction, the PAA-hydrogel-coating-zeolites, which have already been produced in this way, were washed several times with deionized water [51–53]. Then, they were mild dehydrated by low vacuum and, afterwards, they were rehydrated by adding the specific liquid culture medium (meat extract 0.3%, peptone 0.5%, glucose 0.5%). Eventually, the PAA gel-coated zeolites as spherical microbeads were inoculated with pure bacterial inoculum of *B. subtilis*, using a special laboratory tool [53].

5.2. Fungal spores entrapment in radiopolymerized hydrogels

The entrapment of whole microorganisms in different supports is one of the most used immobilization techniques which is defined by a matrix formation around the cells and this has to be carried out only in presence of certain catalytic active cells [73–76].

A notable example of the application of PAA gel immobilization could be also the entrapment of microorganisms spores as it was reported by Stormo and Crawford [77]. The entrapped spores are induced to germinate in the presence of nutrients and a mycelial net develops within the gel. Advantages of this approach are that preparations are obtained with evenly-distributed cells, and pretreatments, such as fragmentation or division of the mycelia are obviously unnecessary. This spore-immobilization technique followed by in situ germination is also applicable to other spore-producing microorganisms [78,79].

In contrast with these applied procedures, various other studies report that immobilization in calcium alginate, for example, is preferable to PAA when the maintenance of cell viability is more important [78]. The immobilization protocol, which uses this material as matrix, involves cells mixing into a sodium alginate solution. Then, this mixture can be dripped into a calcium salt bath, resulting in spontaneous polymerization and the formation of spherical pellets of calcium alginate gel containing the entrapped cells [78–80]. Even though this procedure is mild, with no heating and no toxic required reagents, there were registered some insignificant or unnoticeable enzymatic activities of these microorganisms by re-using of their immobilized cells [80–82].

In addition, one of the main disadvantages of using this type of immobilized cell technology is the increasing resistance to oxygen diffusion of certain immobilization matrices, especially the gels. The oxygen supply inside vessel culture of aerobic bioreactors causes considerable technical difficulties, and, also, the cells immobilization can add to this problem [73,75]. Oxygen use by bacteria and fungi entrapped in alginate beads was investigated by Gosmann and Rehm [73]. The specific rate of oxygen uptake of the immobilized bacterial cells was dependent on the biomass concentration in the gel and the lower cell concentration allowed a maximum respiration. As the concentration was increased, cells used oxygen faster than it could diffuse into the pellets, until, eventually, the absolute oxygen uptake rate of the pellets remained constant [73].

The above studies could illustrate the strategy to increase the oxygen supply in order to immobilize microorganisms cells by enhancing the partial pressure of oxygen in the feed-gas stream. Other techniques include the increasing of oxygen diffusion by reducing the matrix density or increasing the surface-volume ratio by reducing the overall particle size [80].

During our experiments, the isolates have been obtained by making single and mass ascospores of available pure strains of *T. viride* which were grown using the following liquid-nutritive medium: peptone 0.05%, meat extract 0.1%, yeast extract 0.1%, Tween 80 0.3%. The incipient germinated ascospores were placed into complex solutions prepared by mixing collagen with acrylamide monomers in various ratios [53].

In the next stage, after radiopolymerization these ascospores were already entrapped into the meshes of radiopolymerized hydrogels used as immobilization matrices. Finally, these hydrogels were divided into small granules, were washed several times, mildly dehydrated by vacuum, and rehydrated by a specific liquid-nutritive medium supplemented with 3% (g l^{-1}) pure cellulose (Schuhardt)[53].

The immobilized cultures of *T. viride*, in CPAA hydrogels used as immobilization matrices, were maintained for 5 days at 23°C. After this incubation period the immobilized fungal cells have developed a characteristic mycelial net inside the radiopolymerized hydrogels.

5.3. The immobilization of mycelia by natural adherence and surface biofilm growth

It is unanimously accepted the fact that filamentous fungi can exhibit a strong affinity for surfaces of either organic or inorganic materials and adherence properties as well as surface biofilm growth are important features of the natural ecology of these microorganisms. Although the precise mechanisms of fungal adherence are not yet very well known, the beneficial or, sometimes detrimental, consequences of this phenomenon have been well recognized [82].

Fungi, however, often adhere firmly and resist removal by rinsing, being protected by irregularities of the substratum and their attachment having resistance by some properties of their hyphal mycelium [7,43,83]. Many fungal species secrete extracellular polysaccharides with adhesive properties, which could be involved in such cellular adherence on different surfaces. The penetration of hyphae into

porous or open surfaces would appear to serve as an obvious means of this attachment to this type of surface [11,76].

To achieve our experiments regarding the immobilization of vegetative mycelia from some strains of *P. ostreatus* and *P. florida*, by natural adherence and surface film growth on polymeric hydrogels, we used the simple PAA and complex PAA hydrogels, such as CPAA, EPAA and GPAA hydrogels, which had been produced by the preparative procedure previous presented. These polymeric matrices were divided into thin pellets and were rehydrated by a liquid-nutritive medium prepared from: malt extract 0.1%, peptone 0.3%, glucose 0.05%, $(NH_4)_2SO_4$ 0.01%, K_3 PO_4 0.03%. Then, inside the gaps of these hydrogel pellets were placed aseptically small pieces of vegetative mycelium [56].

The immobilization technique had to respect the following steps: (1) the placement of the culture vessel with the PAA hydrogel pellets inside of an aseptic room, under the object lens of the optical microscopy; (2) the punching of the superficial layers of hydrogels by a special tool in order to release the air from the hydrogel gaps and to introduce the inoculum [56]. All these inoculated PAA pellets were maintained at 25°C, for 5 days re-incubation and the liquid-nutritive medium was supplemented with 20% pure cellulose (g/l), as a preliminary carbon source.

On the interface PAA hydrogels-liquid medium there have been registered some changes in fungal cells development on PAA hydrogels (especially, simple PAA and CPAA hydrogels), which determine a specific hyphal morphogenesis of *P. ostreatus* and *P. florida* [81]. In this respect, it can be noticed significant changes in cellular shape and straightened orientation by comparison with the free hyphal cells of the same species grown on agar–agar gels, which were used as control samples. In addition, the immobilized fungal cells have been preserved in these polyacrylamide hydrogels for about 10 months at room temperature without adding any nutritive solutions to maintain the appropriate hydration level of these immobilization matrices during such a long period. Otherwise, after such a long period there were no changes noticed in enzymatic potential of these immobilized microorganisms, when they were tested by biodegradation experiments in continuous systems [56].

6. Immobilized-cell bioreactors designed to be used in continuous cellulose biodegradation processes

Any biotechnological process requires a suitable environment for the growth of pure cultures of certain microorganisms, which can be run free from contamination and under controlled conditions [11,14,56,84,85].

The design of such biotechnological installation must incorporate a device to homogenize the contents, an air supply for aerobic processes, probes to monitor the evolution of cellular growing and the changes in culture medium composition, and regulators to control them. Also, there must be provisioned with particular device for inoculation and sampling, as well as for charging and discharging the culture vessel without any risk of contamination, and in continuous culture it is compulsory to monitor and control the flow rate of the culture medium, the culture volume, and mass, as well as the biomass concentration per unit volume [56,83,84].

The biodegradation experiments were performed by using three types of continuous biotechnological laboratory-scale installations, containing immobilized bacterial and fungal cells on simple or complex PAA hydrogels [85].

6.1. Fixed-bed film bioreactor

In order to study the cellulose wastes biodegradation by consecutive aerobic cultures of immobilized microorganisms there were used fungal cells of *T. viride* and bacterial strains of *B. subtilis* and *B. licheniformis*. The reason for the achievement of a fluidized bed film bioreactor, was to provide an appropriate environment for both bacterial and fungal cultures, in order to produce the cellulose breaking down into low molecular weight compounds which could be used as soil fertilizers [85] (Fig. 1).

6.2. Flow recycling column bioreactor

This bioreactor which was mainly designed for cellulose bioconversion consists in a vertical column interconnected by a closed circuit with an adjacent main reservoir from which is supplied an intermittent nutritive flow inside the culture vessel by an automatic peristaltic pump, connected to this closed system. The constant temperature for fungal growth is assured by a water-heater enclosed circuit, provided with a thermo-regulator which control the temperature level all the time (Fig. 2).

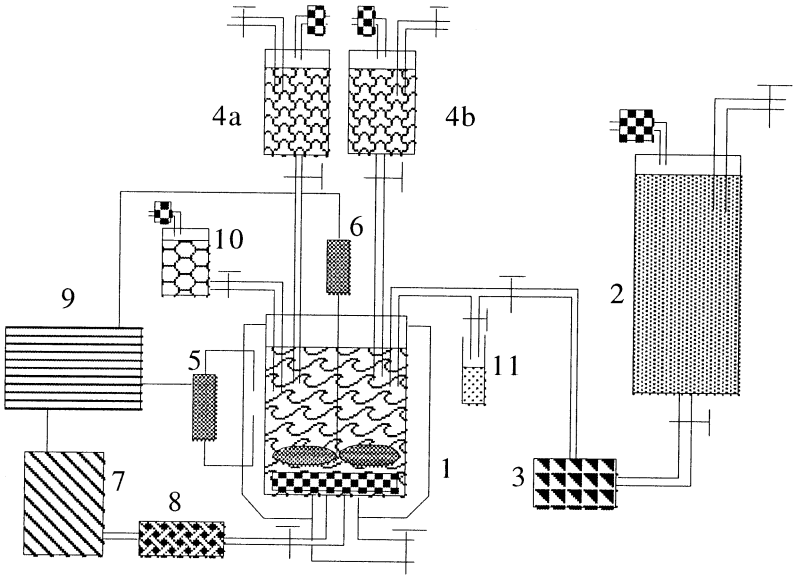


Fig. 1. Schematic figure of a fluidized bed bioreactor. 1, culture vessel; 2, nutrient reservoir; 3, peristaltic pump; 4a, 4b, buffers tanks; 5, water heater; 6, stirrer; 7, air pump; 8, Millipore air filter; 9, automation panel; 10, inoculum reservoir; 11, sample harvester.

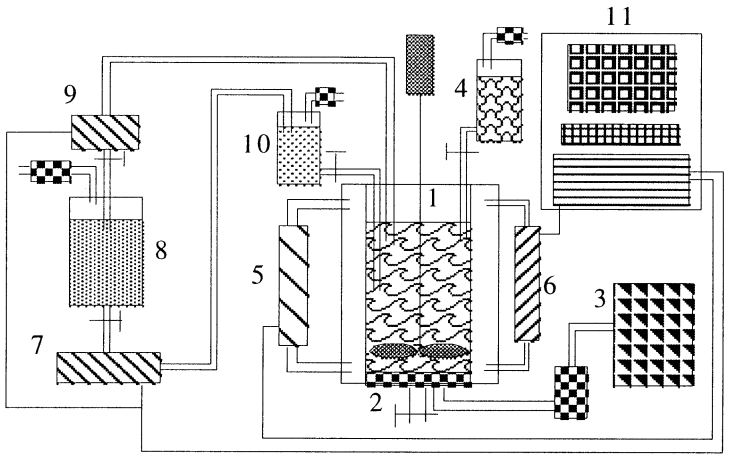


Fig. 2. Schematic figure of a flow recycling column bioreactor. 1, culture vessel; 2, synthetic glass filter; 3, air pump; 4, immobilized cells inoculum reservoir; 5, water heater; 6, refrigerant; 7, peristaltic pump; 8, recycling vessel with immobilized bacterial cells; 9, vacuum pump; 10, buffer tank; 11, automation panel.

In this way, it was achieved an artificial structured ecosystem in which it could be noticed the cellulose concentration gradients at different column levels, the fungal hyphae development on PAA hydrogel variants and at the same time, the enzymatic activity as significant differences from that ones happened in natural conditions [51–53,56].

6.3. Mobile-bed film bioreactor

This type of bioreactor was designed to be used in biodegradation of colloidal cellulose suspensions having as principal subsystems: the culture vessel with deposal multi-flatted beds, the steam-sterilization device and the main reservoir to stock these sterile cellulose suspensions. Each of these beds was designed to be slanted in order to provide the descendent movement of cellulose suspensions from the main reservoir to the culture vessel, both of these bioreactor subsystems being interconnected by an automatic peristaltic pump and, also, to achieve the same fluidity rate of these suspensions inside of the culture vessel. The incubation culture temperature was maintained at a consistent level (23°C), using a water-heater and a thermo-regulator, controlled by a process computer (Fig. 3).

7. Two-phases continuous biocomposting of cellulose wastes using immobilized fungal and bacterial cells

The cellulose wastes biocomposting, as a particular type of biodegradation process, was achieved using immobilized fungal species for continuous production

of oligosaccharides with low molecular weights, which can be further biodegraded or biotransformed into useful compounds by immobilized bacterial cells [86–88]. Firstly, the cellulose wastes were cut down in fragments with an average size of 25 mm and then they were desiccated by hot-air flows and rehydrated by adding aqueous solutions of ammonia salts, such as: 0.1% NH_4NO_3 and 0.3% $(NH_4)_3PO_4$, which were used as nitrogen source for protein biosynthesis. The homogenized cellulose wastes fragments, which have consequently formed the semi-solid cellulose substrata, were finally steam sterilized at 121°C, 1 atm for 25 min and were aseptically added into the culture vessel of a fixed bed bioreactor [51–53,85].

In the first stage of such bioprocess, the ascospores of *T. viride*, which were already immobilized by entrapment in CPAA hydrogel matrices, were added aseptically into the culture vessel of a fixed bed bioreactor and homogenized together with the semi-solid cellulose substratum by a sterile air flow, which further on it was pumped with high pressure into the mixture. For 3–5 days the culture cycle of *T. viride* has almost been achieved by reaching high values of cellulose depolymerization into di- and monosaccharides and using them towards the consecutive protein biosynthesis [88–91].

In the second phase, the immobilized cultures of *B. subtilis* and *B. licheniformis* cells were added over the fungal culture of *T. viride*, in order to amplify the biocomposting process by new conversions of the cellulose depolymerization compounds, already produced, into lower molecular weight compounds which can be used as nutritive organic fertilizers for agricultural fields [92]. In this respect there

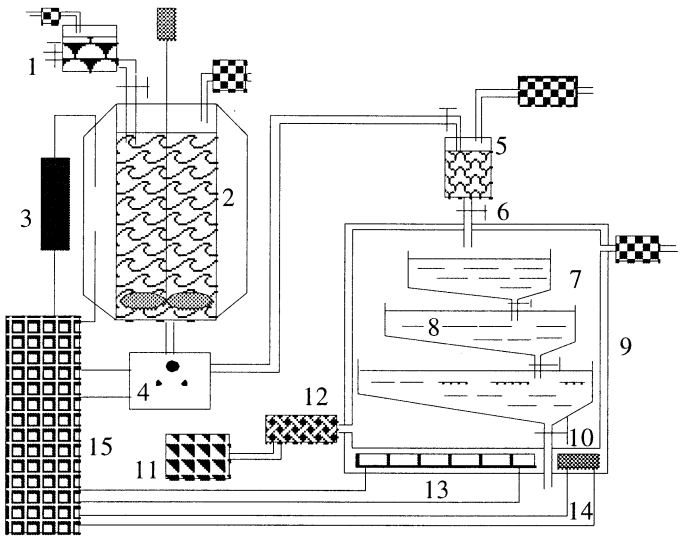


Fig. 3. Schematic figure of a mobile bed bioreactor. 1, cellulose suspensions collector; 2, steam sterilization reservoir; 3, water heater; 4, peristaltic pump; 5, inoculum reservoir; 6, inoculation pipe; 7, culture chamber; 8, deposable multi-flatted beds; 9, incubation room; 10, effluent drain; 11, air pump; 12, Millipore air filter; 13, electric heater; 14, refrigerant; 15, automation panel.

were determined the total reducing sugars contents in order to correlate the experimental date obtained in parallel investigations by dry weight loss measurements of cellulose depolimerization [51–53].

The cellulose wastes biodegradation rates, which have been achieved in two-phases bioprocesses using immobilized fungal and bacterial cells, were represented by dry weight loss measurements, the experimental data being expressed by their mean values, after three replicate experiments. The best cellulose biodegradation rates were achieved by immobilized cells of *B. subtilis* and *B. licheniformis*, using as immobilization matrices EPAA and, respectively, CPAA hydrogels and, by *T. viride* immobilized cells in CPAA hydrogels (Fig. 4 A, B, C).

Concerning the evolution of cellulose depolymerization it could be noticed the significant fact that the highest biodegradation rate was achieved using these fungal and bacterial cells immobilized in CPAA hydrogels, and PAA hydrogel-coated zeolites [92–94].

This procedure has been usually modified in correlation with the biodegradation rate which has been reached at that time of biocomposting process and, for this reason, it should be considered an adaptive bioprocess [94–98].

Furthermore, the final products of this continuous cellulose biodegradation process could integrally be used in crop fertilization, because the hydrogel matrices of immobilized cells, having a great hydrophilic capacity, are obviously useful in so much needed water preservation, with beneficial results in agricultural applications [56,99–103].

8. Cellulose bioconversion by immobilized fungal cells

An efficient method to convert cellulose materials, in order to produce unconventional high-calorie foods or feeds, is the direct conversion by cellulolytic microorganisms [104–116].

Theoretically, any microorganism that can grow as pure culture on cellulose substrata, used as carbon and energy sources, should be considered a potential organism for ‘single-cell protein’ (SCP) or ‘protein rich-feed’ (PRF) production [109,110].

The main aim of our work was focused to establish the enzymatic activity of *Pleurotus* species in continuous biotechnological conversion of cellulose wastes into SCP [113–116] and, also, into so-called PRF [110–112].

Fig. 4. (A) Biodegradation rates in cellulose wastes biocomposting by immobilized *Trichoderma viride* cells. Data shown are representative of three replicate determinations. (B) Biodegradation rates in cellulose wastes biocomposting by immobilized *Bacillus subtilis* cells. Data shown are representative of three replicate determinations. (C) Biodegradation rates in cellulose wastes biocomposting by immobilized *Bacillus licheniformis* cells. Data shown are representative of three replicate determinations.

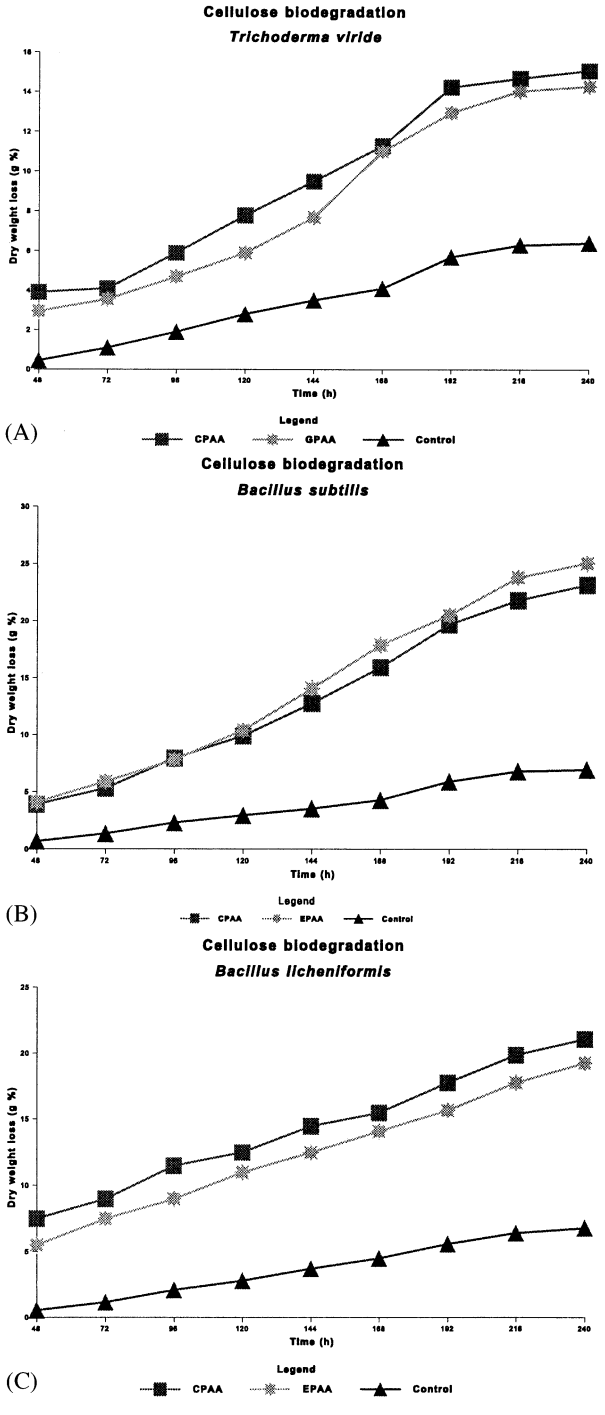

Cellulose biodegradation
Trichoderma viride
Dry weight loss (g %)
Time (h)
Legend
CPAA
GPAA
Control
(A)
Cellulose biodegradation
Bacillus subtilis
Dry weight loss (g %)
Time (h)
Legend
CPAA
EPAA
Control
(B)
Cellulose biodegradation
Bacillus licheniformis
Dry weight loss (g %)
Time (h)
Legend
CPAA
EPAA
Control
(C)

Fig. 4.

8.1. Cellulose bioconversion into SCP

The cellulose bioconversion into SCP has been performed using pure fungal cultures of *P. ostreatus* and *P. florida* strains which were tested for their cellulolytic potential by screening experiments in which there were used different concentrations of pure cellulose solutions. These immobilized microorganisms were aseptically placed inside the culture vessel of continuous biotechnological installations noticing the bioconversion rate of cellulose into protein and using different design variants of continuous biotechnological systems [51–53,85]. The cellulose wastes which were used as substrata in these experiments (such as: wine-producing wastes, grain straws, fruit and vegetable wastes from canned food industry) were pretreated by physical methods before their hydrolysis into usable compounds and bioconversion into protein [110–112,117–121].

Firstly, the cellulose wastes were cut down in small particles with sizes between 1 and 5 mm, secondly, they were dehydrated by hot-air-flow dryers and then, they were made in powdered form by grounding in special mill devices, and thirdly, they were rehydrated by adding aqueous solutions of ammonia salts, such as: 0.1% NH_4NO_3 and 0.3% $(NH_4)_3PO_4$, which were used as nitrogen source for protein biosynthesis [56]. All these homogenized suspensions were finally steam sterilized at 121°C, 1 atm. for 25 min and they were aseptically poured into culture vessel of bioreactor. During this bioprocess, the final biosynthesized product was evacuated from bioreactor, as a filtered biomass suspension, and an equal quantity of fresh substratum was simultaneously added into the culture vessel [85].

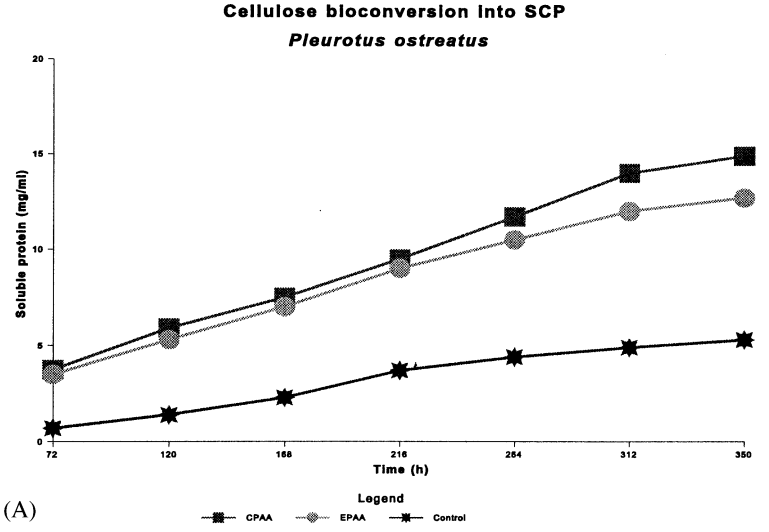
The continuous biotechnological processes were performed using a flow-recycling column bioreactor which was already presented, in the previous subheading, in order to study the influence of immobilization matrices upon cellulolytic activity of fungal cells and soluble protein content, during the bioconversion processes. To analyze the enzymatic activity there were assayed endoglucanase and β-glucosidase, using carboxy-methyl-cellulose as substrata, according to Kubicek procedure [122]. The soluble protein content was determined by extracting three times in 1 N NaOH solution, at 50°C and measuring the protein by a microbiuret procedure [123,124]. The highest soluble protein contents were registered by *P. ostreatus* species, which were immobilized on EPAA and CPAA hydrogels (Fig. 5A,B).

8.2. PRF production from cellulose wastes

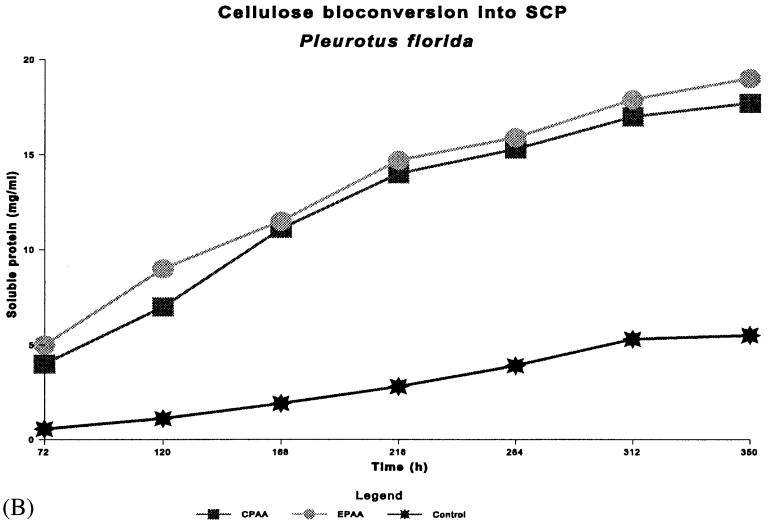
This bioconversion process of cellulose wastes was performed using pure cultures of *P. ostreatus* and *P. florida* strains, immobilized on EPAA and GPAA hydrogel micropellets, which were aseptically placed into the upper part of the culture vessel of a fluidized mobile bed bioreactor, already presented. The substrata were pretreated identically as in the previous bioconversion for SCP production and, after the same steam sterilization treatment, the cellulose suspensions were poured aseptically into the culture vessel of the fluidized mobile bed bioreactor from an adjacent reservoir, both these subsystems being interconnected by an automatic peristaltic pump which was controlled by a process computer. The incubation

temperature was maintained at a constant level of 23°C, using a heat exchanger and a thermo-regulator.

A significant increasing of the contact surface between the immobilized fungal cells and the cellulose substratum was achieved by multi-flatted bed design of the culture vessel and, also, the cellular dynamic during the bioconversion was not affected by shear forces which usually are present during the mixing processes. The

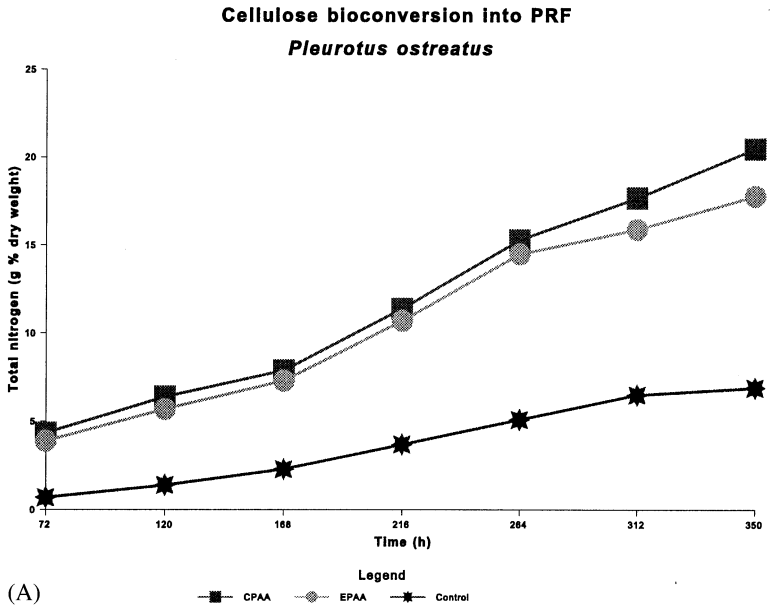


(A)

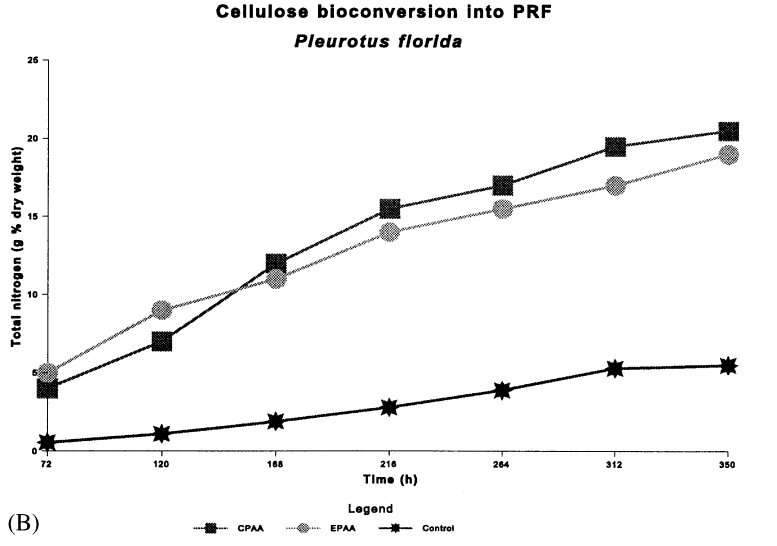


(B)

Fig. 5. (A) Soluble protein biosynthesis during cellulose bioconversion into single-cell protein (SCP) by immobilized *Pleurotus florida* cells. Data shown are representative of at least three replicate determinations. (B) Soluble protein biosynthesis during cellulose bioconversion into SCP by immobilized *Pleurotus ostreatus* cells. Data shown are representative of at least three replicate determinations.



(A)



(B)

Fig. 6. (A) Total nitrogen content of micelial biomass during cellulose bioconversion into Protein-rich feed (PRF) by immobilized *Pleurotus florida* cells. Data shown are representative of at least five replicate determinations. (B) Total nitrogen content of micelial biomass during cellulose bioconversion into PRF by immobilized *Pleurotus ostreatus* cells. Data shown are representative of at least five replicate determinations.

continuity of bioconversion process was assured by a descendent passing of the mixed fungal cells with substratum, from the upper culture bed into the lower one, by a slow gravitational movement through column pipes which were permanent controlled by the automatic electrovalves connected to the process computer.

The both complex PAA hydrogels, as EPAA and GPAA, were inoculated with pure cultures of *Pleurotus* species and they were maintained at 25°C, by a programmed incubator with temperature controller. During bioconversion process, the total carbohydrate concentrations of the growth media were analyzed by the anthrone reagent method [125].

The mycelial protein content was determined as total nitrogen content, by Kjeldahl method [112,126,127] to estimate the rates of cellulose bioconversion into PRF, produced by immobilized cells of *P. ostreatus* and *P. florida*, as it is shown in Fig. 6A,B.

This biotechnology has been already patented in Romania and the final product of this bioconversion was tested and marketed under BIOPROM® trade name, being used as a protein-rich additive for cattle and poultry feed mixtures [128].

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