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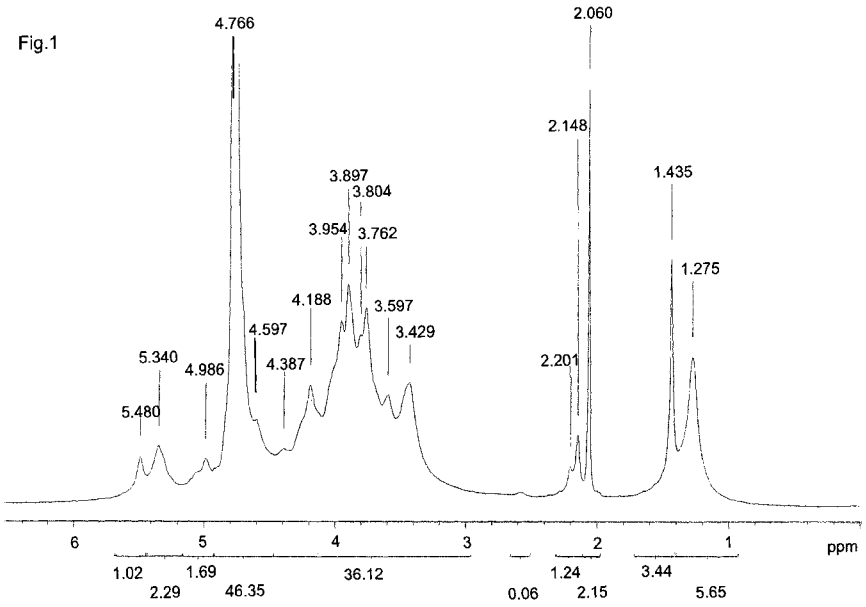
[Continued on next page]

(54) **Title**: PROCESS FOR PRODUCTION OF L-FUCOSE

Fig.1



(57) **Abstract**: The present invention relates to a process for production, on an industrial scale, of L- fucose (or 6-deoxygalactose) from hydrolysis of polysaccharides obtainable by means of aerobic fermentation of a new isolated microbial strain belonging to the family Enterobacteriaceae.

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PROCESS FOR PRODUCTION OF L-FUCOSE

FIELD OF THE INVENTION

The present invention relates to a process for the production, on an industrial scale, of L-fucose (or 6-deoxygalactose) by hydrolysis of a polysaccharide produced by a fermentation process effected by a novel isolated microbial strain belonging to the family *Enterobacteriaceae*.

PRIOR ART

L-Fucose, and fucosylated oligosaccharides and polysaccharides are of great interest to the chemical, pharmaceutical, cosmetic and nutraceutical industry. Fucosylated oligosaccharides and polysaccharides are known to have potential medical applications as anti-tumour and anti-inflammatory agents but also potential cosmetic applications as anti-ageing agent for the skin that is able to improve skin tonicity.

Fucosyl derivatives, for which L-fucose represents the raw material, are of industrial interest for their known antiallergic, hydrating, emulsifying and stabilizing properties.

Another very important use of L-fucose is as a precursor of semisynthetic analogues of the fucosylated oligosaccharides contained in human milk (human milk oligosaccharides). The production of L-fucose industrially is still rather problematic both with respect to chemical and biochemical synthesis, and conversely with respect to the use of bacterial strains that produce L-fucose or polysaccharides containing L-fucose by fermentation.

The known chemical syntheses, which often involve the configuration inversion of a sugar that is readily available, always envisage numerous steps and the use of very expensive reagents (J.-P. Gesson et al. *Tetrahedron Letters*, vol. 33, No. 25, p. 3637-3640, 1992, G. D. Gamalevich et al. *Tetrahedron*, vol. 55, p. 3665-3674, 1999, S. Sarbajna et al. *Carbohydr. Res.,* vol. 270, p. 93-96, 1995).

In US 6,713,287 C.-H. Wong prepares L-fucose enzymatically: in this case the process is based on the use of engineered microorganisms, which express the enzymes necessary for synthesizing L-fucose starting from dihydroxyacetone and lactaldehyde.

The processes for formation of L-fucose by fermentation are also known. Some microbial extracellular polysaccharides (EPS), better known for their properties as thickening, gelling or emulsifying agents, constitute an attractive source of L-fucose. The description of a strain of *Klebsiella* K-63 that produces a capsular polysaccharide containing D-galactose, L-fucose and D-galacturonic acid in a molar ratio of 1:1:1 dates from the end of the 1970s (J.P. Joseleau et al. *Carbohyd. Res.,* vol. 77, p. 183-190, 1979).

In US 4,298,691, G.T. Veeder and K.S. Kang describe a process for producing a heteropolysaccharide S-156 starting from a strain of *Klebsiella pneumonie* ATCC 31646 containing galacturonic acid, D-galactose and L-fucose in a molar ratio of 23:21:26.

EP0102535 describes the fermentation of bacteria of the genera Alcaligenes, Klebsiella, Pseudomonas or Enterobacter for producing extracellular polysaccharides rich in rhamnose or fucose. These deoxy-sugars can, after acid hydrolysis of the polysaccharide, be isolated from the hydrolysate.

Interesting results have been obtained with the use of *Clavibacter michiganensis* which in about 8 days of fermentation is able to produce about 2.4g/l of L-fucose: however, *Clavibacter* is a phytopathogen and so cannot be used industrially (P.T. Vanhooren et al. *Med. Fac. Landbouw. Univ. Gent.*, vol. 62/4a, p. 1271-1276, 1997).

The L-fucose currently on the market is obtained from natural sources, in particular by acid hydrolysis of sulphated polysaccharides such as fucoidans extracted from algae such as *Laminaria*. However, direct extraction from algae is expensive and is moreover subject to seasonal variations in volumes and product quality.

Another source of L-fucose is the bark of trees with a high trunk such as willow, birch and beech. In this case too, L-fucose is extracted by hydrolysis, with low yields and high costs.

It is therefore clearly necessary to provide a process, at least as an alternative, using microbial fermentation, for production of L-fucose that is applicable on an industrial scale and that is efficient and reproducible.

SUMMARY OF THE INVENTION

The present invention solves the aforementioned problems by means of a new bacterial strain belonging to the family Enterobacteriaceae, identified as C2B2, isolated from foliar extract of *Phalaenopsis sp.*

Isolation of this new strain made it possible to develop aerobic fermentation which, surprisingly, leads to the formation of a polysaccharide containing L-fucose in amounts such that, once the polymer has been hydrolysed, the concentration of L-fucose in the fermentation broth is above 3g/l (the amount of L-fucose is evaluated by HPLC after complete hydrolysis of a sample to which 96% sulphuric acid has been added in amounts such as to give a 1.5 M solution, which is then heat-treated at 100°C for 30 minutes).

Then, after several purification steps, crystalline L-fucose can be recovered from the fermentation broth at high yields (above 75%) and with HPLC purity>98%.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig.1 shows the plot of a ^{1}H-NMR spectrum (in deuterated water, performed at 25°C with acetonitrile internal standard at 2.06ppm) of a fucose-containing polysaccharide, produced by a bacterial strain according to the invention (as from example 6)

Fig.2 shows the plot of a ^{13}C-NMR spectrum (in deuterated water, performed at 25°C with acetonitrile internal standard at 1.470ppm) of a fucose-containing polysaccharide, produced by a bacterial strain according to the invention (as from example 6)

Fig.3 shows the plot of a ^{13}C-NMR spectrum (in deuterated water, performed at 25°C with acetonitrile internal standard at 1.470ppm) of the anomeric region of a fucose-containing polysaccharide, produced by a bacterial strain according to the invention (as from example 6)

Fig.4 shows an HPLC plot of a partial hydrolysis mixture of a polysaccharide according to the invention (as from example 7)

Fig.5 shows an HPLC plot of a partial hydrolysis mixture of a polysaccharide according to the invention, which was then treated with ion exchange resins to eliminate the peak at about 11 minutes (as from example 7).

Fig.6 shows an HPLC plot of a complete hydrolysis mixture of a polysaccharide according to the invention (as from example 8)

Fig.7 shows an HPLC plot of a partial hydrolysis mixture of a polysaccharide according to the invention (as from example 9)

DETAILED DESCRIPTION OF THE INVENTION

The C2B2 bacterial strain was isolated from foliar extract of orchids of the genus *Phalaenopsis*. The new strain isolated was identified as belonging to the family Enterobacteriaceae and was designated C2B2. The new strain was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (German Collection of Microorganisms and Cell Cultures), Inhoffenstr. 7 B, D-38124, Braunschweig (Germany) on 23 January 2009 in accordance with the provisions of the Budapest Treaty.

The new microbial strain was assigned the accession number DSM 22227.

The microbial strain according to the invention was characterized by sequencing the ribosomal RNA 16S resulting in a SEQ ID NO: 1 given below:

CCAAGCAGCTTGCTGCTTCGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGG
AAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAAC
GTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGA
TGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGG
TCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCG
CGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGA
TGYGGTTAATAACCGCGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACT
CCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGG
GCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCA
ACCTGGGAACTGCATCCGAAACTGGCAGGCTTGAGTCTCGTAGAGGGGGGTAGAA
TTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGG
CGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAG
GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCC
CTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACG

GCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGC
ATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGA
ACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGC
TGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTATCCTTTGTTGCCAGCGGTYSGGCCGGGAACTCAAAGGAGACTGCCAGTGATA
AACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCT
ACACACGTGCTACAATGGCGCATACAAAGAGAAGCAATCTCGCGAGAGCTAGCGG
ACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGT
CGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTT
GTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTA
ACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGG

The present invention also relates to microbial strains belonging to the family *Enterobacteriaceae*, comprising a sequence rRNA 16S containing a sequence with homology greater than or equal to 93% with SEQ. ID NO: 1; preferably said homology is greater than or equal to 98% with SEQ. ID NO: 1.

The present invention also relates to mutated strains derived from the C2B2 strain. These strains can be obtained by simple selection of strains spontaneously mutated and isolated from a culture of C2B2 or by selection of strains derived from C2B2 by the action of mutagenic factors, such as UV radiation or X-rays, or by the action of chemicals such as ozone, nitrous acid, N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or ethane methane sulphonate (EMS).

Preferaby said derived or mutated microbial strain, comprises an rRNA 16S sequence containing a sequence having homology with SEQ. ID NO: 1 equal or greater than 98%.

The C2B2 bacterial strain according to the invention has the following morphological and biochemical characteristics:

- Microscopic appearance: rods
- Mobility: mobile strain.
- Gram staining: negative staining.
- Culture conditions: 25-35°C, aeration, agitation.
- Culture in agar: colonies of mucoid appearance, pigmented (yellow).

Characterization, both biochemical and by API-test by seeding of the colonies on specific culture media, gave the results described in Table 1.

The present invention also relates to the fermentation process developed for the production of extracellular polysaccharides containing L-fucose and the subsequent isolation of 6-deoxy sugar.

The C2B2 strain is grown by inoculation in any fermentable aqueous medium containing carbon and nitrogen sources and mineral salts.

In general the source of assimilable carbon is represented by one or more sugars (for example glucose, fructose, maltose, sucrose, starch, mannitol, sorbitol, lactose, corn syrup etc.) alone or mixed. Typically the amount of sugars present in the medium is between 2 and 6 wt.%. For a preferred embodiment of the present invention, glucose is a preferred carbon source.

The nitrogen source used can be proteinaceous material of various types (for example yeast extract, soya flour, hydrolysed proteins, corn steep liquor etc.). Typically the nitrogen source is between 0.05 and 0.5 wt.% in the medium.

Various salts that are normally used in nutrient media can be used. Non-limiting examples are: phosphates, sulphates, chlorides, sodium carbonate, potassium, ammonium, calcium and magnesium.

TEST	RESULT
Oxidases	Negative
Catalases	Positive
ONPG	Positive
Arginine hydrolysis	Negative
Lysine hydrolysis	Negative
Ornithine hydrolysis	Negative
Citrate assimilation	Negative
H_2S formation	Negative
Urea hydrolysis	Negative
Indole production	Negative
Voges-Proskauer reaction	Positive
Gelatin hydrolysis	Negative
Glucose fermentation	Positive
Mannitol fermentation	Positive
Inositol fermentation	Negative
Sorbitol fermentation	Positive
Rhamnose fermentation	Positive

Sucrose fermentation	Positive
Melibiose fermentation	Positive
Amygdalin fermentation	Positive
Arabinose fermentation	Positive
Reduction of nitrates	Positive

Table 1

The fermentation can be carried out at temperatures between 25 and 35°C, preferably 30°C, at a pH between 6.0 and 7.5 maintained by means of systems for automatic correction, with aeration and agitation, for a period of 2-5 days.

The formation of L-fucose is monitored by HPLC on samples submitted beforehand to complete acid hydrolysis; macroscopically it is possible to assess the progress of fermentation on the basis of the viscosity, which, in a medium where the expected quantity of polysaccharide is being formed, reaches and even exceeds 2200 cP as described in the literature (P.T. Vanhooren et al. Med. Fac. Landbouww. Univ. Gent., vol. 62/4a, p. 1271-1276, 1997).

Fermentation can be carried out in conventional fermenters by inoculating the nutrient medium with a culture of the C2B2 strain. Prior to inoculation the nutrient medium is sterilized, for example by heat at temperatures of the order of 120°C.

At the end of the fermentation time, the culture suspension is submitted to suitable treatments for the purpose of purification and recovery of L-fucose.

Fermentation conducted according to the procedure described above always provides polysaccharides whose ^{1}H- and ^{13}C-NMR spectra comprise the following principal signals relating to the carbohydrate components (signals obtained according to the protocol described in the present invention):

1) ^{1}H-NMR signals at 1.3ppm (1.275ppm broad singlet, in Fig. 1) and ^{13}C-NMR signals at 15.9ppm (15.989ppm, in Fig. 2): these two signals are diagnostic of the presence of fucose;

2) ^{1}H-NMR signals at 5.5ppm, 5.3ppm and 5.0ppm (respectively 5.480ppm, 5.340ppm and 4.986ppm in Fig. 1) of the anomeric zone in approximate quantitative ratio of 1:2:1 to one another. These signals are compatible and attributable to anomeric signals of glycosides of the alpha type;

3) ^{13}C-NMR signals, one signal at about 104.5ppm (104.558ppm as in Fig. 3) (compatible with the presence of a beta glucuronyl glycoside) and one at about 100.0ppm (100.009ppm as in Fig. 3) (attributable to alpha glycosides).

NMR spectra (not shown) acquired for example at a temperature of 40°C also show anomeric signals at about 4.7ppm and about 4.6ppm in approx. 1:1 ratio and attributable to bonds of the beta type.

The polysaccharides obtained from fermentation of the C2B2 strain preferably have, moreover, ^{1}H-NMR signals at about 1.4ppm (broad singlet) (1.435ppm in Fig. 1) and ^{13}C-NMR signals at about 25.7ppm (25.773ppm in Fig. 2) indicative of the presence of a pyruvate group with a bridge bond between two hydroxyls.

The anomeric zone of the carbon spectrum may, moreover, preferably show from a minimum of 4 to a maximum of 6 signals, between 97 and 105ppm.

The polysaccharides obtained from fermentation of the C2B2 strain can moreover optionally also have other signals attributable to the probable presence of O-acetyl groups such as for example signals at 2.06ppm, 2.148ppm and 2.201ppm in the ^{1}H spectrum (see Fig. 1) and signals at about 20ppm (20.958ppm in Fig. 2) and at about 174ppm (173.866ppm in Fig. 2) in the ^{13}C spectrum.

As a result, therefore, one or more sugars making up the polysaccharide (or mixture of polysaccharides) can be bound to one or more pyruvate groups with a bridge bond between two hydroxyls, and moreover the polysaccharide can be in non-acetylated form or can have one or more acetyls on one or more monomers.

As an example, Fig. 1 and Fig. 2 show, respectively, the ^{1}H- and ^{13}C-NMR spectra of a polysaccharide (or mixture of polysaccharides) obtained as a result of aerobic fermentation of the C2B2 strain as described above.

The spectra were obtained using the model VXR300S NMR Spectrometer (Varian Inc., Palo Alto CA, USA).

Said polysaccharide (or mixture of polysaccharides), if submitted to acid hydrolysis by for example treatment with sulphuric acid at 50-96% (for example carried out at the reflux temperature for 30 min-10 hours) or treatment with trifluoroacetic acid (TFA) at concentrations of 10-60% at the reflux temperature for 20 min-2 hours (in the case of

TFA it is possible to work at pressures above atmospheric, for example up to about 2 atm as is obtainable in autoclave) a hydrolysis mixture is obtained consisting of the following principal monosaccharides, which can be separated by HPLC (Perkin Elmer with Transgenomic Column ICE SEP Ion 300, eluent 0.015 N sulphuric acid, column temperature: 40°C, flow: 0.4 ml/min). (see Fig. 4)

-an HPLC peak corresponding to fucose (retention time of 18.52 minutes)

-an HPLC peak corresponding to galactose (retention time of 15.82 minutes)

-an HPLC peak corresponding to glucose (retention time of 14.63 minutes)

In the case of partial hydrolysis (i.e. in milder conditions such as the conditions given in example 7 and in example 9) of the polysaccharide, a peak may also be found to be present at about 11 min retention time. This peak is called "peak at 11" (in Fig. 4 it can be seen at a retention time of 10.62 minutes). Therefore, for the purposes of the present invention, hydrolysis that gives rise to detection of the peak at 11 by HPLC is called partial hydrolysis.

Based on the NMR analysis of this isolated peak and the enzymatic treatment with β-glucuronidase it is possible to ascribe the structure of a β-glucuronyl galactoside to the aforementioned disaccharide (Z.A. Popper et al., Phytochemistry, 2003, vol 64, p. 325-335), very probably bound 1-3.

If the hydrolysis conditions have given rise to a mixture also containing the disaccharide relating to the "peak at 11", this can be removed by treatment of the partial hydrolysis mixture with ion exchange resins.

As an example, Fig. 5 shows the HPLC plot (Perkin Elmer HPLC with Transgenomic Column ICE SEP Ion 300, eluent 0.015 N sulphuric acid, column temperature: 40°C, flow: 0.4 ml/min) obtained as a result of partial acid hydrolysis. In this case the mixture obtained after hydrolysis was treated with ion exchange resins (Rohm and Haas, Amberlite IR 120 and Amberlite IRA 94S) to remove the peak at 11.

In chromatographic conditions with higher resolution (for example separating the hydrolysis mixtures of the polysaccharide with Perkin Elmer HPLC, eluent 0.015 N sulphuric acid and Transgenomic chromatographic Column ICE SEP Ion 300 thermostatically controlled at 50°C) the presence of another two chromatographic peaks may also be encountered:

- an HPLC peak corresponding to pyruvic acid (with a retention time of 16.70 minutes in Fig. 7)
- an HPLC peak corresponding to glucuronic acid (with retention time of 13.94 minutes in Fig. 7)

The aforementioned hydrolysed polysaccharide (or mixture of polysaccharides) therefore provides mixtures containing mainly L-fucose, D-glucose, D-galactose and preferably also glucuronic acid and pyruvic acid, and from these mixtures it is possible to isolate the desired L-fucose at high yields and purities.

In particular

After partial hydrolysis, the aforementioned polysaccharide has, according to HPLC analysis of the hydrolysate, preferably a molar content of glucose/galactose/fucose/pyruvic acid in the relative proportions **0.5 : 0.5 : 1 : 0.5**

After complete hydrolysis, the aforementioned polysaccharide has, according to HPLC analysis of the hydrolysate, preferably a molar content of glucose/galactose/fucose/glucuronic acid/pyruvic acid in the relative proportions of **0.5 : 1 : 1 : 0.5 : 0.5.**

The proportions of the individual components were deduced (with due approximations) principally from the NMR data (Fig. 1 with signals at 1.275ppm (fucose) and 1.435 (pyruvic acid) in the ratio 1:0.5), from the HPLC chromatograms in Fig. 4 (with calculated molar proportions between fucose (at 18.52 min), galactose (at 15.82) and glucose (at 14.63 min) in the approximate proportions 1:0.5:0.5) and in Fig. 6 (with calculated molar ratios of fucose (at 18.74 min) to glucose (at 14.81 min) in the ratio of about 1:0.5) and analysis of the isolated peak at 11 (data not shown) showing a ratio of galactose to glucuronic acid of 1:1.

It should be noted that the complete hydrolysis of the peak at 11 should cause the liberation of galactose (therefore the content of galactose in a complete hydrolysate is doubled to give a ratio of 1:1 with fucose) and the liberation of glucuronic acid to give a ratio of 0.5:1 with fucose.

Therefore the aforementioned polysaccharides are useful intermediates for preparing L-fucose.

To obtain said L-fucose, the mixture at the end of fermentation is fluidized until a viscosity below 100 cP is reached, by adding an aqueous solution of a strong acid, preferably sulphuric acid, preferably with a concentration between 30 and 70 wt.%, up to a pH between 1.5 and 4.5, preferably between 2.0 and 3.0.

The acidified mixture is then heated at temperatures between 60 and 80°C, for times between 2 and 10 hours, preferably for 4-8 hours.

The first purification step is another crucial point of the process: the polysaccharide is recovered by ultrafiltration since it remains quantitatively in the retentate together with just the cells of the microorganism while all the fermentation by-products (e.g. butanediol, lactic acid etc.) and the salts and the acid added for fluidization are removed almost quantitatively in the permeate. This step provides an excellent solution to the problem of purification of L-fucose, for obtaining a crystalline product of high purity; in fact it allows the sugars constituting the polysaccharide to be separated from all the low molecular weight by-products, for example butanediol, that cannot be removed in the steps described next, and that constitute a great problem for direct crystallization of the product, as they are present in considerable amounts.

The purification process is carried out on the retentate containing the polysaccharide according to known procedures (see for example EP102535, H. Voelskow and M. Schlingmann).

Complete hydrolysis is performed by treatment with a strong acid (for example sulphuric acid, hydrochloric acid, phosphoric acid, trifluoroacetic acid etc.). Sulphuric acid is preferred. Preferably a solution of strong acid at 30-70 wt.% is added to the retentate and it is heated at the reflux temperature for 5-10 hours.

At the end of hydrolysis the mixture is neutralized by adding a base (sodium hydroxide, calcium hydroxide etc.). Calcium hydroxide is preferred.

In a preferred embodiment of the invention, sulphuric acid and calcium hydroxide are used in combination: this leads to precipitation of $CaSO_4$, which is removed by filtration or by centrifugation, thus removing a large part of the salts present in the reaction mixture.

Selective removal of D-glucose and D-galactose and glucuronic acid from the mixture is achieved by techniques known by a person skilled in the art, for example by chromatography or, preferably, by the action of a microorganism that utilizes said carbohydrates as a carbon source for growing, but leaves the L-fucose intact. Preferably, a microorganism is used and even more preferably the solution is inoculated with *Saccharomyces cerevisiae*.

Fermentation is conducted until glucose and galactose and glucuronic acid disappear completely. The solution is then pasteurized at 60-70°C for 10-30 minutes and the yeast cells are removed from the solution of L-fucose by ultrafiltration or microfiltration, and the ultrafiltered or microfiltered solution is deionized by passing over strong cationic and weak anionic ion exchange resins in series.

The deionized solution is concentrated to a syrup and L-fucose (whose HPLC purity in the solution at this stage is above 75%) is crystallized by adding solvent, for example alcohols such as methanol, ethanol, n-propanol, isopropanol or 2-butanone.

The present invention may be better understood from the following examples.

EXPERIMENTAL SECTION

EXAMPLE 1

Isolation and purification of the C2B2 strain

The strain producing the polysaccharide containing L-fucose was isolated from 1 g of foliar homogenate of *Phalaenopsis* sp. (Orchidacea) in saline and diluted serially in the same solution to 10^{-8}.

1 ml of each dilution was seeded at depth in Petri dishes containing 15 ml of TSB medium (Trypticase Soy Broth, Biolife) solidified with 15g/l of agar and left to incubate at 30°C for three days.

The yellow pigmented colonies, clear and of large convex dimensions, that develop on the surface of the agar were then transferred to fresh TSB medium for purification by smearing them for three successive cycles, starting each time from a well isolated colony.

The purity of the strain was also verified by means of observation with the light microscope, while production of the polysaccharide was tested by flask culture on a suitable medium, verifying the increase in viscosity of the culture and determining the percentage by weight of L-fucose, by HPLC on a sample that was submitted to complete hydrolysis

Once purified, the strain was submitted to summary identification by the usual methods of biochemical characterization (API tests) and by sequencing the ribosomal RNA 16S (SEQ ID NO: 1).

The purified strain was stored in glycerol both at –20°C and at –80°C.

EXAMPLE 2

Production of L-fucose in a flask

In a 3-litre flask, 1 litre of culture medium is prepared, composed of:

- calcium carbonate 5g/l
- yeast extract 5g/l
- demineralized water to give a volume of 1 l

Sterilize the medium in autoclave at 121°C for 20 minutes. Cool and add 50 ml of a sterile solution at 50% w/v of glucose: there is a final glucose concentration of 25g/l in the medium.

Inoculate the flask with 0.5 ml of glycerolate stored at -20°C of the C2B2 strain and maintain the culture in growth in the oscillating incubator at a temperature of 30°C for 48 hours.

An aliquot of the fermentation broth was submitted to hydrolysis by adding 96% sulphuric acid in amounts to give a final solution of 1.5 M, which is then heat-treated at 100°C for 30 minutes.

HPLC analysis of the hydrolysate produces the following results (comparing only the content of glucose, galactose and fucose after hydrolysis of the fermentation broth without treatments of purification and isolation of the components)

[Glucose] = 0.09%

[Galactose] = 0.05%

[Fucose] = 0.06%

The molar proportions of Glucose:Galactose:Fucose are thus found to be 1.8 : 1 : 1.3.

EXAMPLE 3

Production of L-fucose at the pilot scale in a 250-l fermenter with feeding of glucose

In a 250-l fermenter, 110 l of culture medium is prepared, composed of:

- demineralized water 110 l
- yeast extract 550g
- calcium carbonate 550g

Sterilize the culture medium by heating it to 121°C for about 30 minutes (pressure 1.2-1.4 bar).

Cool and add 5.5 litres of a sterile solution of glucose at 50% w/v.

Inoculate with 3 litres of preferment prepared according to Example 2 and leave to stand at 30°C, stirring vigorously, blowing-in air from below, at slight pressure, monitoring the glucose concentration. When glucose is absent, add 5.5 litres of a sterile solution of glucose at 50% w/v, monitoring the pH, which must be at 7.0 ± 0.3, and the viscosity of the medium.

After 48 hours from the start of fermentation, add a further aliquot of 5.5 litres of a sterile solution of glucose at 50% w/v. Continue fermentation until the glucose disappears.

The viscosity of the medium at the end of fermentation is 2500 cP.

An aliquot of the fermentation broth is submitted to hydrolysis by adding 96% sulphuric acid in amounts to give a final solution of 1.5 M and is then heat-treated at 100°C for 30 minutes.

HPLC analysis gives the following results: (comparing only the content of glucose, galactose and fucose after hydrolysis of the fermentation broth without treatments of purification and isolation of the components)

[Glucose] = 0.58%
[Galactose] = 0.17%
[Fucose] = 0.34% (concentration in the broth= 3.4g/l)

The molar proportions of Glucose:Galactose:Fucose are found to be: 3.4 : 1 : 2.2

EXAMPLE 4

Production of L-fucose in a 20 m^3 fermenter

In a 3000-litre fermenter, 1000 litres of culture medium is prepared, which will serve as preferment, composed of:

- demineralized water 1000 l
- yeast extract 5 kg
- calcium carbonate 5 kg

Sterilize the culture medium by heating to 121°C for about 30 minutes (pressure 1.2-1.4 bar).

Cool to 35°C and add 50 litres of sterile solution of glucose at 50% w/v. Inoculate 1000 l of the culture medium in sterile conditions with 5 litres of culture prepared according to Example 2.

Once inoculated, the preferment is left at 30°C with stirring, blowing-in air from below and under slight pressure for about 24 hours.

An aliquot of the preferment is submitted to hydrolysis by adding 96% sulphuric acid as in example 2.

HPLC analysis of the hydrolysate shows the following results: (comparing only the content of glucose, galactose and fucose after hydrolysis of the fermentation broth without treatments of purification and isolation of the components)

[Glucose] = 0.28%

[Galactose] = 0.09%

[Fucose] = 0.14% = 1.4g/l

The molar proportions of Glucose:Galactose:Fucose in the preferment are found to be: 3.1 : 1 : 1.7

Prepare the 20 m^3 fermenter, charging:

- demineralized water 15 m^3
- yeast extract 75 kg
- calcium carbonate 75 kg

Sterilize the culture medium by heating to 121°C for about 30 minutes. Cool and add 750 l of a sterile solution of glucose at 50% w/v.

With 600 l of the preferment prepared according to the procedure described above, inoculate 15 m^3 of culture medium and leave to stand at 30°C, stirring vigorously, and blowing-in air from below under slight pressure.

When the glucose is no longer present, add 750 litres of a sterile solution of glucose at 50% w/v, monitoring the pH, which must be at 7.0 ± 0.3 and the viscosity of the medium.

After 48 hours from the start of fermentation, add a further aliquot of 750 litres of a sterile solution of glucose at 50% w/v.

Continue fermentation until the glucose disappears.

The fermentation takes a total of 140 hours.

At the end of fermentation an aliquot of the fermentation broth is submitted to hydrolysis by adding 96% sulphuric acid as in example 2.

HPLC analysis of the hydrolysate shows the following results: (comparing only the content of glucose, galactose and fucose after hydrolysis of the fermentation broth without treatments of purification and isolation of the components)

[Glucose] = 0.27%
[Galactose] = 0.10%
[Fucose] = 0.18%

The molar proportions of Glucose:Galactose:Fucose are found to be 2.7 : 1 : 2.0

The fermentation is then stopped and the ferment is fluidized by adding 50% sulphuric acid to pH = 2.8.

EXAMPLE 5

Recovery, purification and crystallization of L-fucose

15 m^3 of ferment obtained from Example 4 (containing 28 kg of L-fucose) is ultrafiltered for the purpose of separating and concentrating the polysaccharide.

315 kg of 50% sulphuric acid is added to the retentate from ultrafiltration, maintaining the suspension under reflux for 8 hours in a suitable reactor, for complete hydrolysis of the polysaccharide.
After it has been brought back to room temperature, the suspension is adjusted to pH 5.5-6.5, by adding 180 kg of calcium hydroxide.

The precipitate of calcium sulphate that forms after adding calcium hydroxide is removed by centrifugation.
The supernatant is then adjusted to 37°C, and inoculated with 2 kg of lyophilized yeast *Saccharomyces cerevisiae*.
After about 18 hours the glucose and galactose have been consumed.
The solution is then pasteurized at 70°C for 30 minutes and ultrafiltered.

The ultrafiltration permeate is deionized on strong cationic and weak anionic ion exchange resins in series.

At this point the solution is treated with nanofiltration membrane (membrane SR2 Kock Membrane Systems, Milan) and the permeate is then concentrated by evaporation under vacuum to 75°Brix.

The fucose is crystallized by adding 140 l of ethanol: the mixture is heated with stirring to dissolve the syrup in the solvent and is then cooled to crystallize the fucose. After 72 hours the solid is filtered in vacuum and is washed with ethanol. Once dried, the crystalline fucose weighs 22.7 kg (yield = 81%) and has HPLC titre > 99.5% and purity in HPLC area > 99.9%.
Analysis by ^{1}H and ^{13}C NMR and measurement of the rotatory power (value obtained for [alpha] D at 20°C in water, at equilibrium = -75.0° ± 2) confirmed that the product isolated is L-fucose (6-deoxy-L-galactose).

EXAMPLE 6

Isolation of the polysaccharide or mixture of polysaccharides obtained from fermentation of the C2B2 strain and analysis by ^{1}H- and ^{13}C-NMR spectrometry

The fermentation of the C2B2 strain was carried out as in example 4.
30% sulphuric acid was added to the fermentation broth until a pH of 2.9 was reached.
The solution was maintained at room temperature for about 48 hours and then a further amount of 50% sulphuric acid was added to it until a pH of 1.5 was reached.
The solution was then concentrated and diafiltered by ultrafiltration with a polymer membrane with a molecular cut-off of 10 000 Da.
2 litres of retentate were centrifuged at 13 000 rpm for 20 minutes at 20°C and the supernatant was filtered on a dicalite panel and on a filter with porosity of 0.8 μm. The filtration permeate was neutralized to pH 6.9 by adding 32% sodium hydroxide, diafiltered against demineralized water and concentrated by tangential ultrafiltration with

a spiral-wound membrane of 20 000 Da, and finally dried to constant weight in a rotary evaporator (Rotovapor, Buchi).

To 100 mg of sample, dissolved in 0.75 ml of deuterated water, 4µl of acetonitrile was added as internal standard (^{1}H-NMR δ=2.060ppm; ^{13}C-NMR δ=1.470ppm).

The ^{1}H-NMR spectra were recorded on a model VXR300S Varian spectrometer (Varian Inc., Palo Alto CA, USA) with the following settings:

sw (Hz)	4000
tof (Hz)	700
at (s)	4
pw (µs)	pw90
d_1 (s)	10
gain	16
nt	40
lb	0.12
temp (°C)	25

The ^{1}H-NMR spectrum obtained is shown in Fig. 1.

The ^{13}C-NMR spectra were recorded on a model VXR300S Varian spectrometer (Varian Inc., Palo Alto CA, USA) with the following settings:

sw (Hz)	20000
tof (Hz)	0
at (s)	1
pw (µs)	pw90
d_1 (s)	1
gain	32
nt	≥ 10000
lb	5÷20

temp (°C)	25

The ^{13}C-NMR spectra obtained are shown in Fig. 2 and Fig. 3 (where only the anomeric region of the spectrum is presented)

EXAMPLE 7

Purification and partial hydrolysis of the polysaccharide or mixture of polysaccharides obtained from fermentation of the C2B2 strain and analysis by HPLC chromatography

The fermentation of the C2B2 strain was carried out as in example 4.

2.5 litres of fermentation broth, diluted with an equal volume of demineralized water, was centrifuged at 13 000 rpm for 20 minutes at room temperature.

Glacial acetic acid was added to the supernatant until a final concentration of 2% was reached. The sample was then heated at 100°C for 1 hour.

After neutralization with sodium hydroxide to pH 6.5, the sample was centrifuged again at 13000 rpm for 30 minutes and, after dilution 1:1 with water, it was diafiltered against demineralized water and concentrated by tangential ultrafiltration with a spiral-wound membrane with molecular cut-off of 20000 Da.

The concentrate was dried to constant weight in a rotary evaporator (Rotovapor, Buchi).

5 grams of sample was treated with trifluoroacetic acid (TFA) to a final concentration of 16% in autoclave (2 atm) at 121°C for 20 minutes. The solution obtained was exchanged with 100 ml of demineralized water by means of the rotary evaporator (Rotovapor, Buchi) 5 times.

The sample was then submitted to HPLC chromatographic analysis using the Perkin Elmer series 200 HPLC chromatograph, chromatographic column Transgenomic ICE-SEP ION 300 equipped with similar precolumn and refractive index detector with thermostatically controlled cells.

Separation was performed with 0.015 N sulphuric acid as mobile phase and with the column thermostatically controlled to a temperature of 40°C.

The chromatogram is shown in Fig. 4. In addition to the characteristic peaks of monosaccharides obtained after hydrolysis, a peak is seen at 10.62 minutes (so-called peak at 11) (see printout below).

Peak #	Time [min]	Component Name	Concentration %	Area [µVs]	Response factor	Amount [Norm. %]	Area [%]	Delta RT [%]
1	9.848		-7.05e-95	705035	-1.00e+100		4.17	----------
2	10.621	Peak at 11 min	0.374831	3222068	8.5961e+06	18.5	19.08	1. 4789
3	11.765		0.006163	52981	8.5961e+06	0.3	0.31	2.2966
4	11.953		0.008062	69300	8.5961e+06	0.4	0.41	-2.4059
5	12.728	GlucNa	0.051417	441982	8.5961e+06	2.5	2.62	-0.5640
6	13.319		-1.24e-95	123790	-1.00e+100		0.73	----------
7	13.614		0.119755	375802	3.1381e+06	5.9	2.23	-0.6280
8	14.627	Glucose	0.367912	2957295	8.0381e+06	18.2	17.51	-0.0256
9	15.819	Galactose	0.457796	3758336	8.2096e+06	22.6	22.25	-0.0703
10	18.523	Fucose	0.623248	5122742	8.2194e+06	30.8	30.33	-0.0487
11	20.336		0.006238	19575	3.1381e+06	0.3	0.12	0.8710
12	22.168		0.003998	11220	2.8068e+06	0.2	0.07	-2.3443
13	25.862		-2.07e-98	207	-1.00e+100		0.00	----------
14	27.863		0.003977	28951	7.2791e+06	0.2	0.17	-4.1787
15	34.852		-4.35e-99	43	-1.00e+100		3e-04	----------
			2.023395	16889328		100.0	1e+02	

However, the chromatogram given in Fig. 5 shows the HPLC plot obtained in the same conditions on the same sample after elimination of the peak at 11 by passing over ion exchange resins.

In this case the sample obtained after partial hydrolysis was in fact treated with a pair of ion exchange resins Amberlite IR 120 (Rohm and Haas) and Amberlite IRA 94S (Rohm and Haas) to remove the peak at 11 minutes.

(see printout below)

Peak #	Time [min]	Component Name	Concentration %	Area [µVs]	Response factor	Amount [Norm. %]	Area [%]	Delta RT [%]
1	10.608	Peak at 11 min	0.003264	28053	8.5961e+06	0.3	0.30	1.3523
2	12.023		0.026459	227440	8.5961e+06	2.1	2.42	-1.8368
3	12.685	*GlucNa*	0.043995	378183	8.5961e+06	3.6	4.03	-0.8983
4	13.565		0.139027	436280	3.1381e+06	11.2	4.64	-0.9885
5	14.641	Glucose	0.299055	2403819	8.0381e+06	24.2	25.59	0.0689
6	15.835	Galactose	0.326105	2677203	8.2096e+06	26.4	28.50	0.0338
7	18.537	Fucose	0.392409	3225376	8.2194e+06	31.8	34.33	0.0219

8	20.328		0.005591	17544	3.1381e+06	0.5	0.19	0.8350
			1.235903	9393900		100.0	1e+02	

EXAMPLE 8

Purification and complete hydrolysis of the polysaccharide or mixture of polysaccharides obtained from fermentation of the C2B2 strain and analysis by HPLC chromatography

The fermentation of the C2B2 strain was carried out as in example 4.

30% sulphuric acid was added to an aliquot of the fermentation broth to obtain a final pH of the solution of 3.0.

The solution obtained was ultrafiltered by filtration on a ceramic filter with cut-off of 300 000 Da (0.05 micron) and the permeate was concentrated using a nanofiltration membrane (SR2 membrane).

The polysaccharide was then precipitated with methanol (final concentration methanol:water of 3:1).

The precipitate was finally filtered on a paper filter, washed with a solution of methanol:water 3:1 and dried in a stove at 40°C for 8 hours.

100 mg of sample was treated with trifluoroacetic acid (TFA) at a final concentration of 50% in autoclave (2 atm) at 121°C for 60 minutes.

The sample was then submitted to HPLC chromatographic analysis using the Perkin Elmer series 200 HPLC chromatograph, chromatographic column Transgenomic ICE-SEP ION 300 equipped with similar precolumn and refractive index detector with thermostatically controlled cells.

Separation was performed with 0.015 N sulphuric acid as mobile phase and with column thermostatically controlled to a temperature of 40°C.

The chromatogram is shown in Fig. 6 (where the so-called peak at 11 is practically absent).

(see printout below)

Peak #	Time [min]	Component Name	Concentration %	Area [µVs]	Response factor	Amount [Norm. %]	Area [%]
1	10.774	Peak at 11 min	0.012626	20626	9.0508e+06	0.7	0.79
2	11.949		0.001294	2114	9.0508e+06	0.1	0.08
3	12.914	Glucuronic	0.137319	171335	6.9130e+06	7.5	6.55
4	14.806	Glucose	0.320998	484912	8.3698e+06	17.5	18.54
5	16.015	Galactose	0.670134	1044233	8.6335e+06	36.4	39.93
6	17.357		0.002600	4027	8.5828e+06	0.1	0.15
7	18.742	Fucose	0.474430	725582	8.4736e+06	25.8	27.74
8	20.399		0.022124	12531	3.1381e+06	1.2	0.48
9	22.446		0.122837	62229	2.8068e+06	6.7	2.38
10	26.291		0.013841	7840	3.1381e+06	0.8	0.30
11	28.231		0.060735	79793	7.2791e+06	3.3	3.05
			1.838938	2615221		100.0	

EXAMPLE 9

Purification and partial hydrolysis of the polysaccharide or mixture of polysaccharides obtained from fermentation of the C2B2 strain and analysis by HPLC chromatography with column thermostatically controlled to 50°C

The fermentation of the C2B2 strain was carried out as in example 4.

50% sulphuric acid was added to an aliquot of the fermentation broth to obtain a final pH of the solution of 1.5.

The solution was then heated at 97°C for 6 hours.

After cooling to room temperature the solution was neutralized to pH 5.0 by adding calcium hydroxide and centrifuged at 13 000 rpm for 20 minutes.

The sample was then submitted to HPLC chromatographic analysis using the Perkin Elmer series 200 HPLC chromatograph, chromatographic column Transgenomic ICE-SEP ION 300 equipped with similar precolumn and refractive index detector with thermostatically controlled cells.

Separation was performed with 0.015 N sulphuric acid as mobile phase and with column thermostatically controlled to a temperature of 50°C.

The chromatogram is shown in Fig. 7.

(see printout below)

Peak #	Delta. RT [%]	Rel. RT	Time [min]	Component Name	Conc. %	Area [µVs]	Response factor	Amount [Norm. %]	Norm. Area [%]	% s u FCS 100
1	----------	0.505	10.223		-1.30e-95	64529	-1.00e+100		0.5	-2e-93
2	0.13	0.571	11.547	Peak at 11 min	0.514156	2371725	9.3169e+06	17.5	19.9	68.64
3	----------	0.586	11.867		-1.20e-95	59290	-1.00e+100		0.5	-2e-93
4	----------	0.627	12.679		-1.17e-95	58152	-1.00e+100		0.5	-2e-93
5	0.19	0.689	13.943	Glucuronic	0.062781	210634	6.7765e+06	2.1	1.8	8.38
6	----------	0.715	14.470		-3.01e-95	148896	-1.00e+100		1.3	-4e-93
7	----------	0.763	15.440		-8.62e-96	42673	-1.00e+100		0.4	-1e-93
8	-0.02	0.795	16.086	Glucose	0.641156	2599037	8.1875e+06	21.8	21.9	85.59
9	-0.08	0.825	16.700	NaPYR	0.223483	504902	4.5632e+06	7.6	4.2	29.83
10	0.01	0.856	17.320	Galactose	0.522698	2121997	8.1997e+06	17.8	17.8	69.78
11	0.20	0.909	18.398	Galactitol	0.028974	116411	8.1150e+06	1.0	1.0	3.87
12	----------	0.960	19.436		-5.98e-96	29627	-1.00e+100		0.2	-8e-94
13	0.01	1.000	20.237	Fucose	0.749082	3033722	8.1799e+06	25.5	25.5	100.00
14	-0.24	1.059	21.436		0.018087	73250	8.1799e+06	0.6	0 6	2.41
15	0.09	1.150	23.276		0.000946	3106	6.6278e+06	0.0	0.0	0.13
16	0.13	1.287	26.035		0.131079	259132	3.9929e+06	4.5	2.2	17.50
17	----------	1.403	28.397		-6.55e-97	3242	-1.00e+100		0.0	-9e-95
18	----------	1.444	29.228		-6.04e-96	29925	-1.00e+100		0.3	-8e-94
19	0.12	1.534	31.048		0.025082	82704	6.6598e+06	0.9	0.7	3.35
20	0.13	1.598	32.342		0.024077	75815	6.3599e+06	0.8	0.6	3.21
					2.941602	11888768		100.0	100.0	

5

CLAIMS

1. An isolated microbial strain, belonging to *Enterobacteriaceae* family, comprising an rRNA 16S sequence containing a sequence having homology with SEQ. ID NO: 1 equal or greater than 93%; said bacterial strain being able to produce extracellular polysaccharides containing L-Fucose, when subjected to aerobic fermentation.
2. A microbial strain according to claim 1, comprising an rRNA 16S sequence containing a sequence having homology with SEQ. ID NO: 1 equal or greater than 98%.
3. A microbial strain according to claim 2, wherein the rRNA 16S sequence comprises SEQ. ID No: 1.
4. A bacterial strain according to claim 3 deposited at DSMZ under the registration number DSM 22227.
5. A derived or mutated microbial strain obtainable from a bacterial strain according to any one of the claims 1-4, by selecting spontaneously mutated and isolated strains or by selecting strains mutated by the action of mutagenic factors such as UV or X rays, or by the action of chemicals such as ozone, nitrous acid, N-methyl-N-nitro-N-nitrosoguanidine (NTG) or ethane methane sulfonate (EMS); said derived or mutated strain being able to produce extracellular polysaccharides containing L-Fucose, when subjected to aerobic fermentation.
6. A derived or mutated microbial strain according to claim 5, comprising an rRNA 16S sequence containing a sequence having homology with SEQ. ID NO: 1 equal or greater than 98%.
7. Extracellular polysaccharides containing L-fucose, obtainable through aerobic fermentation from any one of the microbial strains according to any one of the claims 1-6.
8. Polysaccharides according to claim 7 containing D-Glucose, D-Galactose, L-Fucose and preferably glucuronic acid and pyruvic acid as well.
9. Polysaccharides according to any one of the claims 7-8, wherein ^{1}H- and ^{13}C-NMR spectra comprise the following characteristic signals:

1. ^{1}H-NMR: 1.3 ppm, 5.0 ppm, 5.3 ppm and 5.5 ppm;
2. ^{13}C-NMR: 15.9 ppm, 100.0 ppm and 104,5 ppm.

10. Use of a polysaccharide or mixture of polysaccharides according to any one of the claims 7-9 for preparing L-fucose.

11. A process for producing at least one polysaccharide according to any one of the claims 7-9, said process comprising the aerobic fermentation of an inoculum of a microbial strain according to any one of the claims 1-6 in any fermentable aqueous medium containing carbon and nitrogen sources and mineral salts; said fermentation conducted at temperatures from 25 and 35°C, and pH from 6.0 and 7.5, with aeration and stirring, over a period of 2-7 days.

12. A process for producing L-Fucose, said process comprising the hydrolysis of at least one polysaccharide according to any one of the claims 7-9.

13. A process according to claim 12, said process further comprising the aerobic fermentation according to claim 11.

14. A process according to claim 13 comprising the following steps:
    a. inoculation into any fermentable aqueous medium containing carbon and nitrogen sources and mineral salts;
    b. fermentation of the mixture obtained from step (a), at temperatures from 25 and 35°C, and pH from 6.0 and 7.5 with aeration and stirring, over a period of 2-7 days;
    c. adding an aqueous solution of strong acid up to pH from 1.5 to 4.5 for fluidifying the mixture obtained from step (b) to obtain a viscosity lower than 100 cps;
    d. heating the mixture obtained from step (c), at temperatures from 60 to 80°C, over time periods from 2 to 10 hrs;
    e. ultrafiltration of the mixture obtained from step (d) to obtain a retentate containing at least one polysaccharide containing L-fucose;
    f. hydrolysis of the retentate, obtained from step (e), by treating with a strong acid, preferably H2SO4, at reflux temperature in an aqueous solvent, to obtain a mixture mainly containing D-Glucose, D-Galactose and L-Fucose;
    g. neutralization of the mixture obtained from step (f), by adding a base,

preferably Ca(OH)2;

h. filtration or centrifugation to eliminate possible precipitated salts followed by a further neutralization carried out as step (g);

i. selectively eliminating Glucose and Galactose from the mixture obtained from step (h), by means of known techniques to obtain a mixture essentially containing L-Fucose;

j. deionization by means of strong cation exchange resins and weak anion exchange resins arranged in series,

k. isolating L-Fucose.

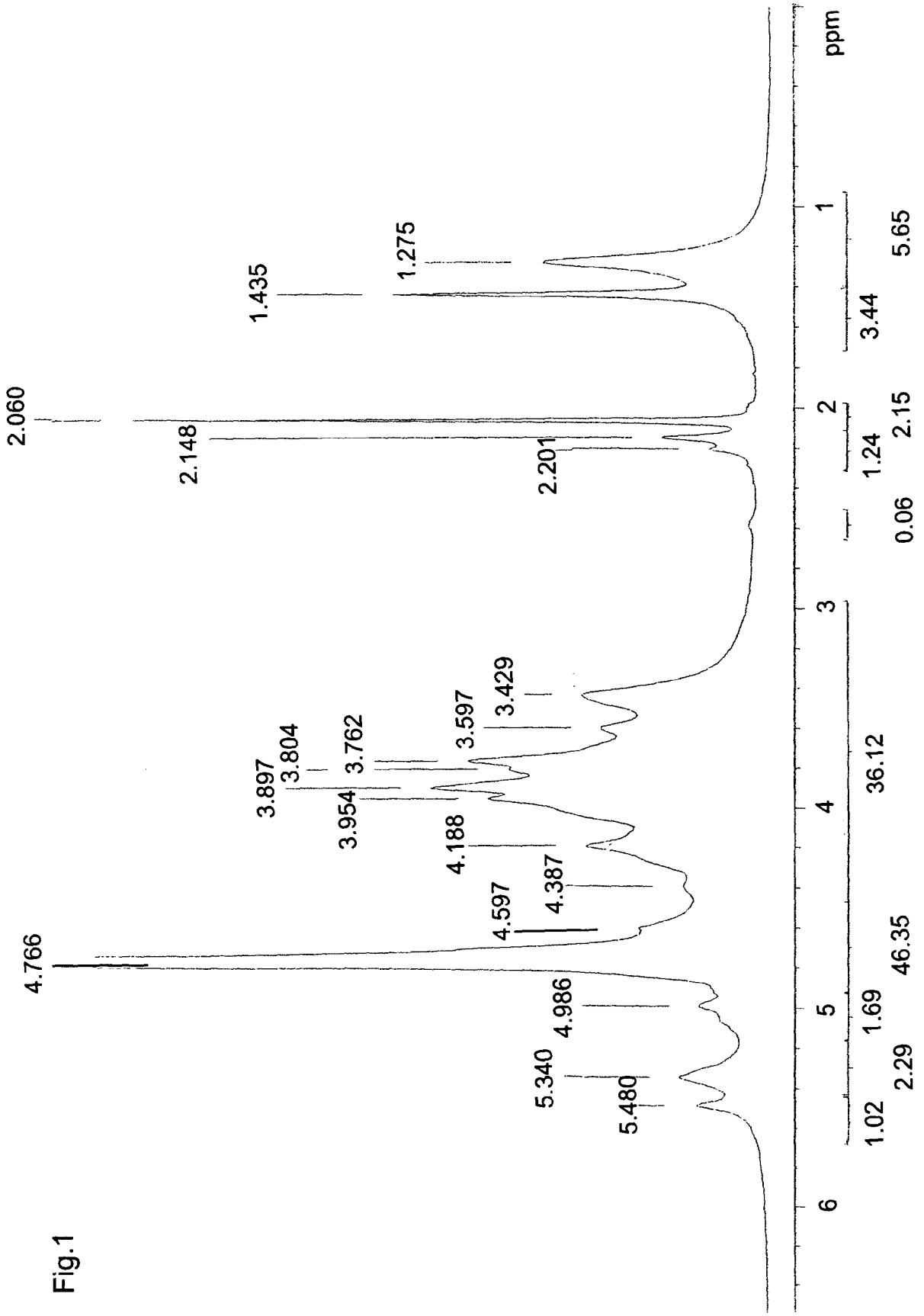
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Fig.1

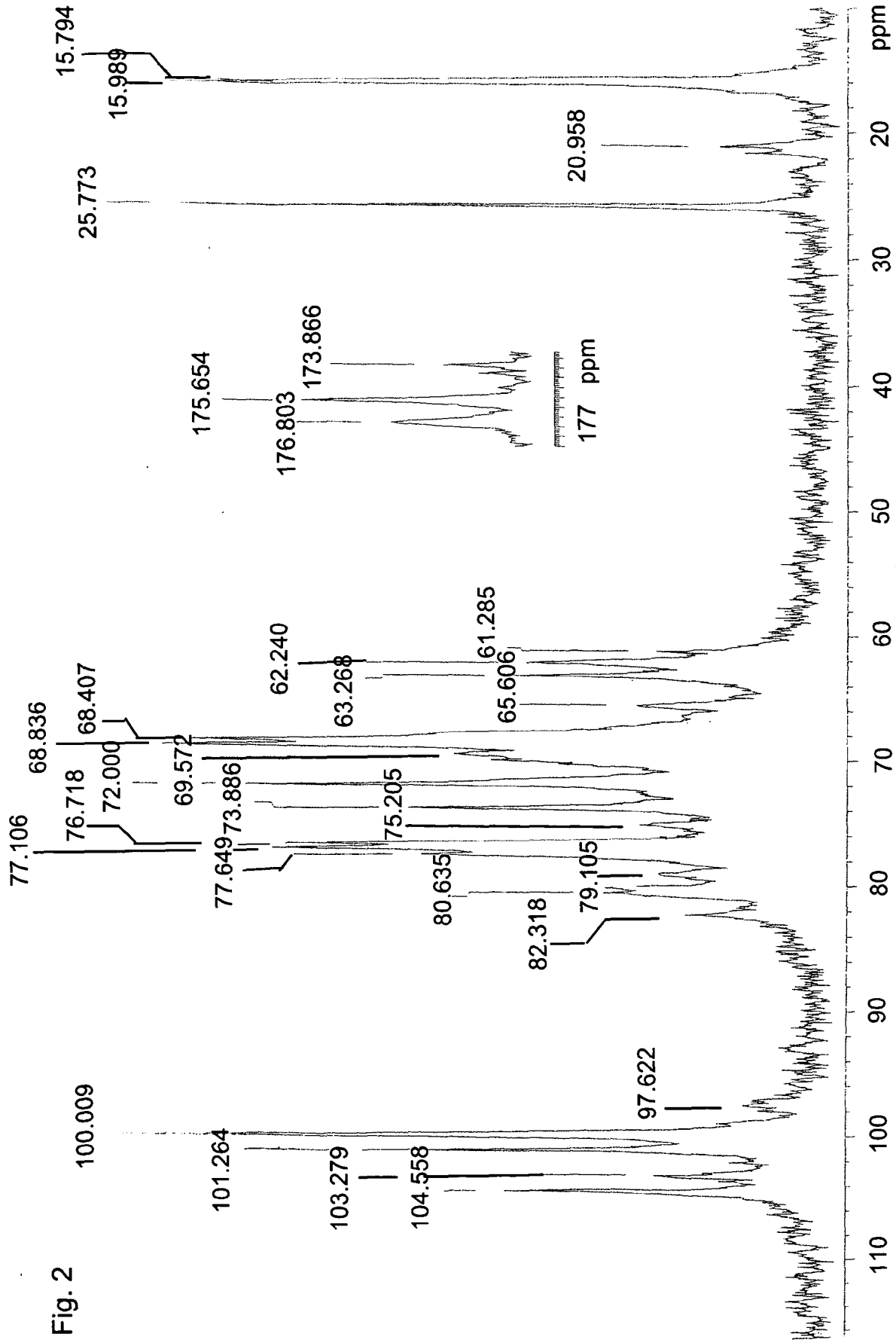
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Fig. 2

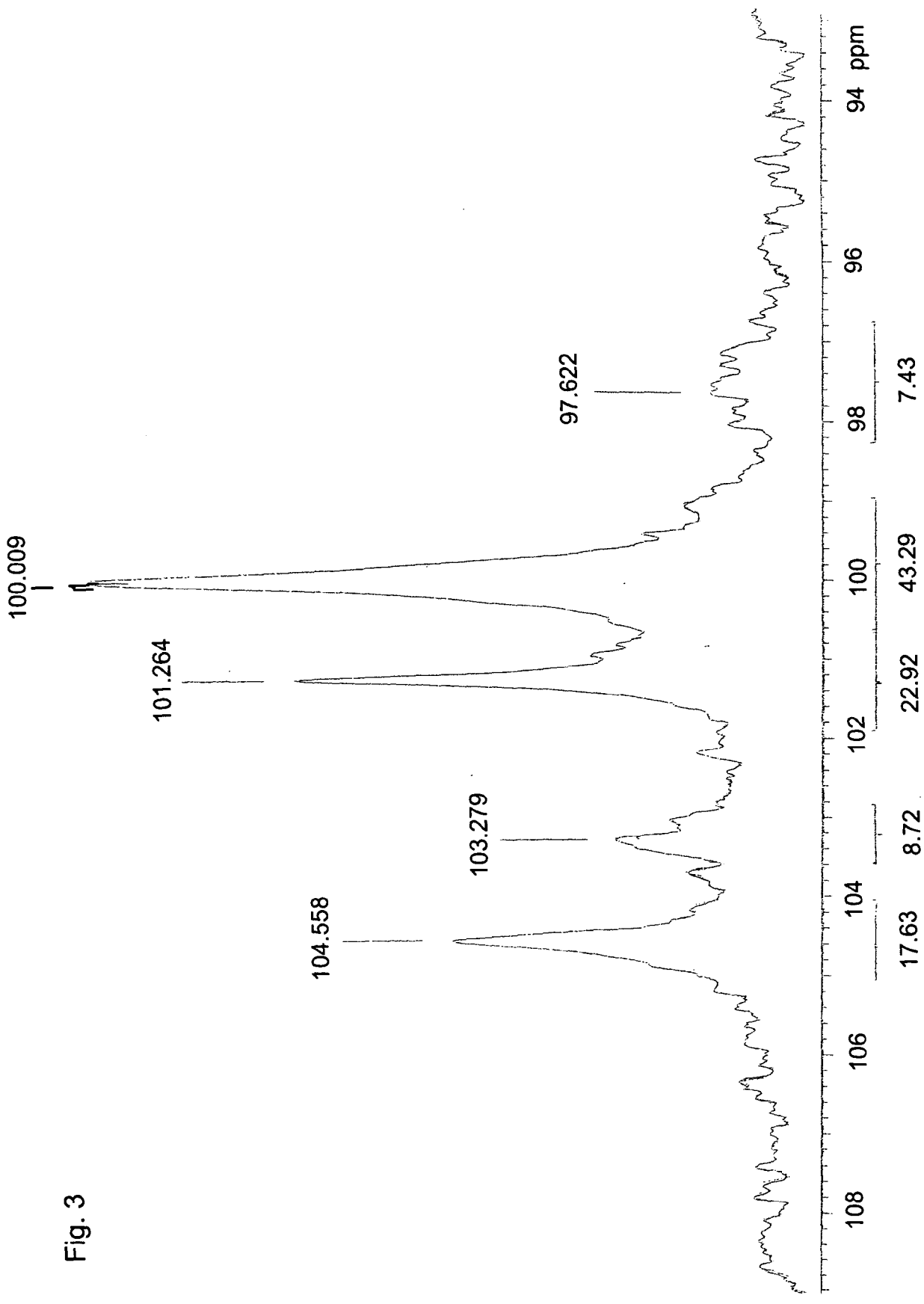
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Fig. 3

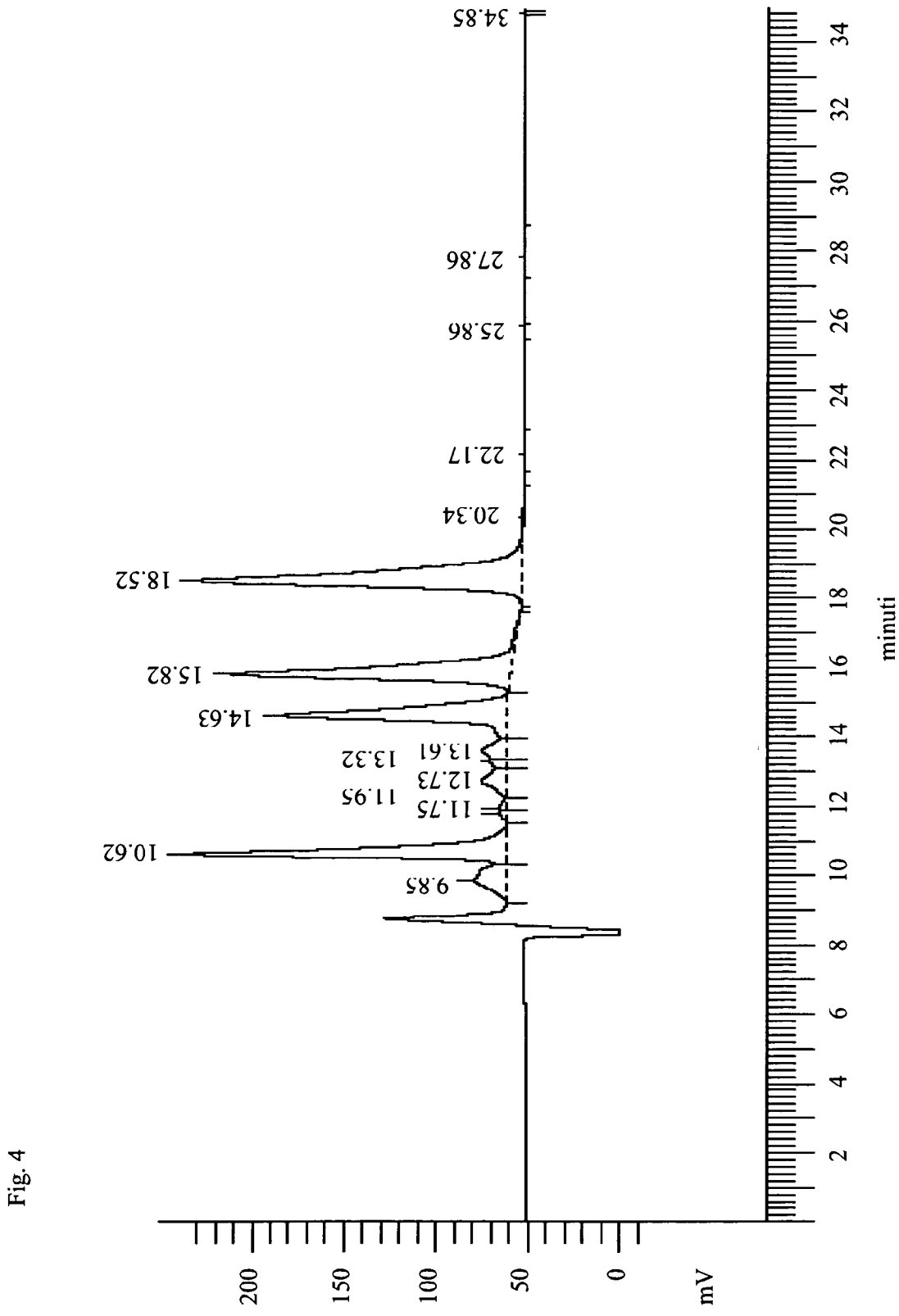
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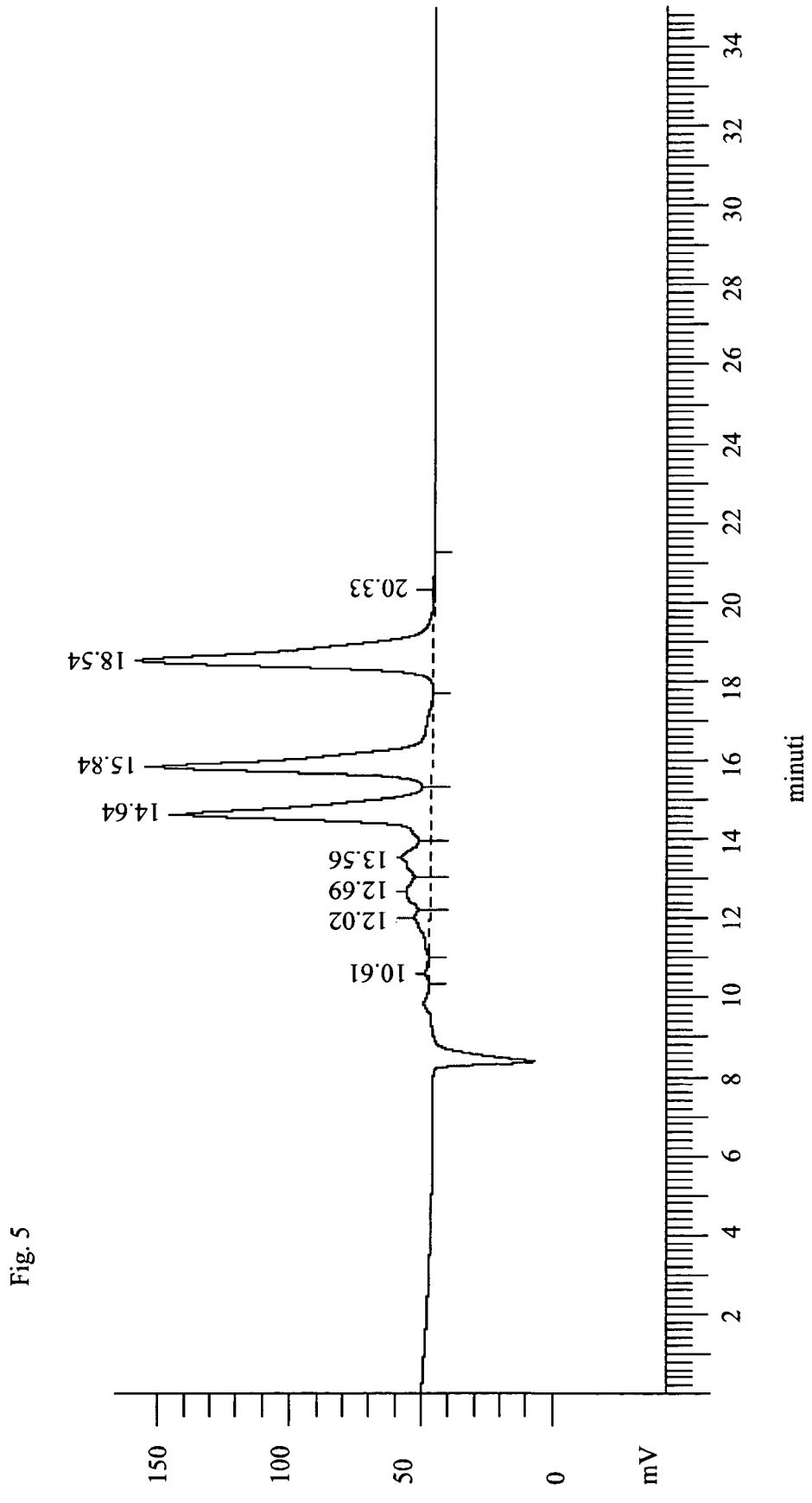
Fig. 4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32
34
minuti

Fig. 5

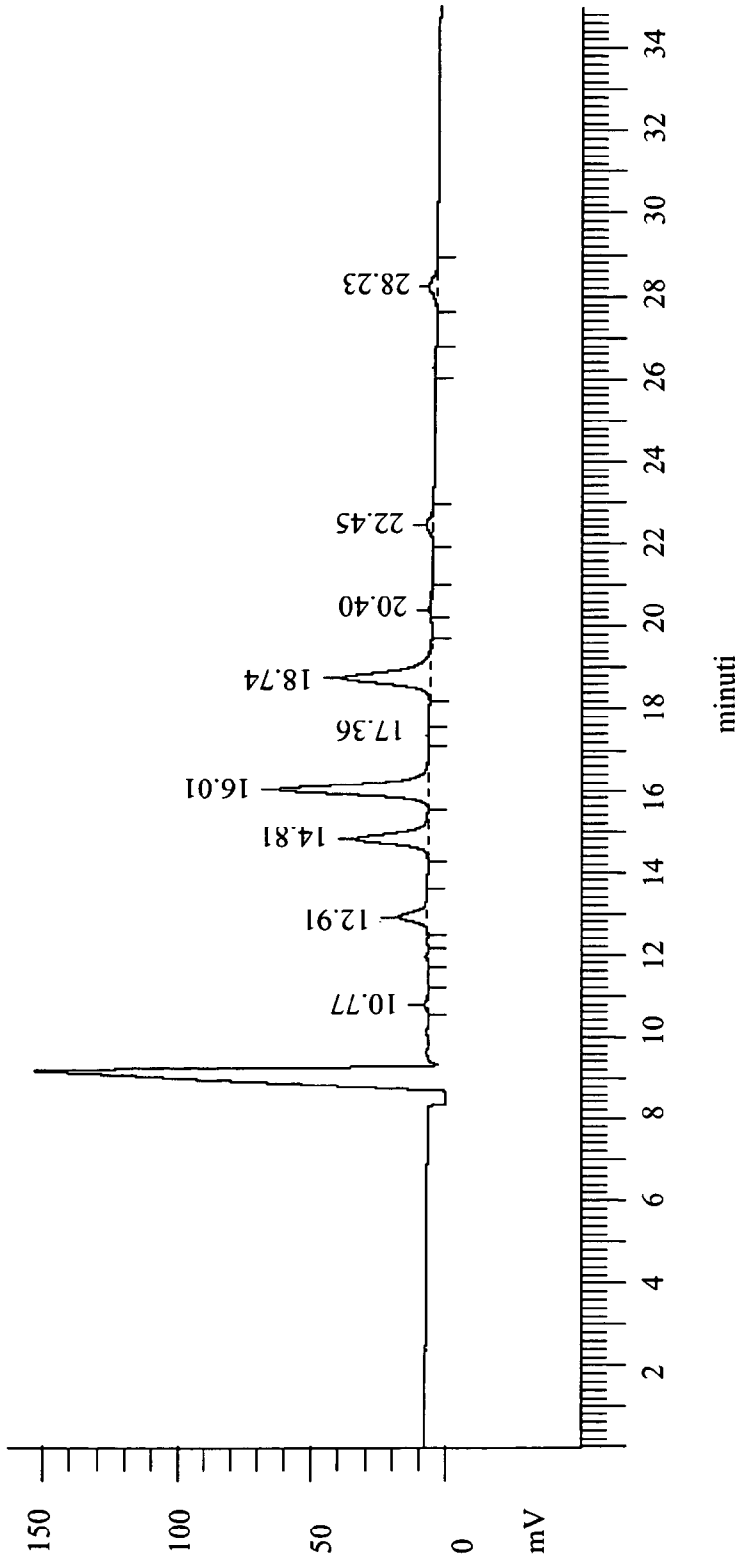
10.77
12.91
14.81
16.01
17.36
18.74
20.40
22.45
28.23
150
100
50
0
mV
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
minuti

Fig. 6

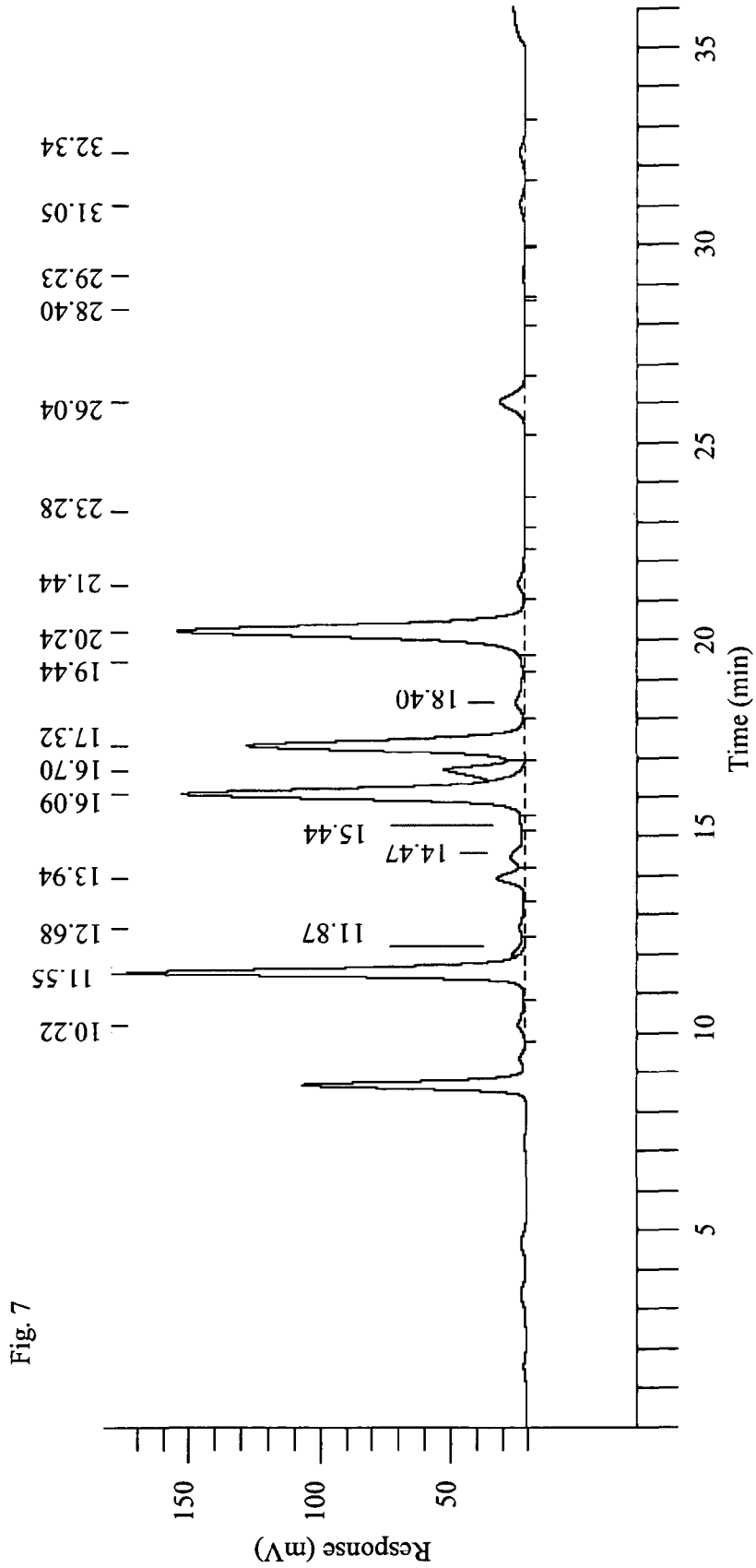
Response (mV)
150
100
50
Time (min)
5
10
15
20
25
30
35
10.22
11.55
12.68
13.94
16.09
16.70
17.32
19.44
20.24
21.44
23.28
26.04
28.40
29.23
31.05
32.34
11.87
14.47
15.44
18.40

Fig. 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/065825

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P19/04 C13K13/00 C12P1/04 C12R1/01 C08B37/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P C12R

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, EMBASE, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VANHOOREN P T ET AL: "L-FUCOSE: OCCURRENCE, PHYSIOLOGICAL ROLE, CHEMICAL, ENZYMATIC AND MICROBIAL SYNTHESIS", JOURNAL OF CHEMICAL TECHNOLOGY AND BIOTECHNOLOGY, BLACKWELL SCIENTIFIC PUBLICATIONS. OXFORD, GB, vol. 74, no. 6, 1 June 1999 (1999-06-01), pages 479-497, XP000860689, ISSN: 0268-2575 page 490, left-hand column, line 1 - page 491, left-hand column, paragraph 2; table 3	1,5,7, 10,12

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

[X] See patent family annex.

* Special categories of cited documents :

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

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"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 November 2011	06/12/2011

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/065825

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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X	RÄTTÖ M ET AL: "Colanic acid is an exopolysaccharide common to many enterobacteria isolated from paper-machine slimes", JOURNAL OF INDUSTRIAL MICROBIOLOGY & BIOTECHNOLOGY ; OFFICIAL JOURNAL OF THE SOCIETY FOR INDUSTRIAL MICROBIOLOGY, SPRINGER, BERLIN, DE, vol. 33, no. 5, 1 May 2006 (2006-05-01), pages 359-367, XP019357800, ISSN: 1476-5535, DOI: 10.1007/S10295-005-0064-1 the whole document	5,7,8,11
X	FR 2 840 920 A1 (BIOCHIMIE APPLIQUEE SOC [FR]) 19 December 2003 (2003-12-19) the whole document	5,7,8,11
X	FR 2 842 201 A1 (PROBEST [FR]) 16 January 2004 (2004-01-16) examples I, II	5,7,8,11
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/065825

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 2011/073874 A2 (73100 SETENTA E TRES MIL E CEM LDA [PT]; CARVALHO FERNANDES DE MIRANDA) 23 June 2011 (2011-06-23) SEQ ID No:1 = NAGENESEQ:AZJ10846;; page 29, last line - page 31, line 2; examples 1,3; sequence 1 -----	1,2,5-8, 11

INTERNATIONAL SEARCH REPORT

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International application No
PCT/EP2011/065825

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