

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
23 April 2015 (23.04.2015)

(10) International Publication Number
WO 2015/055562 A1

(51) International Patent Classification:

A23K 1/06 (2006.01) A23K 1/00 (2006.01)
A23K 1/165 (2006.01) A23K 1/16 (2006.01)
A23D 9/00 (2006.01) C11B 1/00 (2006.01)

(21) International Application Number:

PCT/EP2014/071853

(22) International Filing Date:

13 October 2014 (13.10.2014)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/891,275 15 October 2013 (15.10.2013) US
13192310.4 11 November 2013 (11.11.2013) EP

(71) Applicant: **DSM IP ASSETS B.V.** (heir of the deceased inventor) [NL/NL]; Het Overloon 1, NL-6411 TE Heerlen (NL).

(72) Inventors: **KUMAR, Manoj**; P.O. Box 4, NL-6100 AA Echt (NL). **SMITH, Drew**; P.O. Box 4, NL-6100 AA Echt (NL).

(72) Inventor: **CUMMINGS, Marsha Anne** (deceased).

(74) Agent: **CAZEMIER, Anne Engeline**; DSM Intellectual Property, P.O. Box 4, NL-6100 AA Echt (NL).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: METHOD FOR PREPARING OIL FROM GRAIN MATERIAL

(57) Abstract: The present invention relates to a process for preparing an oil composition from a grain material comprising incubating the grain material with a proline-specific endoprotease and preparing the oil composition. The process may be applied in an ethanol fermentation process.



WO 2015/055562 A1

METHOD FOR PREPARING OIL FROM GRAIN MATERIAL

The present invention relates to a process for preparing an oil composition from a grain material.

5

Background

Crops and grains like corn, soybean, wheat, rye, barley or oat have various contents of carbohydrates, proteins and oils. Depending on the amount of these compounds in the grain, the grains are applied in various food, feed and non-food applications. One of the grains that is used in large scale ethanol fermentation processes is corn, since the major part of the corn grains is starch. When used in ethanol fermentation processes corn can be milled in a wet milling process or a dry grinding process (Bothast and Schlicher, Appl. Microbiol Biotechnol (2005) Vol. 67: 19-25).

In a wet milling process corn is first soaked in water to make it easier to fractionate the various components of the corn. The separated corn proteins and corn oil are used in food and feed and the remaining starch can be converted to ethanol or finds use in various food and feed application.

For large scale ethanol fermentation processes, dry grinding of corn is predominantly applied. In a dry grinding process corn is first ground and then submitted to a liquefaction step, i.e. the ground corn flour is slurried with water, cooked and a heat stable alpha amylase is added to produce a corn mash. After liquefaction, the corn mash is saccharified by adding a glucoamylase which breaks down the starch into glucose. Ethanol fermentation starts by adding a yeast to the saccharified corn slurry. After production, ethanol is distilled off, the residual water is usually recycled and the remaining solids can be sold as distiller's dried grains with solubles (DDGS) to the feed industry.

DDGS contains the unfermented carbohydrates and the remaining oil and proteins from the ground grain. When oil is removed from DDGS it has more value as a feedstock and can also be used as a biodiesel.

Several processes for recovering oil from grains, in particular during ethanol fermentation processes are known.

WO2013/126561 discloses a process for separating oil from an ethanol production process prior to distillation, wherein the oil composition is relatively low in free fatty acids and/or low alcohol esters.

WO2012/036857 discloses a process for grain milling in an ethanol production process, wherein after liquefying grain slurry by liquefying enzymes such as amylases, an oil product stream is separated from the mash.

In another study (S. Majoni, Oil recovery from condensed corn distillers solubles (2009) Graduate Theses, Paper 10846) several enzymes were tested to recover oil from condensed corn distiller's solubles. This study postulated a strong interaction between the corn storage protein zein and oil. Zein is the major storage protein in corn which is rich in glutamic acid, leucine, proline and alanine (Shukla & Chenyan, Industrial Crops & Products 13 (2001) 171-192). Hydrolysis of the zein protein by action of an acid protease ProtexTM 13L or a broad spectrum protease Alcalase 2.4 increased the oil yield.

Despite the developments in enzymatic oil recovery from grains, there is a need for improved oil recovering processes.

The aim of the present invention is an alternative way for efficiently extracting oil from a grain material.

Summary

The present invention relates to a process for preparing an oil composition from a grain containing material incubating the grain material with a proline-specific endoprotease and preparing the oil composition.

Surprisingly, it was found that the yield of an oil composition in a process wherein a proline-specific endoprotease is used was between 10 to 20% higher as compared to a process wherein no proline-specific endoprotease was used.

Detailed description

The present invention relates to a process for preparing an oil composition from a grain material comprising incubating the grain material with a proline-specific endoprotease, and preparing the oil composition.

A grain material in a process as disclosed herein may be maize or corn, barley, wheat, rye and/or oat material. Preferably, the grain material comprises corn.

The terms maize and corn herein are used interchangeably.

Usually the grain material comprises milled or ground grains which may be obtained by wet milling or dry grinding. Wet milling comprises adding water to the grains and

subsequently milling the grains. Dry grinding comprises grinding dry grains. Wet milling and dry grinding of grains are known processes to a skilled person in the art.

A grain material in a process according to the present invention comprises a protein rich in proline. Such proline-rich protein may be a prolamine storage protein such as gliadin in wheat, hordein in barley, secalin in rye, zein in corn or maize, kafirin in sorghum and avenin in oats. A proline-rich protein may be zein when the grain material comprises corn. It was found that when a grain material comprising a proline rich protein was incubated with proline-specific endoprotease an oil composition could be recovered efficiently. Usually a grain material in a process as disclosed herein may further comprise water.

A grain material may further comprise soluble and / or insoluble starch and / or fermentable sugars such as glucose. Such grain material may be obtainable after liquefying and saccharifying the grain material which are known processes to a skilled person in the art and further described herein below.

According to the internationally recognised schemes for the classification and nomenclature of all enzymes from IUMB, oligopeptidases, dipeptidylpeptidases and endoproteases are those enzymes that hydrolyse internal peptide bonds, which are then divided in sub-subclasses on the basis of their catalytic mechanism. A proline specific protease suitable for the purpose of the invention can be an acid-stable and pepsin-stable endoprotease from *A. niger* as disclosed in WO-A-02/45524 and WO-A-2005/027953, which is able to cleave peptides and intact proteins at the carboxyl side of proline residues and which also is able to cleave peptides and intact proteins under very low pH conditions. A proline specific endoprotease as used herein belongs to the S28 family of serine proteases.

A proline-specific endoprotease may be derived from fungi such as *Aspergillus*, for instance *Aspergillus niger* or bacteria such *Flavobacterium*, *Sphingomonas*, *Myxococcus* or *Aeromonas*, for instance *Flavobacterium meningosepticum*, *Sphingomonas capsulata*, *Myxococcus xanthus*, or *Aeromonas hydrophila*, or *Aeromonas punctate*. For example a proline specific endoprotease may be derived from *Aspergillus niger*.

A proline-specific endoprotease in a process as disclosed herein may have at least 70% identity to SEQ ID NO: 2, for instance at least 75, 80, 85, 90, 95, 96, 97, 98,

99% identity to SEQ ID NO:2. A proline-specific endoprotease used in a process as disclosed herein may comprise the amino acid according to SEQ ID NO:2.

The grain material may be incubated with proline-specific endoprotease in an amount of 200 to 4000 PPU/kg wet weight, such as between 500 and 3000 PPU wet
5 weight, or between 1000 to 2000 PPU / kg wet weight grain material.

Incubating a proline-specific endoprotease with a grain containing material may be performed at a pH of between 3 and 6, for instance between pH 4 and 5.

A suitable temperature at which a grain material may be incubated with proline-specific endoprotease may be between 20 and 70 degrees Celsius, such as between
10 30 and 65 degrees Celsius, such as between 40 and 60 degrees Celsius, or such as between 45 and 55 degrees Celsius.

A process as disclosed herein may further comprise fermenting the grain material to produce ethanol and a fermentation residue. Fermenting a grain material to produce ethanol usually comprises a step of liquefying and/or saccharifying the grain
15 material. Liquefying comprises incubating the grain material with enzymes like alpha amylases, wherein grain slurry is obtained. During liquefaction a grain material is usually heated to temperatures above 100 °C and starch comprised in the grain material is broken down into smaller fragments. Saccharification usually takes place after liquefaction and comprises adding a glucoamylase to the grain slurry.
20 Saccharification comprising hydrolyzing starch in a grain material into glucose molecules.

Fermenting a grain material in a process as disclosed herein further comprises adding yeast to a grain material, for instance grain slurry obtained after liquefaction and saccharification, and fermenting the yeast at a suitable temperature to produce ethanol
25 and a fermentation residue. A suitable yeast in ethanol fermentation processes is for instance *Saccharomyces cerevisiae* (Bai *et al*, Biotechnology Advances 2008, Vol 26, p. 89-105). A skilled person in the art knows how to perform an ethanol fermentation process (see for instance Bothast and Schlicher, Appl. Microbiol Biotechnol (2005) Vol. 67: 19-25). Producing ethanol in a process as disclosed herein usually comprises
30 distilling the ethanol from the fermented grain material. The remaining product after ethanol production is a fermentation residue which may also be called whole stillage or distiller's wet grain solubles. The fermentation residue comprises solid grain residues such as unfermented carbohydrates, proteins and oil, and water.

In the event a process as disclosed herein comprises fermenting the grain material to produce ethanol and a fermentation residue, incubating the grain material with proline-specific endoprotease may be performed before, during and/or after the ethanol fermentation process. For instance, the grain material may be incubated with proline-specific endoprotease during liquefying and/or saccharifying the grain material, for instance before the start of an ethanol fermentation process, i.e. before addition of yeast. The grain material may also be incubated with the proline-specific endoprotease during fermenting the grain material by yeast to produce ethanol. Alternatively, proline-specific endoprotease may be incubated with the grain material after fermenting the grain material to ethanol. When ethanol fermentation has been completed, ethanol is usually distilled off before incubating the grain material with proline-specific endoprotease. In this latter case the grain material which is incubated with proline-specific endoprotease comprises the fermentation residue.

A process for preparing an oil composition as disclosed herein may further comprise separating an oil composition from the grain material obtained after incubating with proline-specific endoprotease. Separating an oil composition may be performed in any suitable way, for instance separating may comprising centrifuging the grain material that has been incubated with proline-specific endoprotease.

Alternatively, separating an oil composition may comprise extracting the oil composition from the grain material obtained after incubating with proline-specific endoprotease with a solvent. A suitable solvent for extraction an oil composition from the grain material may be hexane or 2-methylpentane (iso-hexane).

Extracting an oil composition from a grain material comprising a fermentation residue may comprise separating the fermentation residue into a solid phase and a liquid phase. The solid phase may also be called a wet cake and the liquid phase as used herein may be called thin stillage. The oil composition may be extracted from the liquid phase.

In one aspect the present disclosure relates to an oil composition obtainable by a process as disclosed herein. An oil composition prepared by a process as disclosed herein may be used as a feedstock for biodiesel, in animal feed or food grade edible oil.

Definitions

Sequence identity is herein defined as a relationship between two or more amino acid (polypeptide or protein) sequences, as determined by comparing the sequences. Usually, sequence identities or similarities are compared over the whole length of the sequences compared. In the art, "identity" also means the degree of sequence
5 relatedness between amino acid sequences, as the case may be, as determined by the match between strings of such sequences.

Methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to
10 determine identity and similarity between two sequences include BLASTP, publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, MD 20894). Preferred parameters for amino acid sequences comparison using BLASTP are gap open 11.0, gap extension 1, Blosum 62 matrix.

Figures

Figure 1. Oil recovered from wet cake and thin stillage after incubation with proline-specific endoprotease (PEP) and pectinase and without enzyme treatment.

Examples

Materials and methods

Enzymes

Aspergillus niger proline-specific endoprotease (SEQ ID NO: 2; An08g04490) in
25 microgranular form having 40 PPU/g dry weight and available at DSM was used.

One PPU is defined as the amount of enzyme required to release one micromole of pNA from Z-Gly-Pro-pNA in one minute under the defined assay conditions (pH 4.6, T=37°C and at a substrate concentration of 0.37 mM Z-Gly-Pro-pNA).

Pectinase was DSM Cytolase PCL5®. Pectinase activity is expressed in AVJP,
30 wherein one unit AVJP is the enzyme quantity in 1 ml enzyme solution which induces a

variation of viscosity with a speed from which the apparent constant is 0.00027 / minute in the described conditions.

Whole stillage

Corn whole stillage was obtained from an ethanol fermentation plant (One Earth
5 Energy, Gibson City, IL), with dry solid content of 13.6% $\pm$ 0.1.

Example 1

Before incubation, the whole stillage samples were adjusted to pH4.5 with 10N
sulfuric acid. Two hundred grams of whole stillage samples were incubated with
pectinase (100,000 AVJP/kg wet weight) and proline-specific endoprotease from *A.*
10 *niger* (1600 PPU/kg wet weight) at 50 °C for 4 h. A control incubation was performed
with no enzyme addition. Each incubation was conducted in triplicate.

After enzyme incubation whole stillage was centrifuged at 4,200 rpm for 10 min to
obtain thin stillage and wet cake. Thin stillage was placed in a convection oven at 49
°C to evaporate water and was concentrated to 30-40% solid content. The
15 concentrated thin stillage was transferred to a 50 mL centrifuge tube. Hexane (100%,
20 mL) was added followed by 30 min of mixing at 120 rpm at 40 °C. The mixture was
centrifuged at 10,000 rpm for 3 min, and top liquid fraction (hexane and oil solution)
was carefully removed by pipette. The hexane extraction step was repeated three
times. Nitrogen flow injection was used to evaporate hexane to get crude oil. Oil
20 extraction from wet cake followed the same procedure as oil extraction from thin
stillage, except for using 50 mL of hexane. The hexane extraction from wet cake was
repeated twice. The weight of extracted oil from thin stillage and wet cake was
determined gravimetrically.

The results in Figure 1 show that the oil yield from thin stillage after incubation with
25 proline-specific endoprotease was about 20% higher than the oil yield of the control
without enzyme addition. Pectinase did not result in an increased yield of oil.

CLAIMS

1. Process for preparing an oil composition from a grain material comprising incubating the grain material with a proline-specific endoprotease and preparing the oil composition.
5
2. Process according to claim 1, wherein the grain material comprises corn.
3. Process according to any one of the claims 1 or 2, wherein the proline-specific endoprotease is derived from *Aspergillus niger*.
10
4. Process according to any one of the claims 1 to 3 wherein the amount of proline-specific endoprotease is between 200 and 4000 PPU/kg wet weight grain material.
15
5. Process according to any one of the claims 1 to 4 wherein the incubation is performed at a pH of between 3 and 6.
6. Process according to any one of the claims 1 to 5, wherein the incubation is performed at a temperature of between 20 and 70 degrees Celsius.
20
7. Process according to any one of the claims 1 to 6, further comprising fermenting the grain material to produce ethanol and a fermentation residue.
25
8. Process according to claim 7, wherein incubating proline-specific endoprotease is performed before, during and/or after said fermenting.
9. Process according to claim 7 or 8, wherein incubating proline-specific endoprotease is performed after said fermenting.
30
10. Process according to claim 9, wherein the grain material during incubating proline-specific endoprotease comprises the fermentation residue.

11. Process according to any one of the claims 1 to 10, wherein preparing the oil composition comprises extracting the oil composition from the grain material obtained after incubating with proline-specific endoprotease with a solvent.
12. Process according to claim 11 wherein the solvent is hexane.
13. Process according to any one of the claims 7 to 12, comprising separating the fermentation residue into a solid phase and a liquid phase.
14. Oil composition obtainable by a process according to any one of the claims 1 to 13.

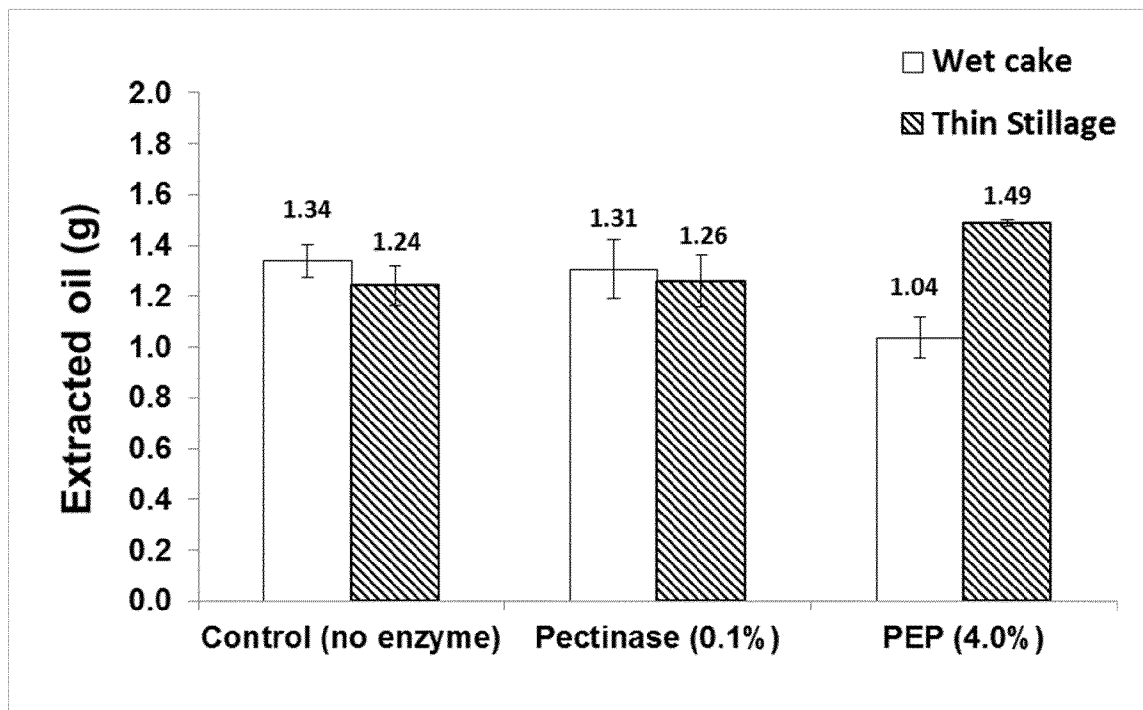


Fig.1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2014/071853

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
 - a. (means)

☐	on paper
☒	in electronic form
 - b. (time)

☒	in the international application as filed
☐	together with the international application in electronic form
☐	subsequently to this Authority for the purpose of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/071853

A. CLASSIFICATION OF SUBJECT MATTER

INV. A23K1/06 A23K1/165 A23D9/00 A23K1/00 A23K1/16
C11B1/00

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)

A23K A23D C11B

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

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

EPO-Internal, FSTA, BIOSIS, MEDLINE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DD 290 912 A5 (ADW DDR [DE]) 13 June 1991 (1991-06-13) claims; examples -----	1-14
X	US 2013/236936 A1 (LEE CHIE YING [US]) 12 September 2013 (2013-09-12) claims -----	1-14
X	US 2010/260918 A1 (WANG HUI [US] ET AL) 14 October 2010 (2010-10-14) claims -----	1-14
X	US 2009/227004 A1 (DALE ROSS [US]) 10 September 2009 (2009-09-10) paragraph [0038]; claims; figure 3 -----	1-14
X	US 2003/180897 A1 (ULRICH JAMES F [US] ET AL) 25 September 2003 (2003-09-25) claims; examples -----	1-14



Further documents are listed in the continuation of Box C.



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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

26 January 2015

Date of mailing of the international search report

12/03/2015

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Vernier, Frédéric

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2014/071853

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DD 290912	A5	13-06-1991	NONE

US 2013236936	A1	12-09-2013	CA 2819842 A1 07-06-2012
			CN 103347615 A 09-10-2013
			EP 2646164 A1 09-10-2013
			US 2013236936 A1 12-09-2013
			WO 2012075481 A1 07-06-2012

US 2010260918	A1	14-10-2010	NONE

US 2009227004	A1	10-09-2009	US 8603801 B1 10-12-2013
			US 2009227004 A1 10-09-2009

US 2003180897	A1	25-09-2003	NONE
