Title: BIOTECHNOLOGICAL PRODUCTION OF CHONDROITIN

Abstract: Chondroitin is produced by culturing a recombinant microorganism which is obtained by inactivation of a gene encoding an enzyme responsible of fructose residues addition to the linear chondroitin polysaccharide in a microorganism producing a fructosylated derivative of chondroitin.

Fig. 3
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Object of the Invention
The present invention relates to a novel recombinant microorganism producing chondroitin and a method for the biotechnological production of chondroitin.

In the present invention the term chondroitin indicates a linear polysaccharide defined as a linear glycosaminoglycan constituted by alternating residues of D-glucuronic acid (GlcUA) and N-acetyl-D-galactosamine (GalNAc) bound by β1-3 (GlcUA → GalNAc) and β1-4 (GalNAc → GlcUA) bonds.

Background of the invention
Chondroitin sulphate is a glycosaminoglycan in which glucuronic acid (GlcUA) and a N-acetyl-D-galactosamine (GalNAc) are linearly and alternatively bound by β1-3 linkage and β1-4 linkage respectively to from a linear polysaccharide chain that is sulphated to varying degrees in its GalNAc residues.

It is present in animals, in cartilages and connective tissue, playing an important role in cell adhesion, tissue regeneration, nerve extension and the like. The production of chondroitin from non-animal sources is an important and desirable step towards the production of non animal-derived chondroitin sulphate.

The available patent literature describes several methods for the production of non animal-derived chondroitin.

In addition, several chondroitin synthase genes, derived from both animals and microorganisms, have been cloned and sequenced.

A method for producing chondroitin has been provided by using a recombinant Gram-positive Bacillus bacterium introduced with a chondroitin synthase gene derived from Pasteurella multocida (US 2007/0281342 A1).

A further invention describes a method for producing chondroitin by introducing both the kfoC and kfoA genes, derived from Escherichia coli O5:K4:H4, into a UDP-glucuronic acid-producing bacterium (WO2008/133350).

Another invention describes an in vitro chondroitin synthesis in an enzymatic system comprising both the Escherichia coli O5:K4:H4 chondroitin synthase and the precursors of reaction (US2009/0263867 A1).

It is known that Escherichia coli O5:K4:H4 is able to produce a capsular polysaccharide (the K4 polysaccharide) having the same backbone structure as that of chondroitin, to which fructose residues are linked to the GlcUA residues...
(see e.g. N. Volpi, Glycoconj. J. (2008) 25:451-457). Therefore, the K4 polysaccharide consists of a repeating trisaccharide unit comprising a D-glucuronic acid (GlcUA) moiety and an N-acetyl-D-galactosamine (GalNAc) moiety linked by a β1-3 (GlcUA → GalNAc) and a fructose residue bound to the C3-hydroxyl group of the GlcUA residue. The fructose residues thus constitute branches of the resulting linear polysaccharide.

Removal of the fructose residues has been achieved by hydrolytic treatment in acid conditions (N. Volpi, Electrophoresis 25, 692-696 (2004)). Although both the Escherichia coli 05:K4:H4 capsule antigen gene cluster and the metabolic pathways leading to the sugars constituting the K4 linear polysaccharide have been extensively studied, the glycosyl-transferase activity responsible for the addition of the fructose moieties to the linear polysaccharide to give the K4 polysaccharide, has so far not been identified.

The novel feature of the present invention is the direct production of high-molecular weight chondroitin by a recombinant microorganism obtained by inactivating a gene encoding an enzyme responsible for the addition of fructose residues to the chondroitin back-bone thus obviating the need to submit the K4 polysaccharide to the hydrolytic removal of the fructose residues bound to the GlcUA moieties to obtain chondroitin.

**Detailed description of the Invention**

An object of the present invention is to provide a recombinant microorganism producing chondroitin, defined as a linear glycosaminoglycan consisting of alternating residues of D-glucuronic acid (GlcUA) and N-acetyl-D-galactosamine (GalNAc) bound by β1-3 linkage and β1-4 linkage respectively, characterized in that in said microorganism a gene encoding an enzyme responsible for the addition of fructose residues to the chondroitin back-bone is inactivated.

Therefore, according to an aspect of this invention it is provided a recombinant microorganism producing chondroitin characterized in that said microorganism is obtained by subjecting a gene originally present therein, which encodes a protein responsible for the addition of fructose residues to the linear chondroitin back-bone, to inactivation, said inactivation including deletion or substitution entirely or part of said gene or disruption by insertion of an additional nucleotide sequence.

According to a preferred aspect of this invention, the recombinant microorganism of the present invention obtained by inactivation of the gene encoding a protein
having a fructosyl-transferase activity is derived from a bacterium that belongs to the species *Escherichia coli*, and preferably belongs to the group 2 of the K antigen that includes well-known serotypes such as K1, K5, K7, K12. Although, according to a representative embodiment of this invention, the recombinant microorganism having the ability to produce chondroitin is derived from *Escherichia coli* 05:K4:H4, any of the microorganisms belonging to the K antigen group, irrespective of whether they share any gene homology with *Escherichia coli* 05:K4:H4, can be employed. Examples of said microorganisms include bacteria belonging to the genera *Haemophilus* such as *H. influenzae* (Branefors-Helander P., Carbohydr. Res., 1981, Jan 15, 88), *Campylobacter* such as *C. jejuni* (McNally DJ, Jarrell HC, Li J, Khieu NH, Vinogradov E, Szymanski CM, Brisson JR., FEBS J. 2005 Sep;272), *Gloeocapsa* such as *G. Gelatinosa* (Raungsomboon S, Chidthaisong A, Bunnag B, Inthorn D, Harvey NW., Water Res. 2006 Dec;40) and *Vibrio* such as *Vcholerae* (Knirel YA, Widmalm G, Senchenkova SN, Jansson PE, Weintraub A-Eur J Biochem, 1997, Jul 1, 247).

More preferably, the bacterium from which the recombinant microorganism of this invention producing chondroitin is derived is *Escherichia coli* serotype 05:K4:H4, which contains the *kfoE* gene, encoding a protein having a fructosyl-transferase activity.

The *kfoE* gene is known to be located within the *E.coli* K4 antigen gene cluster (GenBank AB079602) that contains genes found by the inventors to possess a significant homology with genes from other microorganisms, which are likely to be involved in bacterial capsule production (*T. Ninomiya, N.Sugiura, A. Tawada, K.Sugimoto, H. Watanabe and K. Kimata-JBC, 2002, vol. 277, June 14, N°24, 21567-75).

The bacterium most preferably used to obtain the recombinant microorganism of the present invention is *Escherichia coli* 05:K4:H4, strain U1-41, available from ATCC (American Type Culture Collection, Manassas-Virginia, US) under the accession number ATCC23502.

According to a representative embodiment of this invention the recombinant microorganism is a microorganism producing chondroitin wherein the gene subjected to inactivation is a gene coding for a protein selected from the group consisting of the following (A), (B), (C):
(A) a protein comprising the amino acid sequence of SEQ ID N°2

(B) a protein comprising the amino acid sequence of SEQ ID N°2 modified by deletion, substitution, or insertion of one or more amino acids, and having a fructosyl-transferase activity.

(C) a protein comprising an amino acid sequence having homology of at least 50% with the amino acid sequence of SEQ ID N°2 and having a fructosyl-transferase activity.

The microorganism according to this invention is a microorganism wherein the inactivated gene is the kfoE gene or a DNA selected from the group consisting of the following (a), (b), (c):

(a) a DNA comprising the nucleotide sequence of SEQ ID N°1

(b) a DNA that hybridizes with a DNA comprising the nucleotide sequence complementary to SEQ ID N°1 and encodes a protein having a fructosyl-transferase activity.

(c) a DNA comprising a nucleotide sequence having a homology of at least 50% with the nucleotide sequence of SEQ ID N°1 and encoding a protein having a fructosyl-transferase activity.

An object of the present invention is a microorganism producing chondroitin wherein kfoE inactivation is obtained by modification of its nucleotide sequence, such as by deleting or substituting, entirely or in part, the nucleotide sequence described under (a), (b) or (c) above.

Another object of the present invention is a microorganism wherein kfoE inactivation is obtained by inserting, one or more nucleotides, into the nucleotide sequence described under (a), (b) or (c) above.

According to a most preferred aspect of this invention, the recombinant derivative of Escherichia coli 05:K4:H4 strain Δ1-41 (from now on referred to as E.coli K4) is obtained by inactivation of the kfoE gene, encoding a putative fructosyl-transferase, by means of nucleotide deletion.

The present invention discloses how the disruption of the kfoE gene leads to the direct production of the K4 polysaccharide lacking the fructose residues, i.e. of chondroitin.

According to a further preferred aspect of this invention, the recombinant E.coli K4 of the present invention is obtained by using a method to disrupt chromosomal
genes in which PCR primers provide the homology to the targeted gene \cite{Datsenko and Wanner, PNAS 2000 N 2 vol. 97, 6640-6645}.

The recombinant \textit{E. coli} K4 strain of the present invention has been subjected to the inactivation of the chromosomal \textit{kfoE} gene first by substituting most of its nucleotide sequence with an exogenous kanamycin resistance gene ("first genetic modification") and then by deleting the inserted gene using a FLP recombinase expression vector ("second genetic modification").

The recombinant \textit{E. coli} K4 strain obtained after the first genetic modification, referred to as \textit{E. coli} K4 (AkfoE/kan$^R$) has been deposited on April 30, 2010 at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstrasse 7B, 38124 Braunschweig, Germany, according to the Budapest Treaty, under the accession number DSM23578.

The recombinant \textit{E. coli} K4 strain obtained after the second genetic modification, referred to as \textit{E. coli} K4 (AkfoE) has been deposited on May 26, 2010 at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstrasse 7B, 38124 Braunschweig, Germany, according to the Budapest Treaty, under the accession number DSM23644.

The inactivation of the \textit{kfoE} gene was achieved by means of 3 successive bacterial transformations firstly with the Red Recombinase expression plasmid (pKD46), secondly with a DNA fragment derived from a template plasmid (pDK4) suitably modified to provide homology with the \textit{kfoE} gene and thirdly with the helper plasmid expressing the enzyme FLP recombinase (pCP20).

In order to obtain the first genetic modification of \textit{E. coli} K4, both the pKD46 plasmid (GenBank: AY048746) and the linear DNA fragment have been used.

The pKD46 plasmid, used in the first step of \textit{E. coli} K4 transformation consists of 2,154 nucleotides from phage \textit{lambda} and of the gene encoding resistance to ampicillin. This plasmid promotes an enhanced rate of recombination when using linear DNA fragments.

The linear DNA fragment used in the subsequent transformation of \textit{E. coli} K4 has been obtained by PCR using several pairs of primers that include homology extensions to both the \textit{kfoE} gene and the template plasmid pKD4 carrying a kanamycin resistance cassette (GenBank: AY048743).

This procedure was able to generate a linear DNA fragment carrying a kanamycin resistance cassette, having the \textit{kfoE} 5' and 3' homologous termini at its ends.
In one embodiment of this invention, bacterial transformation was effected by electroporation that was selected due to its ability to generate easily double transformants that could be recovered from plates containing both ampicillin and kanamycin.

However, although electroporation is the preferred technique, this result could be achieved by any known transformation method such as calcium chloride transformation or dextran-DEAE transformation.

With the aim to verify the correct location of the substitution of the original DNA sequence with the kanamycin resistance cassette in the transformants of E.coli K4 (AkfoE/kanR), several PCR amplifications have been performed, using 2 nearby locus-specific primers pairs: the first primers pair was able to demonstrate the formation of a new junction between kfoE remaining 5’ terminus and the inserted kan gene, while the second primer pair was able to demonstrate the formation of a new junction between the inserted kan gene and kfoE remaining 3’ terminus.

The helper plasmid used to remove the kanamycin resistance cassette ("second genetic modification") was plasmid pCP20, carrying the yeast FLP recombinase gene and an ampicillin resistance gene. Both pKD46 and pCP20 plasmids are temperature-sensitive vectors that were subsequently removed from transformant strains of E.coli K4 following growth at 43°C.

A sequencing analysis has been performed on E.coli K4 (AkfoE/kanR) to confirm the substitution of the kfoE gene, wholly or in part, with the kanamycin resistance cassette. Likewise, a sequencing analysis has been performed on E.coli K4 (AkfoE) to verify the subsequent deletion of the kanamycin resistance cassette, resulting in the final production of the AfoE-disrupted bacterial strain.

The method used for the successful construction of a recombinant E. coli K4 capable of producing a non-glycosylated variant of a natural glycosaminoglycan is of general applicability and can be advantageously applied to other glycosylated products where it is desirable to prevent such glycosylation. In conclusion, a general method was developed for obtaining microorganisms capable of producing non-glycosylated variants of natural glycosaminoglycans.

Another object of the present invention is to provide a method for the biotechnological production of chondroitin comprising the following steps:

1) culturing in a suitable medium the recombinant microorganism
and

(2) recovering the chondroitin produced from its broth culture.

Any recombinant microorganism capable of producing chondroitin obtained according to the method described above to inactivate a gene encoding an enzyme responsible of fructose residues addition to chondroitin may be used in the culturing step.

According to a preferred embodiment of this invention, a recombinant bacterium obtained from E.coli K4 such as E. coli DSM23644, is employed as the recombinant microorganism having the ability to directly produce chondroitin.

The method adopted for the cultivation of the bacterium E. coli DSM23644 is a general method applicable to the cultivation of members of the genus Escherichia. Said method is based on the preferred, but not exclusive, use of a cultivation medium containing per liter: $\text{K}_2\text{HPO}_4 \ 3\text{H}_2\text{O} \ 9.7 \text{ g}$, $\text{KH}_2\text{PO}_4 \ 8 \text{ g}$, sodium citrate $5\text{H}_2\text{O} \ 0.5 \text{ g}$, $\text{MgCl}_2 \ 7\text{H}_2\text{O} \ 0.2 \text{ g}$, casamino acids $20 \text{ g}$, $(\text{NH}_4)_2\text{SO}_4 \ 20 \text{ g}$, glucose $2 \text{ g}$, yeast extract $10 \text{ g}$. Higher levels of chondroitin production can be achieved by suitably modifying the composition of the basal medium and/or providing further nutrients to the culture by means of substrate feeds.

Culture conditions are defined in order to maximize bacterial growth and chondroitin production. Typically, cultivation is carried out at temperatures between $25^\circ\text{C}$ and $40^\circ\text{C}$ for $8 \text{ h}$ to $72 \text{ h}$.

The supernatant is collected preferably by centrifugation and used for the subsequent purification and characterization of the chondroitin produced.

Chondroitin purification was achieved according to an adaptation of the methods described by Rodriguez and Jann (Eur.J.Biochem. 117, 117-124, FEBS 1988).

Briefly, the method adopted to purify chondroitin is based on several steps of precipitation starting from the culture supernatant and a final drying under vacuum. The identity of the product recovered can be ascertained by a number of methods, preferably by a combination of methods providing evidence of the structure of the polysaccharide chain and of the absence of fructose residues.

The absence of fructose from the purified product can be advantageously verified by means of an acid hydrolysis of the product, in conditions known to release fructose from native K4 polysaccharide, followed by a specific assay for any fructose released as a consequence. This test consistently showed that the
polysaccharide recovered from cultures of the bacterium *E. coli* DSM23644 contains no fructose, in contrast with the native K4 polysaccharide obtained from the *Escherichia coli* 05:K4:H4 strain u1-41 that consistently produced a polysaccharide containing fructose in the amounts expected from the structural formula of the K4 polysaccharide.

A further confirmation of the identity of the product recovered from the cultures of the bacterium *E. coli* DSM23644 was obtained by subjecting the product to digestion with the enzyme Chondroitinase ABC, which is known to completely degrade to disaccharides the fructose-free chondroitin polysaccharide, but not the native K4 polysaccharide. In other words, Chondroitinase ABC is unable to digest the native K4 polysaccharide. Chondroitinase ABC digestion experiments of the product recovered from cultures of the bacterium *E. coli* DSM23644 yielded the amounts of the disaccharide product expected from a complete digestion, thus confirming the nature of the polysaccharide backbone and in particular the absence of fructose residues.

According to one embodiment of the present invention, to confirm the function of *kfoE* as the gene coding for the K4 fructosyl-transferase activity, a recombinant plasmid carrying wild type *kfoE* nucleotide sequence has been constructed and introduced into *E. coli* K4 strain (AkfoE) to mediate the complementation of lost function.

Briefly, *kfoE* gene has been amplified and cloned into pTrcHis plasmid (Invitrogen Corporation, 5791 Van Allen Way PO Box 6482 Carlsbad, California) within Ncol and BamHI restriction sites. The construct pTrcHis-*kfoE* has been used to transform by electroporation the recombinant *E. coli* (AkfoE) and the transformants have been selected at 37°C, on plates containing 100 µg/mL ampicillin.

*E. coli* (AkfoE) transformants carrying the construct pTrcHis-cfoE have been cultured and K4 polysaccharide purified according to Rodriguez and Jann (*Eur.J.Biochem. 117, 117-124, FEBS 1988*) and in order to quantify the fructose present in the product recovered, free fructose was determined both before and after hydrolysis with 0.2 M trifluoroacetic acid for 1 h at 99 °C. Free fructose assayed before and after hydrolysis has been taken as the fructose bound to the starting K4 molecule.
The product recovered from the culture of *E. coli* DSM23644 transformed by pTrcHis-/cfoE showed the presence of bound fructose, confirming that in this strain the loss of fructosyl-transferase activity was complemented by plasmid.

**Description of the drawings.**

Fig. 1 shows schematically the genetic modifications to which *Escherichia coli* 05:K4:H4 strain U1-41 was subjected resulting in the construction of *E. coli* K4 (AkfoE/kanR) and *E. coli* K4 (AkfoE):

a) DNA fragment carrying a kanamycin resistance cassette (kanamycin), flanked by two FRT (Flippase Recognition Targets) recombination sequences; the kanamycin resistance gene is derived from pKD4 plasmid template by using P1 and P2 priming sites.

b) Detailed structure of the K antigen gene cluster of *E. coli* 05:K4:H4 strain U1-41, where kfoD and kfoF are the flanking genes of kfoE.

c) *E. coli* K4 (AkfoE/kanR) chromosomal DNA showing the disruption of the kfoE gene by substituting a fragment of original DNA with the kanamycin resistance gene (kanamycin).

d) *E. coli* K4 (AkfoE) chromosomal DNA showing the final deletion of most of the kfoE gene.

Fig. 2 shows the results of PCR amplification carried out on 3 *E. coli* K4 (AkfoE/kanR) transformants to verify the sequence of the 3' and 5' kfoE remaining flanking regions:

- Lanes 1 and 10 show the molecular weight marker (1 Kb ladder);
- Lanes 2-4: PCR product of residual kfoE 3' terminus of the 3 transformants;
- Lanes 6-8: PCR product of residual KfoE 5' terminus of the 3 transformants;
- Lanes 5 and 9: PCR product of *Escherichia coli* 05:K4:H4 strain U1-41 obtained by using the 3' and 5' pairs of primers respectively.

Fig. 3 shows a chromatogram of the polysaccharide produced by *E. coli* DSM23644 analyzed by Capillary Electrophoresis after digestion with Chondroitinase ABC, where the unsaturated Δ-disaccharide (Adi-0S), typical of chondroitin digestion with Chondroitinase ABC is shown (peak 8).

Fig. 4 shows a 13C-NMR spectrum of the chondroitin produced by *E. coli* DSM23644 obtained according to example 3.

**Examples**

**Example 1:**
Construction of *E. coli* K4 (*AkfoE/karP*) strain

The construction of the linear DNA fragment (Fig. 1a) carrying both *kan*\(^R\) gene and *kfoE* homologous termini was achieved by PCR using the pKD4 vector as a template and the following PCR primers:

- **OL1 51**: atgtcttaataatgtctggttcctatgttcaacaagaatgtgtaggctggagcttc (SEQ ID N°3)
- **OL1 52**: tcatactgcagcctccttaaaaatttcatataatctaaatgcacatatgaatatcctcct (SEQ ID N°4)

In each oligonucleotide sequence, the first 40 nucleotides provide *kfoE* gene homology and the remaining 20 nucleotides provide pKD4 template plasmid homology (P1 and P2 priming sites).

PCR was performed on 120 ng of template DNA according to the following conditions:

- 94°C x 3 min, (94°C x 1 min, 40°C x 1 min, 68°C x 2 min) x 5 cycles, (94°C x 1 min, 59°C x 1 min, 68°C x 2 min) x 30 cycles, 68°C x 10 min, 4°C x 10 min.

The PCR product was gel-purified and the bacteria were transformed.

*Escherichia coli* 05:K4:H4 strain U1-41 (Fig. 1b) was prepared and transformed by electroporation with the pKD46 plasmid according to Datsenko and Wanner (PNAS, June 2000, vol 97, N°12, 6640-6645) then plated onto ampicillin-containing medium.

Ampicillin-resistant transformants were identified and isolated.

Two transformants were verified by plasmid extraction and PCR using the following primers and conditions:

- **OL1 49**: ccactcataaatcctcatagag (SEQ ID N°5)
- **OL1 50**: ccacttacttctgtacaacgat (SEQ ID N°4)

at 94°C x 3 min, (94°C x 1 min, 43°C x 1 min, 68°C x 2.5 min) x 30 cycles, 68°C x 10 min, 4°C x 10 min

The PCR product was analyzed by 0.8% agarose gel electrophoresis and a product with a size of 1799 base pairs was identified, in complete accordance with the expected product size.

One of the two pKD46 transformants was submitted to a subsequent electroporation, using the DNA fragment carrying both the kanamycin resistance cassette and the *kfoE* homologous termini.
Plate selection on media containing both ampicillin and kanamycin was used to isolate recombinants carrying the substitution of most of kfoE nucleotide sequence with the kanamycin resistance gene. Three double transformants were verified by PCR amplification of both kfoE 3' and 5' flanking regions, using the appropriate following primers:

- OL1 53: aatccgacgggaccttagatt (SEQ ID N°7)
- OL1 42: aacctgttgcaggctcaag (SEQ ID N°8)
- OL1 43: gcttttcccctgtccagat (SEQ ID N°9)
- OL1 54: gctaatgtatatgattgccaggt (SEQ ID N°10)

At 95°C x 5 min, (94°C x 1 min, 47°C x 1 min, 68°C x 2 min) x 30 cycles, 68°C x 10 min, 4°C x 10 min

PCR products were analyzed by 0.8% agarose gel electrophoresis and two products with a size of 1773 base pairs for 3' terminus amplification and 769 base pairs for 5' terminus amplification of kfoE gene respectively were identified, in complete accord with the expected products size (Fig. 2).

In order to verify the orientation of the kanamycin resistance gene and to ensure the correct direction of gene transcription, a further analysis of transformants was carried out by sequencing analysis of E. coli K4 (AkfoE/kanR) (Fig. 1c), using the following oligonucleotides:

- OL1 53: aatccgacgggaccttagatt (SEQ ID N°7)
- OL1 54: gctaatgtatatgattgccaggt (SEQ ID N°10)

The resulting nucleotide sequence is identified by SEQ ID N°14.

**Example 2:**

**Construction of E. coli K4 (AkfoE) strain**

In order to obtain the E. coli K4 strain (AkfoE) lacking the kanamycin resistance cassette and carrying a deletion of most of the kfoE gene with the attendant loss of function, a further transformation of E. coli K4 strain (AkfoE/kanR) with the pCP20 plasmid was performed.

After the electroporation step, the transformants were selected on media containing ampicillin at 30°C and then colony purified.

Putative transformants were grown on non-selective plates at 43°C and then tested for loss of all antibiotic resistances.

E. coli K4 strain (AkfoE) transformants were verified by sequencing of both kfoE flanking 3' and 5' remaining termini (Fig. 1d), using the following oligonucleotides:
Example 3:

Cultivation of *E.coli* DSM23644 and chondroitin analysis

Cultivation of *E.coli* DSM23644 was carried out according to Rodriguez and Jann (Eur.J.Biochem. 117, 117-124, FEBS 1988).

Briefly, vegetative stage of culture was realized starting from 0.5 ml of thawed culture stock, inoculating a flask containing 20 ml of broth culture consisting per liter of: 9.7 g K$_2$HPO$_4$, 3H$_2$O, 8 g KH$_2$PO$_4$, 0.5 g sodium citrate 5H$_2$O, MgCl$_2$, 7H$_2$0 0.2 g, casamino acids 20 g, ammonium sulphate 20 g, glucose 2 g, yeast extract 10 g, incubated at 37°C for 16h, with shaking at 180 rpm and 2.5 cm of displacement.

The subsequent cultivation stage was carried out in batch culture, in a 500 ml-baffled flask containing 85 ml of broth culture as described above, inoculated with 0.05% of vegetative culture prepared as described above and incubated at 37°C for 48 h with shaking at 180 rpm and 25 cm of displacement.

At the end of the incubation the culture was harvested by centrifugation and the supernatant was subjected to purification in order to isolate and characterize the produced chondroitin.

Chondroitin purification was achieved according to an adaptation of the methods described by Rodriguez and Jann (Eur.J.Biochem. 117, 117-124, FEBS 1988).

Briefly, the polysaccharide was precipitated from the culture supernatant by Cetavlon (alkyl-trimethylammonium bromide, CAS N°71 92-88-3), extracted with NaOH 0.5M at 3°C, neutralized and subsequently purified by 3 cycles of precipitation with 80% ethanol.

A final step of purification was carried out with 90% cold phenol pH 6.8 to precipitate contaminating proteins thus recovering the aqueous phase by centrifugation. The purified chondroitin was recovered from the aqueous phase by precipitation with 80% ethanol and drying under vacuum.

Several analytical approaches were used to ascertain the nature of the chondroitin produced.
The first approach was based on the presence or absence of fructose in the product recovered from the culture after acid hydrolysis carried out with 0.2M trifluoroacetic acid for 1 h at 99°C. In order to quantify the fructose present in the product recovered, free fructose was determined both before and after hydrolysis. Fructose was assayed enzymatically using the EnzyPlus Sucrose/D-Glucose/D-Fructose kit supplied by BIOCONTROL (BioControl Systems Inc. 12822 SE 32nd Street Bellevue, WA 98005, United States). The difference between the free fructose present after hydrolysis and that present before hydrolysis was taken as the fructose bound to the starting K4 molecule. The product recovered from the culture of *E.coli* DSM23644 showed no presence of bound fructose, confirming that this strain produces a fructose-free polysaccharide. The absence of bound fructose from the polysaccharide recovered from cultures of *E.coli* DSM23644 as described above was confirmed by enzyme digestion with Chondroitinase ABC. It was further demonstrated that the purified chondroitin when digested with Chondroitinase ABC yielded the unsaturated Δ-disaccharide (Adi-0S) typical of chondroitin digestion as confirmed by Capillary Electrophoresis (CE), using the Micellar Electrokinetic Chromatography (MECK) technique (Fig.3). The confirmation of the Adi-0S structure was obtained by the use of the appropriate Δ-disaccharide reference standard (equivalent electrophoretic elution). The quantitative determination of the Adi-0S obtained was achieved by means of an external calibration curve.

Finally, the purified chondroitin polysaccharide produced by *E.coli* DSM23644 was characterized by C\(^{13}\) NMR (Fig.4). This technique showed that the product in question was spectrally identical with the product obtained after the removal of fructose from the native K4 polysaccharide by acid hydrolysis. **Example 4:**

**Plasmid-mediated complementation of kfoE function.**

In order to verify the function of *kfoE* as the gene coding for the K4 fructosyltransferase activity, a recombinant plasmid carrying wild type *kfoE* nucleotide sequence was constructed and introduced into *E. coli* K4 strain (AkfoE) to mediate the complementation of lost function.
The pTrcHis plasmid (4400 bp - Invitrogen Corporation, 5791 Van Allen Way PO Box 6482 Carlsbad, California) was used to introduce the amplified and gel-purified kfoE gene (1569 bp) into suitable cloning sites.

70 ng of pTrcHis vector digested by Ncol and BamHI restriction enzymes and 75 ng of kfoE gene having compatible PciI/BamHI digested ends were subjected to a ligation reaction at 25°C for 15 min. Then 50 µL of Escherichia coli DH5a competent cells (Invitrogen Corporation, 5791 Van Allen Way PO Box 6482 Carlsbad, California) were electroporated with 5 µL of ligation mixture and five transformants were selected at 37°C, on plates containing 100 µg/mL ampicillin.

After colony purification, the constructed plasmid pTrcHis-kfoE was extracted and digested by Mfe I restriction enzyme, which was able to cut the DNA construct within the inserted kfoE sequence.

By means of gel electrophoresis analysis, 3 of 5 transformants after Mfe I digestion showed the expected length of 5887 bp and the sequences analysis confirmed the correct insertion of kfoE gene.

The verified pTrcHis-kfoE construct was used to transform by electroporation the recombinant E.coli DSM23644 and transformants were selected on plates containing 100 µg/mL ampicillin.

Selected transformants were cultured according to the conditions described in Example 3 and K4 polysaccharide was purified according to Rodriguez and Jann (Eur.J.Biochem. 117, 117-124, FEBS 1988).

In order to quantify the fructose present in the product recovered, free fructose was determined both before and after hydrolysis with 0.2 M trifluoroacetic acid for 1 h at 99 °C. Fructose was assayed enzymatically using the EnzyPlus Sucrose/D-Glucose/D-Fructose kit.

The difference between the free fructose present after hydrolysis and that present before hydrolysis was taken as the fructose bound to the starting K4 molecule.
The product recovered from the culture of *E.coli* DSM23644 transformed by pTrcHis-/cfoE showed the presence of bound fructose, confirming that in this strain the loss of fructosyl-transferase activity was complemented by plasmid.
CLAIMS

1. A recombinant microorganism producing chondroitin, defined as a linear glycosaminoglycan constituted by alternating residues of D-glucuronic acid (GlcUA) and N-acetyl-D-galactosamine (GalNAc) bound by β1-3 (GlcUA → GalNAc) and β1-4 (GalNAc → GlcUA) bonds, characterized in that in said microorganism a gene encoding an enzyme responsible for the addition of fructose residues to the linear chondroitin backbone is inactivated.

2. A recombinant microorganism producing chondroitin according to claim 1 wherein a gene originally present which encodes a protein responsible for the addition of fructose residues to the linear chondroitin backbone is inactivated, by deletion or substitution entirely or in part of said gene or disruption by insertion of an additional nucleotide sequence.

3. A recombinant microorganism according to any one of claims 1 and 2 wherein the inactivated gene is a gene encoding for a protein responsible for the addition of fructose residues to the linear chondroitin backbone selected from the group consisting of:
   (A) a protein comprising the amino acid sequence of SEQ ID N°2;
   (B) a protein comprising the amino acid sequence of SEQ ID N°2 modified by deletion, substitution, or insertion of one or more amino acids, and having a fructosyl-transferase activity; and
   (C) a protein comprising an amino acid sequence having homology of at least 50% with the amino acid sequence of SEQ ID N°2 and having a fructosyl-transferase activity.

4. A recombinant microorganism according to any one of claims 1 to 3 wherein the inactivated gene is the kfoE gene or a DNA selected from the group consisting of:
   (a) a DNA comprising the nucleotide sequence of SEQ ID N°1;
   (b) a DNA that hybridizes with a DNA comprising the nucleotide sequence complementary to SEQ ID N°1 and encodes a protein having a fructosyl-transferase activity; and
   (c) a DNA comprising a nucleotide sequence having a homology of at least 50% with the nucleotide of SEQ ID N°1 and encoding a protein having a fructosyl-transferase activity.
5. A recombinant microorganism according to any of claims 3 and 4 wherein the gene is inactivated by deletion or substitution entirely or in part of its nucleotide sequence, or insertion of one or more nucleotides into its nucleotide sequence.

6. A recombinant microorganism according to claim 5 wherein the inactivated gene is the *kfoE* gene.

7. A recombinant microorganism of claim 6 wherein the *kfoE* gene is inactivated by entire or partial substitution thereof with a kanamycin resistance cassette and its subsequent removal, resulting in the entire or partial deletion of the *kfoE* gene.

8. A recombinant microorganism according to any of claim 1 to 7, which is derived from a bacterium belonging to a genus selected from *Escherichia*, *Haemophilus*, *Campylobacter*, *Gloeocapsa* and *Vibrio*.

9. A recombinant microorganism according to any one of claims 1 to 8, wherein said microorganism is derived from a bacterium belonging to the species *Escherichia coli*.

10. A recombinant microorganism according to claim 9, which is derived from a serotype of the species *Escherichia coli* belonging to the group 2 of K antigen.

11. A recombinant microorganism according to claim 10, which is derived from *Escherichia coli* 05:K4:H4 u1-41 strain.

12. A recombinant microorganism according to any of claims 1 to 11 which is *Escherichia coli* DSM23644.

13. A method for biotechnological production of chondroitin, comprising the following steps:
   a. cultivating in a suitable medium the recombinant bacterium of any of claims 1 to 12
   b. recovering and purifying the chondroitin present in the microbial culture.

14. A method for biotechnological production of chondroitin according to claim 13 where the recombinant microorganism is *Escherichia coli* DSM23644.


16. A biotechnologically produced chondroitin of claim 15 obtainable by the method of claim 13 where the recombinant microorganism is *Escherichia coli* DSM 23644.
Fig. 1

a) 5′ kfoE P1 FRT kanamycin FRT P2 3′ kfoE

b) kfoD 5′ kfoE kfoE 3′kfoE kfoF

kfoE 5′ and 3′ homologous recombination

SEQ ID N°14

c) kfoD 5′kfoE P1 FRT kanamycin FRT P2 3′kfoE kfoF

FRT recombination

d) kfoD 5′kfoE P1 FRT P2 3′kfoE kfoF

SEQ ID N°13
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12R1/19 C08B37/08 C08B37/00 A61K31/737 C12N1/00
C08L5/08

ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C08B A61P A61L C12R A61K C12N

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, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category* Citation of document, with indication, where appropriate, of the relevant passages Relevant to claim No.


A page 451, col umn 2, paragraph 2

page 452, col umn 1, paragraph 3

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

X See patent family annex.

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

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Date of the actual completion of the international search 1 August 2011

Date of mailing of the international search report 11/08/2011

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<td>A</td>
<td>page 2, paragraph 4 - paragraph 6</td>
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<td>A</td>
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<td>06-11-2008</td>
<td>CA 2684883 AI</td>
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