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(57) Abstract: The subjects of the present invention are a method for the production of bacterial cellulose, a method of immobilising the bacteria *Bacillus subtilis,* synthesizing a protease, a method for the production of immobilised biocatalysts, an application of bacterial cellulose produced in a stationary culture of an *Acetobacter xylinum* strain as a wound dressing, a method of modifying cellulose membranes in order to produce wound dressings. In general, the present invention relates to the synthesis of cellulose which forms a gelatinous, elastic membrane on the surface of a liquid medium in stationary conditions. The present invention also relates to the application of bacterial cellulose as a carrier for the production of immobilised biocatalysts, as well as a method for the application of bacterial cellulose as a wound dressing in the treatment of extensive 1, 2 and 3 degree burns and surgical incisions, which is used in the form of cellulose membranes of arbitrary size and shape. The subjects of the present invention are used in the production of wound dressings in the form of cellulose membranes of arbitrary size and shape.

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A METHOD FOR THE PRODUCTION OF BACTERIAL CELLULOSE

The subjects of the present invention are a method for the production of bacterial cellulose, a method of immobilising the bacteria *Bacillus subtilis*, synthesizing a protease, a method for the production of immobilised biocatalysts, an application of bacterial cellulose produced in a stationary culture of an *Acetobacter xylinum* strain as a wound dressing, a method of modifying cellulose membranes in order to produce wound dressings. In general, the present invention relates to the synthesis of cellulose which forms a gelatinous, elastic membrane on the surface of a liquid medium in stationary conditions. The present invention also relates to the application of bacterial cellulose as a carrier for the production of immobilised biocatalysts, as well as a method for the application of bacterial cellulose as a wound dressing in the treatment of extensive 1, 2 and 3 degree burns and surgical incisions, which is used in the form of cellulose membranes of arbitrary size and shape. The subjects of the present invention are used in the production of wound dressings in the form of cellulose membranes of arbitrary size and shape.

Cellulose is an unbranched homopolysaccharide, composed of β-D-glucopyranose units connected by β-1,4-glycosidic bonds. Bacterial cellulose (BC) is a highly crystalline cellulose, with a high I_α [33; 10] fraction content. The glucan chains synthesized by bacteria bind to form elementary cellulose sub-fibrils some 1,5 nm wide These are some of the thinnest naturally appearing fibrils, comparable only with the elementary cellulose fibrils discovered in the cambium of some plants, and the mucus from *Cydonia oblonga* [18].

Bacterial cellulose is synthesized by several genera of bacteria, of which the best known are strains of the vinegar-producing genus *Acetobacter*.

The synthesis of type and cellulose by *Acetobacter xylinum*, as well as by other organisms capable of it, is composed of at least two stages: 1) polymerisation of glucose molecules to form a linear β-1,4 glucan, 2) and the binding and crystallisation of individual polymer chains to form larger structural units.

Electron microscopy of negative-stained cellulose produced by an *Acetobacter xylinum* [14] culture has shown the hierarchic character of spatial cellulose accumulation. Firstly, 10-15 β-1,4-glucan chains form a subfibril some 1,5 nm wide. The subfibrils bind and form microfibrils (3,0- do 3,5 nm) composed of numerous parallel chains. In the next stage, 50-80 micrifibrils bind into loose structures 40-60 nm wide, known as ribbons, which contain around 1000 individual glucan chains [8].

Biochemical analyses of the regulatory mechanism of cellulose synthesis have shown that cellulose synthase activity is allosterically activated by the presence of an effector, the cyclic nucleotide, diguanylic acid monophosphate (c-di-GMP) [25]. Wong et al. [34] and Ben Bassat et al. [1] proved, that the biosynthesis of bacterial cellulose is related to the activity of four genes, *bcsA* (2261 base pairs), *bcsB* (2405 base pairs), *bcsC* (3956 base pairs) and *bcsD* (467 base pairs), which form the cellulose synthase operon 9217 base pairs long. The functions of the proteins coded by each of these genes have not been fully explained yet, but some information has been acquired pertaining to their potential roles, using the complementary method in a damaged cellulose synthase gene [34]. An analysis of the gene coding the catalytic subunit (*cesA*), analogous to the gene *bcsB* [34; 1], was also presented by Saxen et al. [28], who then [29] identified the gene coding the regulatory subunit (*cesB*). However, the work of Saxen [28, 29] showed that the positions of the first two genes in the cellulose synthase operon are precisely opposite to that demonstrated by Wong et al. [34], suggesting that the first of these genes codes the catalytic subunit of cellulose synthase. The functions of the expression products of *bcsC* and *bcsD* have not been fully explained as of yet.

Industrial-scale BC production is still limited, primarily due to difficulties in the selection of highly active strains, capable of the biosynthesis of cellulose in large cultures and lacking the ability to metabolise glucose to gluconic acids, as well as due to the relatively high costs of the culture medium.

Selection of strains characterised by high BC productivity was performed using traditional screening, through activity determination in monocultures. In their research, Toyosaki et al. [32] isolated 2096 *Acetobacter* strains from a large variety of plant samples, (fruit, flowers, sugar cane, nuts). From among 412 strains forming a cellulose membrane on the medium surface, one strain was selected, *Acetobacter sp.* BPR2001, characterised by particularly effective BC synthesis in mass culture. From 1500 monocultures originating from grapes, Kojima et al. isolated the *Acetobacter* strain *S-35*, characterised by highly elevated

productivity in the presence of glucose, as well as fructose, synthesizing 3.3 g/l/day, in a stationary culture [15].

The search for the most effective carbon source took into account not only its cost, but also its metabolism, since some metabolites may cause inappropriate conditions for cellulose synthesis. Jonas and Farah [13] compared various mono-, di- and polysaccharides, alcohols, organic acids and other compounds as substrates for cellulose synthesis. The best results were obtained using media containing D-arabitol or D-mannitol, rather than glucose. In these trials, the amount of cellulose formed was about 6 and 4 times higher respectively, in comparison to a trial using glucose. It was shown that xylulose and fructose are the metabolites of these substrates, and thus the pH of the culture is maintained at the same level. The productivity obtained in these studies was nevertheless much lower than that obtained by Masaoka et al. [19], who using lower glucose concentrations obtained 0.6 g BC/g glucose/day following 2-4 of culture. Recently published results of research into the effects of various carbon and nitrogen sources on BC synthesis show that saccharose, glucose and mannitol with hydrolysed caseine , peptone, glutamate or ammonium sulphate as a nitrogen source were optimal for cellulose biosynthesis, Raman et al. [23].

In most of the published research, the efficiency of BC synthesis in a medium with glucose or fructose was lower than the efficiency of the above mentioned, and ranged from 0.02 g BC/g/day to 0.07 g BC/g/day [13]. Among other carbon compounds used in this process, citrate, gluconate and lactate should be mentioned. Geyer et al. [7] showed that citrate can me metabolised by *Acetobacter*, but only when the medium contained both citrate and glucose, where the latter will be utilised first, and the citrate after it has been depleted. It was also noted that citrate has a beneficial effect on the synthesis of cellulose. Other authors indicate gluconates as one of the possible carbon sources in cellulose [13]. Masaoka et al. [19] show that gluconates accumulated in the medium, up to a concentration of 20 g/l, were utilised by the bacteria. Matsuoka et al. [20] observed that lactate (0.15% v/v) added to a medium containing fructose, yeast extract and peptopne as a nitrogen source, exhibits a stimulating effect on the production of bacterial cellulose. In order to produce lactate into the culture medium, mixed cultures were maintained, containing bacteria producing acetic acid and lactic acid. The best results were obtained using strains of *Lactobacillus*, *Leuconostoc* and *Pediococcus*. These bacteria were also cultured in the presence of the yeast *Sacharomyces* in order to hydrolyse saccharose using β-fruktofuranosidase. This method helps increase the efficiency of BC synthesis, because after 14 days of mass culture some 8,1 g cellulose were obtained from 1 litre of medium, as compared to thr 6,4 g/l obtained without using *Lactobacillus* [31].

Ethanol may also be one of the stimulating factors of BC synthesis [21]. The results from a continuous culture of *Acetobacter xylinum* using a medium containing fructose and ethanol show that an ethanol concentration of 10 g/l significantly increases BC synthesis

efficiency, whereas at 15 g/l it is inhibitory. Based on these results, it may be surmised that just as for lactate, the ethanol acts as a source of energy accumulated as ATP, and is not a substrate for cellulose synthesis. ATP activates fructokinase, and inhibits glucose-6-phosphate dehydrogenase, thereby inhibiting the conversion of 6-phosphoglucose to 6-phosphogluconates. It was also noted that the addition of ethanol to the culture medium helped prevent the spontaneous mutation of the *Acetobacter sp.* A9 strain into the *Cel*$^-$ form, which is incapable of synthesizing cellulose. The precise mechanism driving this phenomenon is not understood, however.

Research into the effects of vitamins on BC synthesis has shown that the best stimulators are pirydoxin, nicotinic acid, p-aminobenzoic acid, and biotin [12, 20]. Fontana et al. [5] successfully used plant extracts, particularly from black tea, which contained components which stimulate cellulose synthesis. Mathematical methods are also used to optimise the culture medium, which has led to a large increase in the efficiency of cellulose synthesis [4, 6].

Analysis of the effect of pH on the synthesis of cellulose by *Acetobacter xylinum* has shown a pH optimum between 4.0 and 7.0. depending on the specific requirements of the strain [13]. Masaoka et al. [19] determined that a pH between 4,0 - 6,0 is optimal for BC synthesis, where 80% of the product accumulates from pH 3,5 to 7,0.

In a majority of the research cited, the optimal temperature for the biosynthesis of cellulose, the optimal temperature was found to be from 25-30°C, though a temperature of 28-30°C was used most often [9, 13, 7, 11, 14].

Bacterial cellulose biosynthesis can be maintained both in stationary cultures and in liquid mass cultures. Generally, the selection of the method to be employed is dependent upon the planned use of the synthesized polymer [16]. The control over the process is greatly complicated in a stationary culture by a membrane which forms on the surface of the medium, which in turn limits access to the medium. BC synthesis in stationary conditions can occur using a single-step procedure (a medium inoculated with a 5-10% inoculate) or a two-step procedure [2]. The latter also includes a liquid culture stage in order to amplify the biomass, and then a continuation in a stationary culture [22].

Large scale cellulose production in constantly mixed cultures is complicated by a series of difficulties, the greatest of which is the culture's instability characterised by a tendency for the strains to spontaneously mutate into inactive forms, the so called *Cel*$^-$ [30]. An unstable strain can be successfully used in a stationary culture, in which bacterial growth and cellulose synthesis occurs at the air-medium phase border. *Cel*$^-$ cells are favoured in a shaken culture, in which the growth of *Cel*$^+$ bacteria is limited by the rate of oxygen dissolution and cell aggregation by the synthesized cellulose (which limits oxygen access).

Bacterial cellulose may also be produced in a continuous stationary culture [26]. *Acetobacter xylinum* cells are in this case cultured on trays, on SH medium. Following 2-3

days of synthesis, the cellulose membrane produced on the medium's surface is continuously fed into a bath in a solution of sodium dodecyl sulphate (SDS) in order to inactivate the cells, and is then rolled onto a special roller. This process was maintained for several weeks, at a rate of 35 mm/h, and periodic replenishment of the medium (every 8-12 hours), in order to maintain optimal culture conditions. In this way, a 5 m long cellulose belt was produced which indicates the possible utility of this method in industrial processes.

Another described method of cellulose synthesis is an *Acetobacter* culture in horizontal bioreactors equipped with w rotating rollers, on which the produced polymer accumulated [27]. The observed bacterial growth was accelerated, and the efficiency of cellulose biosynthesis was almost doubled when compared to a stationary control culture.

Bacterial cellulose has great utility in medicine, particularly as a wound dressing and for artificial organs. This is due to properties such as being highly crystalline, high mechanical strength, an ability to absorb fluids, and excellent histocompatibility with living tissue, particularly with blood. specially purified cellulose membranes produced in a stationary culture may be a ready wound dressing, which meets all standards for modern dressing materials [17, 5]. It is biocompatible, porous, elastic, easy to apply and easy to store. It maintains optimum moisture during the healing of a wound, and may be sterilised using high temperature. Cellulose membranes are also excellent media for the immobilisation of various bioactive substances, to accelerate healing processes. Remembering the latest problems and controversies surrounding products of animal origin, collagen bandages can be replaced with cellulose ones. To date, several reports have been made regarding positive results in clinical tests of bacterial cellulose as a burn trauma dressing, trophic rash dressing, or as a biomaterial for skin transplantation [3, 13, 24].

The properties of bacterial cellulose open up many possible and economically feasible practical applications, if improved culturing methods are taken into account with improved strains of *Acetobacter*, which utilise inexpensive and easily available materials.

Patent descriptions PL 171952 (published 1995.02.06) and application P-317139 (submitted 1996.11.20) relate to a method of producing bacterial cellulose membranes through a surface culture of *Acetobacter xylinum*, on a glucose-based medium. This solution is based on the isolated *Acetobacter xylinum* strain P23, of the genus *Acetobacter*, a typically aerobic species characterised in its ability to produce acids from an incomplete oxidation of carbohydrates and alcohols, which exists as oval rods, singly or doubly or arranged into chains, and has an optimal growth temperature of 28-30°C at a pH of 4 -6.5 on solid media containing glucose, yeast extract, peptone, and agar. In liquid media, it forms a dense mucus-like membrane. The acids it produces as temporary or terminal products are released into the culture environment. In the presented solution, the isolated *Acetobacter xylinum* P23 strain is incubated in a liquid medium. A method according to the present invention produces

cellulose membranes containing 90 - 97% α-cellulose. Membranes produced by this method may be used as a dressing in surgery and dermatology.

Patent description US5472859 (published in 1995.12.05) describes a method for the enzymatic production of new forms of cellulose and another for *in vitro*. In the first method, cellulose is synthesized in a reaction catalyzed by endoglucanase, in the presence of an active form of saccharide substrate in an organic solvent environment. The second method relates to the synthesis of cellulose in a reaction catalyzed by glycosyl transferase, in the presence of UDPG, in an aqueous environment in conditions conducive to polymerisation and crystallisation of parallel glucan chains from the enzyme/mycelle complex.

Patent descriptions US5975095 (published in 1999.11.02), US5962278 (published in 1999.10.05), US6110712 (published in 2000.08.29) present a solution relating to the synthesis of cellulose by *Acetobacter xylinum* subsp. *nonacetoxidans*, which do not oxidize acetates and lactates, or oxidize them to an insignificant extent.

Patent descriptions US6429002 (published in 2002.08.06), US6329192 (published in 2001.12.11), US5144021 (published in 1992.09.01), US5079162 (published in 1992.01.07), US4863565 (published in 1989.09.05) present a solution relating to the production of bacterial cellulose in a shaken environment, in which over 70 h at least 0.1g/L was produced. The cellulose produced was strongly crosslinked, and very resilient.

Patent description US5955325 (published in 1999.09.21) presents a solution relating to microbiological cellulose with a high water content, produced in a disc fermenter.

Patent description EP0792935 (published in 2003.03.05) presents a process of cellulose production in a fermenter, at a CO_2 partial pressure of 10.13 kPa (0.10 atm) or less in the gaseous phase of the fermenting chamber.

Patent description JP54041321 (published in 1979.04.02) presents a method for the production of a dressing for skin lesions, which has excellent local adherence and long-term activity, using hydroxypyrocellulose and polyacryllic acid salts and their active ingredient.

Patent description EP0918548 (published in 1997.06.02) presents a solution relating to the application of oxidized cellulose, preferentially oxidized regenerated cellulose (ORC) and their complexes with proteins such as collagen, in order to provide a protective dressing for chronic wounds.

Patent description WO8602095 (published 1986.04.10) relates to a method of producing a cellulose film as an artificial skin to be used in grafting. The method of producing the cellulose film encompasses the preparation of a culture medium, in which the nutrient media are nitrates and carbohydrates, which is inoculated with *Acetobacter xylinum*. It also encompasses incubation at a temperature ensuring bacterial activity during the time the film is being produced, and removal of the film for dehydratation under tension.

Patent descriptions US4788146 (published 1988.11.29), and US4588400 (published 1986.05.13) relate to the application of cellulose membranes as dressings for wounds and

abrasions, as well as thermal wounds. They are produced in a culture of *Acetobacter xylinum*, to a thickness of 0.1 do 15 mm or more and are sterilised following removal, while they are still highly hydratated.

Despite the above mentioned studies on the production of bacterial cellulose, and the application bacterial cellulose as wound dressing material, a real need still exists to: obtain effective tools for the synthesis of bacterial cellulose, to select highly active, stable bacterial strains, to optimise the culture medium including its economic aspects to perfect the technologies, and to perfect the resulting material, which will broaden its possible applications.

The goal of the present invention is to provide the means which could be used to synthesise bacterial cellulose, and to obtain it for the production of wound dressings possessing certain, defined qualities determined by their intended usage.

Unexpectedly, the embodiment of the above stated goal and a solution to the problems described in the state of the art regarding: the production of bacterial cellulose in membrane form, the application of bacterial cellulose for the production of biocatalysts, the production of formulations which allow bacterial cellulose to be used as a wound dressing for extensive burns; have been achieved by the present invention.

The subject of the present invention is a method for the production of bacterial cellulose, characterised in that a culture of *Acetobacter xylinum* is maintained on an appropriate medium for the production of a cellulose surface membrane, which is then separated from the culture liquid, and further purified.

Preferentially, this process encompasses after the further purification the sterilisation process.

Preferentially, this process encompasses the initial culture phase, as well as the proper production phase.

Preferentially, this process encompasses the initial culture phase, in which the culture medium is inoculated with *Acetobacter xylinum* bacteria and they are cultured to achieve a density of ca. $5x10^7$ cfu/ml, and then this inoculum is used to inoculate the production culture.

Preferentially, the culture medium is characterised by the following composition by weight: 10 - 30 parts glucose, 2.5 – 12.5 parts yeast extract, 2.5 – 12.5 parts peptone, 1.25 – 6.25 parts $MgSO_4 \times 7H_2O$, 1.25 - 7 parts Na_2HPO_4, 0.5 - 3 parts citric acid, 5 – 100 parts ethanol to 1000 parts distilled water.

Preferentially, the culture medium used in the initial culture is inoculated with 5 - 10% (v/v) of the inoculum.

Preferentially, the method of preparing the inoculum consists of inoculating culture medium with a bacterial culture at an amount of 5 - 10%, maintained in this medium at maximum 48 h, at a temperature of 27-33°C.

Preferentially, following intensively mixing the culture with the formed membrane, the bacterial suspension is used to inoculate the production medium.

Preferentially, prior to the proper production culture, the inoculated medium is preincubated in the bioreactor.

Preferentially, the preincubation of the inoculated medium is performed with constant shaking.

Preferentially, the inoculated medium is preincubated up to 24 h at a temperature of 27 -33°C.

Preferentially, the inoculated medium is thoroughly mixed and poured into the bioreactor, and cultured for 5-10 days.

Preferentially, the proper production culture is maintained without shaking.

Preferentially, the membranes produced are removed and thoroughly purified.

Preferentially, the membranes produced are thoroughly cleaned, by rinsing in water, by boiling in a 1 - 3% NaOH solution for 0.5 - 2 hours, rinsing again to remove all traces of NaOH, treating with 0.5 - 3% acetic acid, rinsing with tap water and then distilled water.

Preferentially, the membranes produced are rolled, pressed, and wringed, to remove excess water, packed into sealed foil and sterilized by irradiation, using doses of 20 - 25 kGy.

Preferentially, the carbon source in the production medium for the synthesis of bacterial cellulose is a byproduct of industrial production of glucose.

Preferentially, the composition of the production medium for the synthesis of bacterial cellulose is based on byproduct constituents of plant origin.

Preferentially, the process encompasses preparing the production medium for the synthesis of bacterial cellulose, in which the carbon source is byproduct material containing 45 - 70% glucose, 10 - 15% isomaltose, 5 - 6 gentiobiose or glucose-fructose syrup (1:1) or molasses containing 40 - 60% saccharose and glucose from 5 - 25% or the effluent from the dextran microbiological synthesis.

Preferentially, the effluent from the microbiological synthesis of dextran contains 10 - 15% fructose, 0.05 - 2% glucose and 1 - 6% saccharose or glycerol.

Preferentially, the nitrogen source in the culture medium is corn steep liquor.

Preferentially, in the culture medium 3 - 5 parts yeast extract and 3 - 5 parts peptone from the culture medium are replaced with 15 - 25 parts of corn steep liquor.

Preferentially, the liquid following the end of culturing is reused to produce the medium.

Preferentially, the culture medium is supplemented with 3 - 8 parts carboxymethylocellulose (CMC) with a molecular mass between 90.000 – 250.000.

Preferentially, the 3 - 5 parts yeast extract and 3 - 5 parts peptone from the culture medium are replaced by introducing the inoculate liquid as a nitrogen source.

Preferentially, the cellulose biosynthesis in the stationary culture is maintained in such conditions, that the S/V ratio (bioreactor surface/volume) is 0.5-0.9 cm^{-1}.

Preferentially, the stability and synthesis of cellulose in a stationary culture is warranted by 0.5 - 3% ethanol present in the medium.

The subject of the present invention is a method of immobilising *Bacillus subtilis* bacteria, characterised in that the immobilisation carrier is a mixture of polyvinyl alcohol (PVA) with a mass of 72000, at a concentration of 10 - 12% and pulverised bacterial cellulose at 0.12 - 12%.

Preferentially, the mixture is cryo-gelled into spherical forms through repetitive freezing at temperatures between –17 and -25°C and defrosting at ca. 20°C, in vegetable oil.

The subject of the present invention is a method of producing immobilised biocatalysts, characterised in that the bacterial cellulose synthesized by the isolated strain of *Acetobacter xylinum*, P_{30}, belonging to the genus *Acetobacter*, in a stationary culture, is used to produce a carrier to immobilise the yeast *Saccharomyces cerevisiae* and the bacteria *Bacillus subtilis*.

Preferentially, the bacterial cellulose is synthesized in a liquid medium containing 10 - 30 parts glucose, 10 - 30 parts corn steep liquor, 5 - 15 parts ethanol, at a temperature of 27 - 33°C over 5 - 10 days, and subsequently cleaned with 1 - 3% NaOH at a temperature of 80-100°C, for 30-120 min and rinsed with water.

The subject of the present invention is an application of bacterial cellulose produced in a stationary culture of an *Acetobacter xylinum* strain as a wound dressing, particularly for burns and surgical wounds.

Preferentially, the bacterial cellulose is used in the form of cellulose membranes of arbitrary shape and size.

Preferentially, dressings made of bacterial cellulose secure the surface of wounds against excessive extranefrous water loss.

Preferentially, bacterial cellulose dressings ensure a moist environment and seal the surface of the wound.

Preferentially, the modified cellulose material is used.

The subject of the present invention is a method of modifying cellulose membranes, characterised in that it consists of selective oxidation of bacterial cellulose using oxidative agents.

Preferentially, the cellulose membranes are produced in the presence of CMC.

The subject of the present invention is a method of modifying cellulose membranes, characterised in that it consists of the saturating the membrane with a solution of 1-6 % glycerol, 0.5 – 4 % PEG 400, 0.01 – 1% chlorohexidine.

The subject of the present invention is a method of modifying cellulose membranes, characterised in that it consists of the production of composites consisting of cellulose and monomers and/or polymers and/or nanofibres and/or textiles during culturing.

Preferentially, the composite is the additional binding agent for bioactive substances promoting healing.

Preferentially, the composite increases the pressure applied to the wound.

Preferentially, the composite elongates the drying-out time of the dressing.

The attached figures facilitate a more thorough explanation of the nature of the present invention.

Figure 1 shows a deep 2b burn of moderately thick skin of the forearm, hand and fingers of the right hand with the cellulose dressing.

Figure 2 shows the application of a cellulose dressing. The dressing covers all of the back of the neck, back and loin area.

Below are listed example embodiments of the present invention defined above.

Production of cellulose membranes using modified culture media (examples 1-9)

Example 1.

The P1 inoculation medium containing the following constituents by mass: 20 parts glucose, 5 parts yeast extract, 5 parts peptone, 2.5 parts $MgSO_4x7H_2O$, 2.7 parts Na_2HPO_4, 1.15 parts citric acid, 10 parts of ethanol, to 1000 parts distilled water; is inoculated with 5% (v/v) of a suspension of *Acetobacter* bacteria ($5x10^7$ units/ml) maintained in that medium no longer than 7 days, at a temperature of 4°C. It is incubated for 2 days at a temperature of 30°C, when, following intensive mixing, the suspension is used to inoculate production medium of the same composition at a rate of 5% of production medium volume. The whole volume is then preincubated for 24 h at a temperature of 30°C, and it is then transferred into bioreactors of an appropriate volume, such that the S/V ratio (surface/volume) is 0.7 cm^{-1}. Using a bioreactor with a surface area of 10 000 cm^2, 14 dm^3 of culture medium is prepared, which following inoculation and preincubation is used to maintain the production culture proper in the bioreactor in stationary conditions over 7 days, whence the 5mm thick membranes formed are cleaned with tap water. They are then treated with 1% NaOH at a temperature of 100°C, for 1 hour, again rinsed in tap water, and then treated with 1%acetic acid, once again in tap water, and finally in distilled water. Excess water is removed through wringing, and then the membranes, with ca. 90% of their water removed, are placed in sealed foil bags and sterilised by irradiation using a dose of γ rays at 25 kGy.

Using the P1 medium it is possible to obtain ca. 3.5 g cellulose dry mass from 1 dm^3 of medium, and its protein content will not exceed 3%.

Example 2

An inoculate obtained as per Example 1 was used to inoculate sterile production medium composed of the following by mass: effluent from glucose crystallisation in an amount corresponding to 2% glucose, 20 parts maize mash and 10 parts ethanol in 1000 parts distilled water. Then, the procedure was as per Example 1 and as a result a mass of cellulose comparable to Example 1 was obtained.

Example 3

An inoculate obtained as per Example 1 was used to inoculate sterile production medium containing as a carbon source glucose-fructose syrup (1:1) in an amount corresponding to 1% glucose and 1% fructose and the remaining components like in medium P1. Then, the procedure was as per Example 1 and as a result a mass of cellulose ca. 15% higher than in Example 1 was obtained.

Example 4

An inoculate obtained as per Example 1 was used to inoculate sterile production medium, in which the fructose source utilised was 50 parts by mass of fructose effluent, a byproduct of separation of dextrans from a *Leuconostoc mesenteroides* culture and 2 parts ethanol and 5 parts of yeast extract, and distilled water to 1000 parts. Then, the procedure was as per Example 1 and as a result a mass of cellulose ca. 20% higher than in Example 1 was obtained.

Example 5

An inoculate obtained as per Example 1 was used to inoculate sterile production medium, in which the carbon source was molasses in an amount such that the saccharose contained therein amounted to 50 parts by mass, and 5 parts yeast extract as the nitrogen source, 10 parts ethanol, and distilled water to 1000 parts. As a result a mass of cellulose ca. 75% of the mass of cellulose from Example 1 was obtained.

Example 6

An inoculate obtained as per Example 1 was used to inoculate sterile P1 production medium, in which 5 parts of yeast extract and 5 parts peptone were replaced with 20 parts maize mash, not altering the remainder of the components. Then, the procedure was as per Example 1 and as a result a mass of cellulose ca. 10% higher than in Example 1 was obtained.

Example 7

An inoculate obtained as per Example 1 was used to inoculate sterile P1 production medium, in which 50% was liquid from a 7 day culture. Then, the procedure was as per Example 1 and the results were comparable to Example 1.

Example 8

An inoculate obtained as per Example 1 (10% v/v) was used to inoculate sterile P1 production medium, which was missing yeast extract and peptone, leaving the other components unchanged. The nitrogen source in this case was contained in the inoculate sample, 100 parts by weight. Then, the procedure was as per Example 1 and as a result a mass of cellulose ca. 70% of the mass from Example 1. The membrane produced in these conditions was white and more clear, which made the purification process much less labour intensive.

Example 9 The effect of CMC on the mass of produced cellulose membranes

The bacteria were cultured for 7 days on a modified SH medium, containing an addition of 1% ethanol and 0.05% $MgSO_4x7H_2O$, at a temperature of 30°C, in 500 ml Erlenmeyer flasks containing 100 ml of medium enriched with 1.0% CMC1 (90.000) or CMC2 (250.000). After purification according to the method presented in Example 1, the membranes produced during this period were air-dried to a solid, dry mass, which varied between 0.55 g depending on the degree of CMC polymerisation. In the presence of CMC1, at an increasing concentration in the range 1%, the masses of the membranes produced were, respectively, 0.55 g, whereas they were 0.31 g without CMC or 0.50 g with CMC2.

Application of bacterial cellulose for the production of immobilised biocatalysts (examples 10-12)

Example 10

0.12% of bacterial cellulose powder is added to a 10% polyvinyl alcohol solution (molecular mass 72 kDa and polymerisation degree 1600) along with a suspension of *Bacillus subtilis* at 7,6 x 10^9/ml. The mixture obtained is cryo-gelled into spherical shapes using 5 stages of freezing at -20°C and defrosting at 20°C, in vegetable oil. In parallel a control is run without bacterial cellulose.

To test the mechanical resistance of such biopreparations they were suspended in water and shaken at 150 rpm, at a temperature of 55°C, while measuring their dissolution times. The dissolution time of the sample with bacterial cellulose lengthened to 45 min, whereas for samples without cellulose, the time was 10 min.

In order to determine the proportion of bacteria released from the carrier, biopreparation samples obtained as per Example 10 undergo triple rinsing in 30 min, using 1 ml aliquots of

physiological saline. The suspension of released bacteria is swabbed onto medium solidified with agar, and counted after 24 h of culture at 37°C. After the third cycle of rinsing, 10 times less bacteria were released than from the sample without cellulose.

Immobilised biopreparations produced as per Example 10 are used in the biosynthesis of proteolytic enzymes, introducing 4% of biocatalyst into 50 cm^3 of sterile culture medium and incubating it at 30°C, for 66 h, constantly mixed at 220 rpm. The culture cycle was repeated 3 times using the same biopreparations, following washing with physiological saline. In each case the serine protease activity was 17% higher when the preparation contained bacterial cellulose.

Example 11

The cellulose membrane obtained as in Example 6 is ground down to a particle size of 2-3 mm, which are sterilised (120°C, 20 min.) and introduced at a rate of 1 g s. s. into a 4 hour shaken culture of the yeast, *S. cerevisiae* in 100 ml of medium containing: 10 parts saccharose, 5 parts of yeast extract, 0.5 parts $(NH_4)SO_4$, 0.7 parts $MgSO_4x7H_2O$, 0.5 parts KH_2PO_4, 0.5 parts NaCl, distilled water to 1000 parts (pH=4,8). The culture is continued for the next 4 hours, after which the suspension of cellulose along with adsorbed yeast cells is separated from the medium, and following thorough a wash, used in the process of saccharose hydrolysis, using a 5% saccharose solution and the biopreparation at a ratio of 1:5 by weight. One cycle lasted 10 hours, and there were 10 cycles. The highest degree of saccharose inversion, 97%, was achieved during the third cycle.

Example 12

The cellulose membrane obtained as in Example 6 was initially ground, and then particles 2-3 mm in diameter were homogenized at 13000 rpm. The pulp obtained was centrifuged at 5000 rpm. and the wet mass was added at a rate of 10% to a 2% sodium alginate solution. 1g of wet yeast pulp was introduced into 20 cm^3 of this mixture, thoroughly mixed, and dripped into an ice-bath chilled 2% $CaCl_2$ solution, with constant mixing. The spherical portions of the biopreparation were kept in the 2% $CaCl_2$ solution at a temperature of 4°C for 12 hours.

The produced biopreparation was used for continuous saccharose hydrolysis, using a 50% solution thereof. The process took place in a vertical reactor at a temperature of 40°C, with a 0.04 cm^3/min substrate flow-through rate. After 30 days, the saccharose inversion was 80% when the carrier contained cellulose and 65% when it did not.

This biopreparation was also used in the hydrolysis of 50% saccharose, using a periodic method of 6 cycles of 8 hours, and the preparation at 1 g per 18 g substrate. In every cycle, the degree of saccharose inversion was 25-30% higher using the biopreparation with the cellulose carrier, than when a non-cellulose carrier was used..

Application of cellulose membranes as filters (examples 13-14)

Example 13

A cellulose membrane prepared as per Example 6, after 90% of the water was removed, was used in the form of a circle 10 cm in diameter to filter samples of apple juice, using a vacuum filter apparatus. In the comparative method of traditional filtration, 100 cm^3 of juice was supplemented by 0.5 ml of 1% gelatine solution, and 0.5 ml 3% solution of siliconic acid, and then filtered through a filter containing Hyflo Super Cel diatomaceous earth. The filtered juices were subjected to stability testing by heating to a temperature of 80°C, chilling, and freezing to -18°C for one hour, and then defrosted at room temperature. The comparative analysis is presented in Table 1.

Table 1

Indices	**Juice after traditional clarification**	**Juice after filtration through cellulose membranes**
Extract [%]	11,5	11,5
Overall acidity [g maleic acid in 1 l]	3,35	3,38
Overall polyphenyls [g/l]	0.24	0.27
Clarity [% $T_{620\ nm}$]	96	99
Colour [A_{420nm}]	0.24	0.21
Following stability test		
Clarity [% $T_{620\ nm}$]	92	95
Colour [A_{420nm}]	0.25	0.27

As the results indicate, the juice clarified via the cellulose membranes was characterised by a higher clarity before and after the stability test, when compared to the control. Following the stability assay, the juice samples filtered through the membranes exhibit a higher colour intensity.

Example 14

Using a culture medium like in Example 6, *Acetobacter xylinum* were cultured in a bioreactor 100x15x8,5 cm, which dimensions corresponded to the UF filtration module from Amicon for an RA 2000 device. The purified membrane, following removal of 90% of its water, was used as a filtration module to filter wine which exhibited a secondary, opalescent haze. The obtained samples of wine retained full clarity during the 6-month study period, maintained at room temperature.

Example 15

Water-retention ability of membranes synthesized in the presence CMC and centrifuged

Membranes produced as per Example 9, following purification, were centrifuged at 176g for 15 min. It was determined that membranes spun at 176g, and produced in the presence of CMC1 at concentrations of 0.5% and 1% retain ca. 30% water, and with CMC2 at the same concentration, ca. 40% water, whereas without CMC only 15% water.

Example 16

Water retention ability during drying at 37°C of membranes modified with CMC

Cellulose membranes produced according to Example 9 in the presence of 0.5% CMC with a m.m. of 90000 were purified, and dried at a temperature of 37°C. Following 3 hours of drying, the membrane produced in the presence of CMC lost ca. 17% of its water, whereas without CMC it lost 23%, and following 22 hours this was respectively 92,5% and 99%.

Example 17

Modification of cellulose membranes using sodium periodate ($NaIO_4$) and their hydroscopic properties

Cellulose membranes produced as per Example 1, were immersed in a sodium periodate solution, most preferentially 1%, at a temperature of 22°C for 2 hours, and were then washed with distilled water to remove all traces of the periodate. The product obtained contained 2,75 mmol of aldehyde groups / g of dry mass and was 2 times more hydroscopic than unmodified membranes.

Example 18

Application of membranes oxidized with $NaIO_4$ to immobilize lysozyme.

Membranes oxidized under conditions described in Example 17 were immersed in a 0.02% lysozyme solution, with an activity of 16800 U/mg, for 4 hours, at room temperature, and shaken at 100 rpm. They were then washed with distilled water to rinse away any unbound protein. Compared with unmodified cellulose, oxidized cellulose binds about 2 times as much protein, meaning ca. 60%.

Example 19

The cellulose membrane according to Example 1, was purified and excess water was removed (to 50% of initiall mass). It was then soaked in a solution containing 0,1% chlorohexidine, 6% glycerol, 4% PEG 400. The resulting material retained its elasticity following drying at room temperature, did not cling to skin, was easy to remove without need of prior moistening, and moreover exhibited bacteriocidal properties.

Example 20

A membrane produced as in Example 1, following 4 days of culture, was used as a matrix for the application of sterile material (i.e. cotton cloth) with dimensions 2 cm less than the dimensions of the membrane, and the culture was maintained a further 4 days. During this time, a membrane is formed over the cloth, and it becomes sealed between the two membrane layers which fuse at the edges around the cloth. The membrane formed over the cloth is tightly bound to it, whereas a pocket is formed between the cloth and the matrix, which was filled with water or physiological saline in order to increase the mass of the material, and thus its exerted pressure, as well as to elongate its drying time. This pocket may also be filled with bioactive substances which may penetrate the wound and accelerate healing.

Example 21

Application of cellulose dressings to treat surgical incisions and burns in animals

40 female Wistar rats were used as experimental animals, aged 8-10 weeks with body masses ranging from 152 to 190g (average 178g).

The animals were divided into 4 groups of 10:

Group A – rats with a surgical incision treated without dressing

Group B – rats with a surgical incision treated with a cellulose dressing

Group C – rats treated with a burn treated without dressing

Group D – rats treated with a burn treated with a cellulose dressing

The wound surface was an area of 2x3cm. Such a wound surface comprises from 1,9% to 2,5% (on average 2%) of body surface. In the case of burns, such a wound is considered light. The wounds were located on the dorsal side of the animal, below the shoulder blades. In the groups treated with a cellulose dressing, sterile cellulose patches were used according to Example 1. One hour prior to the treatment, the animals were pre-medicated and then following depilation a surgical wound was produced by removing a patch of skin with a scalpel, to its complete depth, or by producing a burn wound through the application of a steel plate 19x28x1mm heated to 500^0C. This method rendered a III degree burn with a surface of 2x3 cm. The wound penetrated the entire thickness of the skin. The wound in group A was left to heal without a dressing. In group B, cellulose dressings were used, exchanged every alternate day. The burns in group C were treated without dressings. In group D, dressings were used as in group B. In order to prevent the animals from affecting the dressings, a plaster

cast was put on their torso. After treatment, the animals were kept in individual cages. They received an analgesic in their water and standard feed.

To quantify the results, macroscopic examination criteria were used. The macroscopic and microscopic examinations of wound healing were performed after 7 and 21 days on 5 animals from each group. The animals were euthanised with an overdose of ether through inhalation. Material for microscopic observation was collected through the excision of skin, along with the area of healing or healed wounds. The material was fixed in 10% formaldehyde. Postfixation, paraffin sections were made which were stained with haematoxilin and eosin and examined microscopically.

All rats survived the observation period. The results showed that for surgical wounds, the application of a cellulose dressing accelerated the healing and reconstruction of the epithelium, caused a lesser degree of inflammatory fluid seepage, and encouraged the preliminary formation of skin features. In the case of burns, application of the cellulose dressing caused: faster scab formation, more advanced epithelium formation, and preliminary formation of skin features.

Example 22 Clinical studies

Application of a bacterial cellulose dressing in the treatment of burns

Experimental group „A" encompassed 41 patients hospitalised at the Centre for Burn Treatment in Siemianowice Śląskie, from the first day following IIA, IIB and/or III degree skin burns to 9-18% of the entire body surface area, of both sexes, aged 18-70 years old. These patients were diagnosed free of colagenosis, diabetes, uraemia, malignant tumours and were never treated with cytostatic drugs, radiation, immunosuppression and had not received blood or blood product transfusions. The local treatment of the burn consisted of applying dressings of bacterial cellulose in 10-day cyclesafter cleaning and disinfecting the burned surface. The cellulose membranes were produced according to Example 1 and sterilised through irradiation. The doctor-in-charge tightly covered the wound surface in sterile conditions [Fig. 1, 2] and additionally secured it with a 3% Braunol dressing. Control group „B" was composed of 12 patients selected according to the same criteria as above, locally treated according to standard methods, using dressings of 3% Braunol, 3% boric acid solutions, and salicylate paste in 10 day cycles. Following cleaning and local preparation of the wound, both groups were treated with (depending on medical indication) a skin graft of moderate thickness. For all patients in groups A and B, the Parcland crystalloid rule was

applied during the first day of hospitalisation, and as of the second day, systemic water loss was measured using the haematocrit.

Before the wound was cleaned on the 5th and 10th days of treatment, smears for microbiological analysis were taken, and urine samples were taken on the 1st day. All patients were treated with targeted antibiotics starting on the 5th day of hospitalisation (as indicated medically). In both groups blood morphology and the haematocrit were measured, as were the sodium and potassium levels were in peripheral blood, total blood protein, and albumin levels on the 1st, 5th and 10th days of treatment. Photographs of the wounds were taken on the same day. On the 10th day, excisions were made in both groups for histo-pathological analysis. All cellulose dressings from group A were evaluated on day 1 and day 10 of the treatment. The 10 day treatment observation cycle allowed to repeat until such time as the wound was fit for an autogenic skin transplant of moderate thickness. Graft healing was evaluated and documented photographically during the 2nd, 7th and 12th day following the operation. The number of 10 day intervals of preparatory treatment were determined, as was the total time of healing for the wound covered with the graft.

The research showed that:

- Wounds treated with a cellulose dressing are well insulated from the environment. The dressing protects the wound from infection and tissue fluid loss.
- The dressings reduce the subjective sensation of pain in the burn patients.
- The dressings are well tolerated by the patients, and must be kept on the wounds until the completion of epithelium formation.
- The dressings maintain a moist environment and create opportunities for further moistening.
- In shallow burns, the dressings accelerate epithelium formation, and in deep burns they accelerate necrosis demarcation.
- The possibility to produce a cellulose dressing of arbitrary chape and size enables the whole wound to be covered with one dressing.
- The speed at which the dressing is applied to the wound post-trauma, while maintaining asepsis is conditional the effectiveness of the treatment (without scarring).

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Patent Claims

1. A method of producing bacterial cellulose, characterised in that *Acetobacter xylinum* bacteria are cultured in an appropriate medium for the production of a surface cellulose membrane, which is then isolated from the culture liquid and further purified.
2. A method according to Claim 1, characterised in that the process encompasses after the further purification the sterilisation process.
3. A method according to Claim 1, characterised in that the process encompasses the initial culture phase, as well as the proper production phase.
4. A method according to Claim 1, characterised in that the process encompasses the initial culture phase, in which the culture medium is inoculated with *Acetobacter xylinum* bacteria and they are cultured to achieve a density of ca. $5x10^7$ cfu/ml, and then this inoculum is used to inoculate the production culture.
5. A method according to Claim 1, characterised in that the culture medium is characterised by composition by weight: 10 - 30 parts glucose, 2.5 – 12.5 parts yeast extract, 2.5 – 12.5 parts peptonu, 1.25 – 6.25 parts $MgSO_4$ x $7H_2O$, 1.25 - 7 parts Na_2HPO_4, 0.5 - 3 parts citric acid, 5 - 100 parts ethanol to 1000 parts distilled water.
6. A method according to Claim 1, characterised in that the culture medium used in the initial culture is inoculated with 5-10% (v/v) of the inoculum.
7. A method according to Claim 1, characterised in that the method of preparing the inoculum consists of inoculating culture medium with a bacterial culture at an amount of 5 - 10%, maintained in this medium at maximum 48 h, at a temperature of 27-33°C.
8. A method according to Claim 1, characterised in that following intensively mixing the culture with the formed membrane, the bacterial suspension is used to inoculate the production medium.

9. A method according to Claim 1 , characterised in that prior to the proper production culture, the inoculated medium is preincubated in the bioreactor.
10. A method according to Claim 1, characterised in that the preincubation of the inoculated medium is performed with constant shaking.
11. A method according to Claim 1, characterised in that the inoculated medium is preincubated up to 24 h at a temperature of 27-33°C.
12. A method according to Claim 1, characterised in that the inoculated medium is thoroughly mixed and poured into the bioreactor, and cultured for 5-10 days.
13. A method according to Claim 1, characterised in that the proper production culture is maintained without shaking.
14. A method according to Claim 1, characterised in that the membranes produced are removed and thoroughly purified.
15. A method according to Claim 1, characterised in that the membranes produced are thoroughly cleaned, by rinsing in water, by boiling in a 1 - 3% NaOH solution for 0.5 - 2 hours, rinsing again to remove all traces of NaOH, treating with 0.5 - 3% acetic acid, rinsing with tap water and then distilled water.
16. A method according to Claim 1, characterised in that the membranes produced are rolled, pressed, and wringed, to remove excess water, packed into sealed foil and sterilized by irradiation, using doses of 20 - 25 kGy.
17. A method according to Claim 1, characterised in that the carbon source in the production medium for the synthesis of bacterial cellulose is a byproduct of industrial production of glucose.
18. A method according to Claim 1, characterised in that the composition of the production medium for the synthesis of bacterial cellulose is based on byproduct constituents of plant origin.
19. A method according to Claim 1, characterised in that it encompasses preparing the production medium for the synthesis of bacterial cellulose, in which the carbon source is a byproduct material containing 45 - 70% glucose, 10 - 15% isomaltose, 5 - 6 gentiobiose or glucose-fructose syrup (1:1) or molasses containing 40 - 60% saccharose and glucose from 5 - 25% or the effluent from the dextran microbiological synthesis.
20. A method according to Claim 1, characterised in that the effluent from the microbiological synthesis of dextran contains 10 - 15% fructose, 0.05 - 2% glucose and 1 - 6% saccharose, or glycerol.
21. A method according to Claim 1, characterised in that nitrogen source in the culture medium is corn steep liquer.
22. A method according to Claim 1, characterised in that the 3 - 5 parts yeast extract and 3 - 5 parts peptone from the culture medium are replaced with 15 - 25 parts of corn steep liquor.

23. A method according to Claim 1, characterised in that the liquid following the end of culturing is reused to produce the medium.
24. A method according to Claim 1, characterised in that the culture medium is supplemented with 3 - 8 parts carboxymethylocellulose (CMC) with a molecular mass between 90.000 – 250.000.
25. A method according to Claim 1, characterised in that the 3 - 5 parts yeast extract and 3 - 5 parts peptone from the culture medium are replaced by introducing the inoculate liquid as a nitrogen source.
26. A method according to Claim 1, characterised in that the cellulose biosynthesis in the stationary culture is maintained in such conditions, that the S/V ratio (bioreactor surface/volume) is 0.5-0.9 cm^{-1}.
27. A method according to Claim 1, characterised in that the stability and synthesis of cellulose in a stationary culture is warranted by 0.5 - 3% ethanol present in the medium.
28. A method of immobilising *Bacillus subtilis* bacteria, characterised in that the immobilisation carrier is a mixture of polyvinyl alcohol (PVA) with a mass of 72000, at a concentration of 10 - 12% and pulverised bacterial cellulose at 0.12 - 12%.
29. A method according to Claim 28, characterised in that the mixture is cryo-gelled into spherical forms through repetitive freezing at temperatures between –17 and -25°C and defrosting at ca. 20°C, in vegetable oil.
30. A method of producing immobilised biocatalysts, characterised in that the bacterial cellulose synthesized by the isolated strain of *Acetobacter xylinum*, P_{30}, belonging to the genus *Acetobacter*, in a stationary culture, is used to produce a carrier to immobilise the yeast *Saccharomyces cerevisiae* and the bacteria *Bacillus subtilis*.
31. A method according to Claim 30, characterised in that the bacterial cellulose is synthesized in a liquid medium containing 10 - 30 parts glucose, 10 - 30 parts corn steep liquor, 5 - 15 parts ethanol, at a temperature of 27 - 33°C over 5 - 10 days, and subsequently cleaned with 1 - 3% NaOH at a temperature of 80-100°C, for 30-120 min and rinsed with water.
32. An application of bacterial cellulose produced in a stationary culture of an *Acetobacter xylinum* strain as a wound dressing, particularly for burns and surgical wounds.
33. An application according to Claim 32, characterised in that the bacterial cellulose is used in the form of cellulose membranes of arbitrary shape and size.
34. An application according to Claim 32, characterised in that dressings made of bacterial cellulose secure the surface of wounds against excessive extranefrous water loss.
35. An application according to claim 32, characterised in that bacterial cellulose dressings ensure a moist environment and seal the surface of the wound.
36. An application according to Claim 32, characterised in that modified celulose material is used.

37. A method of modifying cellulose membranes, characterised in that it consists of the selective oxidation of bacterial cellulose using oxidative agents.
38. A method according to Claim 37, characterised in that cellulose membranes are produced in the presence of CMC.
39. A method of modifying cellulose membranes, characterised in that it consistes of saturating the membrane with a solution of 1-6 % glycerol, 0,5 – 4 % PEG 400, 0,01 – 1% chlorohexidine.
40. A method of modifying cellulose membranes, characterised in that it consists of the production of composites consisting of cellulose and monomers and/or polimers and/or nanofibres and/or textiles during culturing.
41. A method according to Claim 40, characterised in that the composite is the additional binding agent for bioactive substances promoting healing.
42. A method according to Claim 40, characterised in that the composite increases the pressure applied to the wound.
43. A method according to Claim 40, characterised in that the composite elongates the drying-out time of the dressing.

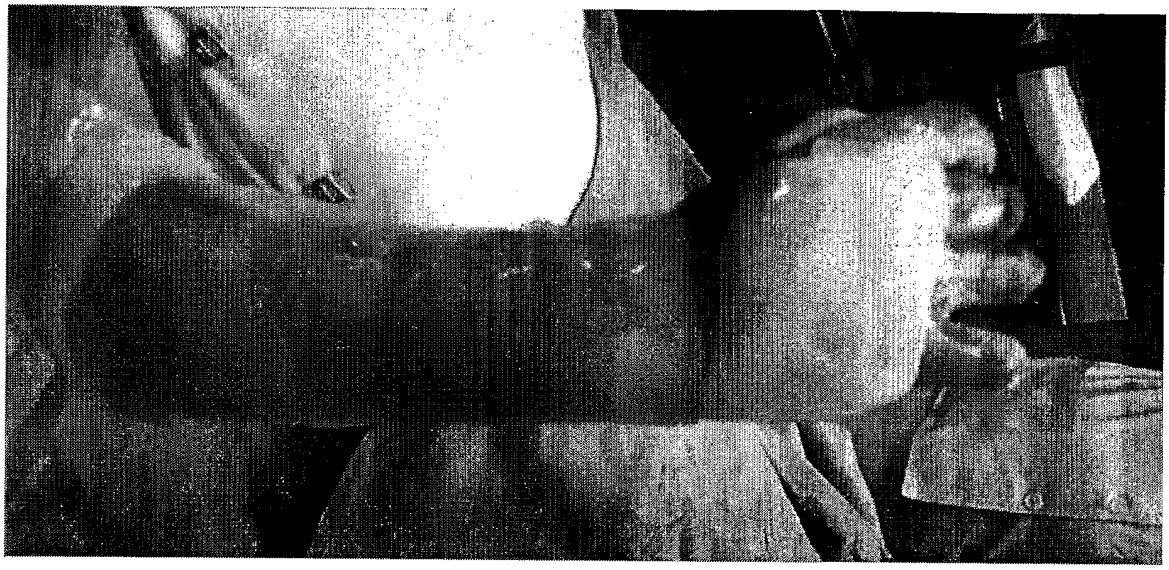

Fig. 1

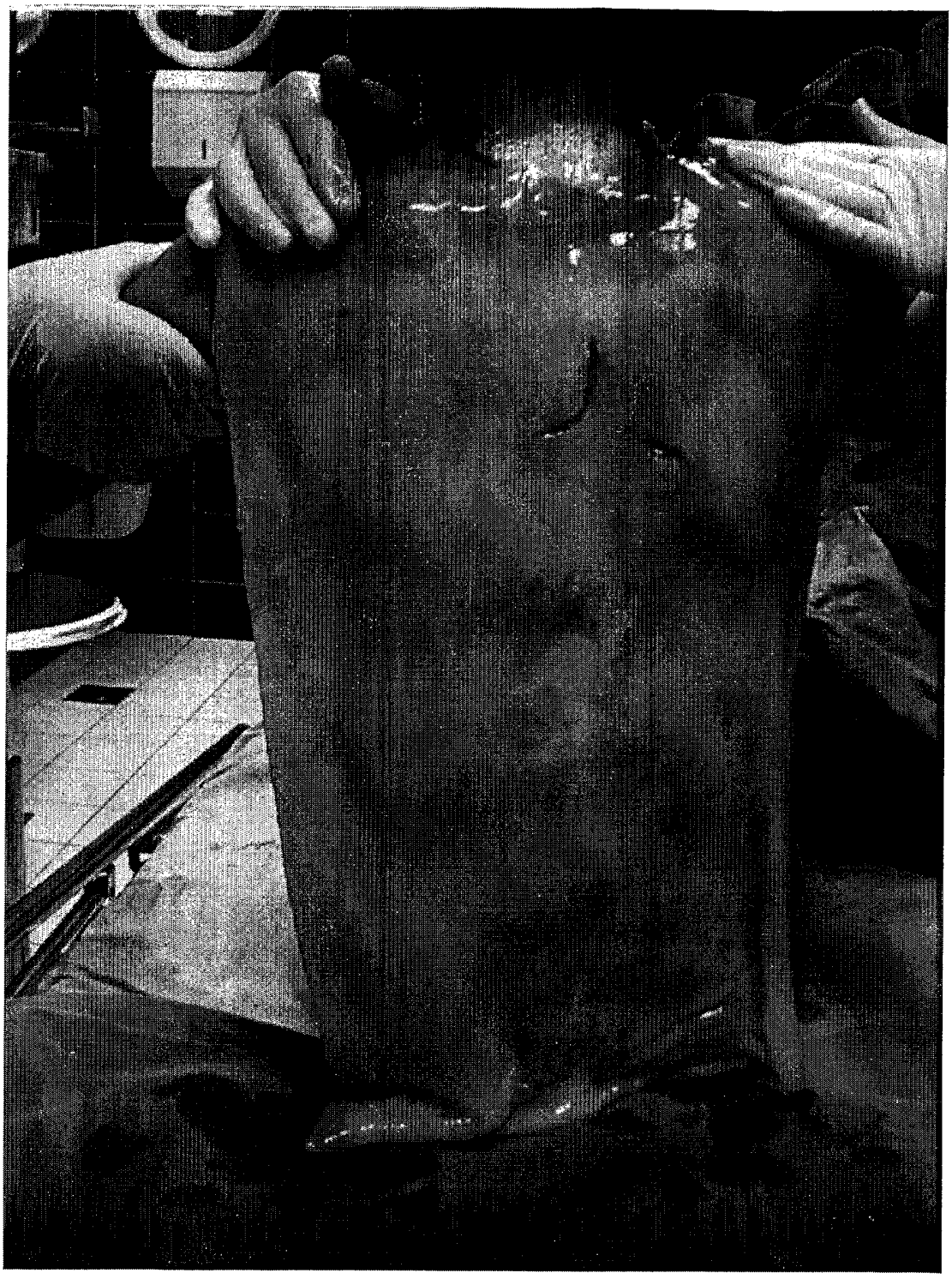

Fig. 2

INTERNATIONAL SEARCH REPORT

International Application No
PCT/PL2004/000051

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P19/04 C12N11/08 C08B1/00 A61L15/28

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)
IPC 7 C12P C12N C08B

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 practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, PAJ, FSTA, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KRYSTYNOWICZ A ET AL: "THE EVALUATION OF USEFULNESS OF MICROBIAL CELLULOSE AS A WOUND DRESSING MATERIAL" MEDEDELINGEN VAN DE FACULTEIT LANDBOUWWETENSCHAPPEN UNIVERSITEIT GENT, GENT, BE, vol. 65, no. 3A, 2000, pages 213-220, XP009032540 ISSN: 0368-9697 the whole document ----- -/--	1-23, 25-27, 32-36

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

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"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
21 October 2004	08/11/2004

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, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Lejeune, R

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Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/PL2004/000051

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KRYSTYNOWICZ A ET AL: "Factors affecting the yield and properties of bacterial cellulose" JOURNAL OF INDUSTRIAL MICROBIOLOGY AND BIOTECHNOLOGY, vol. 29, no. 4, October 2002 (2002-10), pages 189-195, XP008037200 ISSN: 1367-5435 the whole document	1-16, 25-27, 32-36, 40-43
X	SZCZESNA-ANTCZAK M ET AL: "Bacillus subtilis cells immobilised in PVA-cryogels" BIOMOLECULAR ENGINEERING, ELSEVIER, NEW YORK, NY, US, vol. 17, no. 2, January 2001 (2001-01), pages 55-63, XP004257826 ISSN: 1389-0344 the whole document	28-31
X	SERAFICA G ET AL: "Inclusion of solid particles in bacterial cellulose" APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 58, no. 6, May 2002 (2002-05), pages 756-760, XP002301763 ISSN: 0175-7598 the whole document	40-43
X	EP 0 346 507 A (UNIV TEXAS) 20 December 1989 (1989-12-20) the whole document	1,3,4,6, 7,37,38, 40,42,43

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/PL2004/000051

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 32-36 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/PL2004/000051

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0346507 A	20-12-1989	AU 611159 B2	06-06-1991
		CA 1339913 C	16-06-1998
		US 4942128 A	17-07-1990
		EP 0346507 A1	20-12-1989
		AT 123532 T	15-06-1995
		AU 1770088 A	21-12-1989
		DE 3853946 D1	13-07-1995
		JP 1320994 A	27-12-1989