



(51) International Patent Classification:

C12Q 1/04 (2006.01) *D06M 16/00* (2006.01)
C12Q 1/22 (2006.01) *G01N 33/34* (2006.01)
C12Q 1/34 (2006.01) *D06M 101/06* (2006.01)
C12Q 1/68 (2006.01)

(21) International Application Number:

PCT/IB2015/058278

(22) International Filing Date:

27 October 2015 (27.10.2015)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

1451333-7 7 November 2014 (07.11.2014) SE

(71) Applicant: STORA ENSO OYJ [FI/FI]; P.O. 309, FI-00101 Helsinki (FI).

(72) Inventors: PARTTI-PELLINEN, Kirsi; Puumiehentie 6, FI-55400 Imatra (FI). RÄSÄNEN, Jari; Västäräkinkuja 15, FI-55420 Imatra (FI). HÄRMÄLÄ, Kielo; Vasamatie 6, FI-56100 Ruokolahti (FI). KETTUNEN, Anu; Paulatie 12, FI-03100 Nummela (FI). RIIHINEN, Kalle-Juhani; Rummunyöjäkatu 8 as 60, FI-02600 Espoo (FI).

(74) Agent: KLING, Susanna; Stora Enso AB, Group IP, Box 9090, S-650 09 Karlstad (SE).

(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))
- with information concerning incorporation by reference of missing parts and/or elements (Rule 20.6)

(54) Title: IMPROVED METHOD FOR DETERMINATION OF MICROORGANISMS

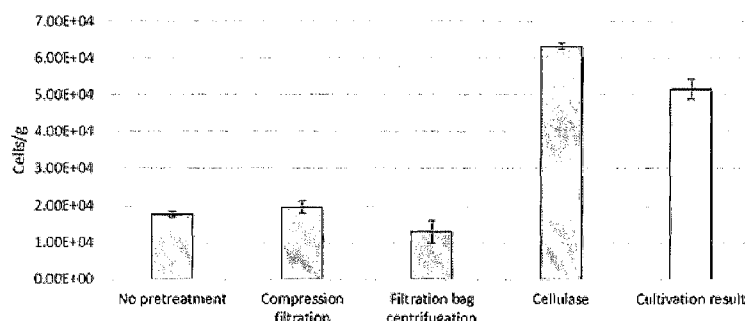


Fig. 5

(57) Abstract: The present invention relates to determination of the microorganism content in material comprising cellulose within the pulp and paper industry. The material comprising cellulose is enzymatically pretreated and microorganisms are determined using PCR based technology.

IMPROVED METHOD FOR DETERMINATION OF MICROORGANISMS

Field of invention

This invention relates to a method for determination of the microorganism content in material comprising cellulose within the pulp and paper industry.

5 Description of related art

Paper and board are important packaging materials used in many fields of industry. Packaging paper or board can provide physical or chemical protection to the object(s) enclosed; they can also e.g. provide information and enhance marketing and portion control of the objects. Paper and board have
10 also a long history in the food industry in a multitude of uses including packaging. As a packaging material, paper often comes in direct contact with food. Paper and board containers are used for dry food, liquids and frozen products. Paper wrapping is commonly used also for fast food and candies, pharmaceuticals packaging, cigarette packaging, luxury packaging and high-
15 class graphical products. The packaging protects the content against the action of hazardous factors of the environment thus prolonging the useful life of the packed contents. The hygiene requirement for consumer packaging board products is extremely high.

20 Paper and board are usually mainly composed of cellulose fibres, calcium carbonate, starch and hemicellulose. All these are natural components and can serve as growth media for contaminating microorganisms. The circulation of water having a high content on nutrients and starch is a common origin of microbial infection.

25

Traditionally, microbial purity of a substance has been evaluated by using microbiological methods requiring cultivation of a sample of the material or surface to be analyzed. The composition and culturing conditions (temperature, time, medium) have impact on the accuracy of the method. The
30 sample for quantitative analysis has to be diluted to such an extent that one can count single colonies formed by cells. There may be dozens or at most a few hundreds of cells in the studied sample. The colony count in parallel samples may vary a lot and, consequently, the reproducibility is bad.. Thus, the accuracy or reliability is poor.. In addition, microbial methods are typically labor

intensive and time consuming and thus not optimal for follow-up (monitoring) the purity of paper and board packages.

5 Polymerase chain reaction (PCR) based methods of identifying a microorganisms in a papermaking process including a paper sheet are known from e.g. US 8 613 837 and US2013189152.

10 As the present methods are labor intensive, time consuming and their accuracy and sensitivity are not satisfying there is a need for improved methods of determining microbiological purity of paper and board material.

Objects and summary of the invention

15 An object of this invention is to provide an improved method for determination of contaminating microorganisms in material comprising cellulose within the pulp and paper industry. This object is achieved by the present invention as will be described and claimed below.

The first aspect of the invention is a method for the determination of a microorganism content in material comprising cellulose within the pulp and paper industry. Characteristic features of said method are given in the characterizing part of claim 1.

20 The second aspect of the invention is the use of cellulase in the pretreatment of material comprising cellulose within the pulp and paper industry before determination of microorganisms using quantitative PCR.

25 The method allows for simple and accurate follow up of the microbiological purity of material comprising cellulose within the pulp and paper industry and also of the resulting paper and board materials, such as consumer packaging materials, especially food packaging materials. Thereby the hygiene of packaging material is enhanced since possible contaminations can be detected at an early stage.

30

Short description of the Figures

Figure 1 shows a comparison of qPCR analysis for bacteria in inoculated board suspended in a buffer and subjected to a compression filtration and traditional cultivation for the determination of microorganisms.

- 5 **Figure 2** shows a comparison of qPCR analysis of bacteria in inoculated board suspended in a buffer and subjected to a bag centrifugation filtration and traditional cultivation for the determination of microorganisms.

- 10 **Figures 3a and 3b** shows a comparison between the method according to the present invention and a traditional cultivation based method in the determination of microorganisms in two different papers or boards.

Figure 4 shows a comparison of the method for the determination of microorganisms in board firstly using qPCR without enzymatic pretreatment, secondly according to the present invention and thirdly using a traditional cultivation based method.

- 15 **Figure 5** shows a comparison of microorganism determination using traditional cultivation, qPCR without enzymatic pretreatment, compression filtration as pretreatment, filtration bag centrifugation as pretreatment and the method according to the present invention using two different mixed commercial cellulase preparations.

20 Detailed description of the invention

- The inventors have surprisingly found that the PCR based determination of microbiological purity of material comprising cellulose within the pulp and paper industry can be remarkably enhanced by enzymatic pretreatment of the sample. The enzymatic pretreatment allows quantitative detection using PCR
25 also when the levels of contaminating microorganisms are very low.

According to the invention the determination of a microorganism content in material comprising cellulose within the pulp and paper industry comprises the steps of:

- 30 (a) providing a suspension of a sample of the material comprising cellulose; and

- (b) treating the suspension of step (a) with one or more enzyme preparation(s) having cellulase activity; and
- (c) separating the microorganisms from the enzyme treated suspension of step (b); and
- 5 (d) isolating nucleic acids from the separated microorganisms of step (c); and
- (e) conducting a PCR analysis on the isolated nucleic acids of step (d), wherein the PCR result indicates the microorganism content of the sample.

- 10 As known within the art a positive PCR result indicates the presence of a certain nucleic acid and thus a certain microorganism whereas qPCR is used for the determination of microorganism levels.

- 15 In this connection "a microorganism content" should be understood to cover both the qualitative (e.g. identifying the microbial strain) and quantitative (e.g. the amount of colony forming units of bacteria) microorganism content of the sample. Determination of the quantitative and qualitative microorganism content in a sample material can be done independently or together. The qualitative content can be measured using primers specific to certain genera or
- 20 species. Presence of the PCR product indicates presence of the microorganism and can be determined using gel electrophoresis or preferably using analytics of qPCR equipment. Quantitative determination is performed using qPCR equipment and known controls, where necessary. Either broad-range primers or specific primers can be used.

- 25 According to this invention the phrase "material comprising cellulose within the pulp and paper industry" means cellulose containing materials within the pulp and paper industry including raw materials, cellulosic material during the manufacturing process and the end products. Examples of such materials comprising cellulose (cellulosic materials) are, without limiting to those, wood
- 30 chips, mechanical pulp, and various papers and boards. Pulp, various papers and boards are preferred materials. Consumer board is particularly preferred and packaging board for food and liquids is the most preferred material comprising cellulose.

In this connection the term "consumer board" includes general packaging board, food and non-food packaging board, liquid packaging board, pharmaceuticals packaging board, cigarette board and graphical board. The paper or board to be tested for microbiological purity can be single-ply or multiply as well as coated or uncoated. Paper or board contains cellulosic fibres and optionally a varying amount of hemicellulose and optionally other constituents and/or coatings. The hemicellulose content or coating can have an effect on the optimal composition of the enzyme preparation. The preferred paper and board material is consumer packaging board, especially packaging board for liquids and food material.

The suspension can be taken directly from an aqueous phase of the manufacturing process. However, when the suspension is derived from a process or an aqueous product it must be ensured that the solution is suitable for the activity of enzyme preparation in the following steps. If necessary, the buffer has to be changed.

A sample of the potentially contaminated material comprising cellulose may be suspended in a solution, typically a buffer solution suitable for activity of the enzyme preparation to be used. The paper or board material can be spliced before the suspending step e.g. by using scissors. Usually the step of suspending the material of paper or board is enhanced by mechanical grinding and/or e.g. vortexing. One example of a preferred method of suspending a sample can be found in Standard Methods for the Examination of Dairy Products, 17th edition, APHA publications. Samples derived from pulping processes may be subjected to enzyme treatment without mechanical grinding. However, in some cases the suspension buffer has to be changed in order to meet the needs of enzymatic activity in the following step.

Thereafter, a gravimetric amount of a, preferably homogenized, suspension is contacted with an enzyme preparation containing one or more cellulase and optionally hemicellulase activities. Typically the enzyme preparation is added to the suspension.

The suspension is treated with cellulolytic enzymes in order to enhance separation of microorganisms and fibrous material. Enzyme preparation according to this invention contains at least one enzyme having cellulase

activity. Optionally the enzyme preparation further contains hemicellulase activities. The enzyme preparation can also be a mixture of enzymes having cellulase activities and optionally one or more hemicellulase activities. Enzyme preparation containing several cellulase activities is preferably enriched with
5 one or more endoglucanase activities. Cellulase enzymes can be endocellulases such as endoglucanases (EC 3.2.1.4) or exoactive cellobiohydrolases (EC 3.2.1.91), without restricting to those, and they can have also other cellulolytic and/or hemicellulolytic activities or any mixture of those. Endoglucanases are preferred. Endoglucanase activities of the cel5
10 family are especially useful. Hemicellulases can be e.g. xylanases (EC 3.2.1.8) or mannanases or any mixtures thereof. An example of a commercially available enzyme mixture useful in the present invention is Ecostone L900 (AB Enzymes, Finland), which is a cel5 enriched *Trichoderma* cellulase mixture.

Enzymatic treatment is gentle for living microorganisms and reduces the need
15 for extensive mechanical grinding. It enhances separation of microorganisms and fibres in the paper or board substrates whereby the sensitivity and accuracy of the determination is enhanced.

Preferably, the Enzyme preparation is dosed in a slight excess in order to ensure proper separation of microorganisms and fibres in the material
20 comprising cellulose. The conditions including e.g. pH, temperature and incubation time for enzymatic treatment (defined as step (b) above) should be suitable for the enzyme preparation activity. Enzymatic treatment also shows better repeatability than mechanical methods of maceration. After the enzymatic treatment, the suspension allows separation of the microorganisms
25 from the cellulose containing material using e.g. filtration bag centrifugation.

The amount of contaminating microbiological impurities (the amount of microorganisms) in pulp derived from a properly functioning manufacturing process, or from paper or board obtained using a properly functioning manufacturing process, is typically very low. Thus it is necessary to separate
30 the microorganisms from the remaining fibres. In principle any known method for recovering the microorganisms can be used but filtration bag centrifugation is the most preferred. A centrifuge filter bag will recover living microorganisms but remaining residual fibres will be discharged. A suitable pore size for the

filter bag can be selected by a person skilled in the art and may be e.g. 50 micrometers.

Thereafter, the separated cells can be treated by propidium monoazide (PMA). PMA penetrates the membranes of dead cells and reacts with nucleic acids, resulting in DNA which cannot be amplified by PCR. Thus only the DNA from viable cells can be determined.

Then nucleic acids of the separated microorganisms are isolated. Again, any known method can be used. One example of a suitable protocol is described in Rinttilä *et al.*

Finally, the isolated nucleic acid material is conducted to a PCR analysis, usually to quantitative PCR (qPCR). qPCR allows both quantitative and qualitative determination of contaminating microorganism(s). A person skilled in the art is able to select suitable PCR primers for e.g. total bacterial determination; for example primers for amplifying 16S rDNA region are usable in the determination of the total bacterial content. It is also possible to use primers specific for certain genera or species of microorganisms (a qualitative analysis, optionally with also a quantitative result). Samples with amplification curve exceeding the threshold cycle before a non-template control and a valid melting curve indicate the presence of microorganisms. A quantitative PCR result is calculated using standards with a known amount/concentration of microorganisms.

When compared to traditionally used methods of cultivation qPCR is remarkably faster and not as labor intensive as cultivation. The method of the invention is also very sensitive and enables detection of small changes in microbial purity of the material. For example a liquid packaging material is deemed to be microbiologically pure when the total bacterial count per gram of liquid packaging board is less than 250 colony forming units (cfu/g) (Standard Methods for the Examination of Dairy Products, 17th edition, APHA publications). Thus sensitivity and low detection limit are essential for usability of the method.

Improved accuracy, specificity and usability of the determination method allows continuous follow-up of the produced material on the manufacturing site and

easy follow up or random testing before using the packaging material. Thus, the method according to the invention improves the safety and hygiene of consumer packaging. In addition, microbiological impurities in manufacturing processes are harmful to the process and the resulting product. Early detection
5 of a possible increase in microbial count makes it easier to e.g. prevent formation of microbial deposits.

The method described here is suitable for determination of any type of microorganism. The method is especially suitable for determination of bacteria, especially *Bacillus* species and species within *Bacillus* D group. It is also
10 possible to determine contaminating fungi and mold.

The use of cellulase enzyme in pretreatment of a material comprising cellulose within the pulp and paper industry before determination of microorganisms using quantitative PCR is also within scope of this invention. Cellulase enzyme can be used alone or in combination with other cellulase enzymes and/or with
15 one or more hemicellulase enzyme(s). Both mixed cellulase preparations and so called whole cellulase preparations having several hydrolytic activities, as well as cellulases having only one or two main activities are usable within the scope of this invention. As known in the art the type of the cellulase is dependent on the type of the fibrous material. In a most preferred use paper or
20 board material is pretreated with endoglucanase enzyme before the quantitative determination of microorganisms.

The invention is illustrated by the following non-limiting examples. It should be understood that the embodiments given in the description above and the examples are for illustrative purposes only, and that various changes and
25 modifications are possible within the scope of the invention.

Examples

Example 1: Inoculation of packaging board

A bacterial strain belonging to *Bacillus* D group was grown in TSB (tryptic soy broth) overnight at +37 °C. To ensure viability, bacteria were harvested during
30 the exponential growth phase. From the primary growth broth, a volume of 5 ml was transferred to 500 ml of fresh broth and incubated at +37 °C for 6 hours. Bacterial cells were divided into 10 X 50 ml falcon tubes and harvested by

centrifugation. All 10 bacterial pellets were re-suspended into a 5 ml sterile citric acid buffer (0.05 M, pH 5.0) and combined together and filled up to 200 ml ('Bacterial stock'). Bacterial stock was added to a board-buffer mixture at 20 µl for Low and 200 µl for Mid-Low inoculum.

- 5 A packaging board was inoculated with two levels of *Bacillus* strains belonging to *Bacillus* D group (Low and Mid-Low). Culturing was carried out according to FDA standard protocol (Standard Methods for the Examination of Dairy Products, 17th edition, APHA publications). Serial dilution of *Bacillus* grown as explained above was used to inoculants; High-Level was 1:10 dilution, and
10 following Mid-High, Mid-Low and Low were diluted respectively so that Low level inoculant was diluted 1:10 000.

Example 2. Propidium monoazide treatment

- For propidium monoazide treatment, a pellet containing bacterial cells was dissolved with a hand vortex to 1980 µl of 0.9% NaCl. The resulting liquid was
15 transferred into 2 ml Eppendorf tube and 20 µl of PMA (propidium monoazide) reaction solution was added. The tubes were manually shaken to mix the samples and then incubated for 5 min in dark @ RT without shaking. After incubation, the tubes were inverted to homogenize the samples, placed sideways on cold elements under 500W lamp and illuminated for 5 min.
20 Thereafter, the samples were centrifuged at 20000 x g at +4°C for 10 min (Eppendorf Centrifuge 5804 R) and the supernatant was poured away. After centrifugation, the pellet was dried in vacuum desiccator at +45°C for 20 minutes and dissolved into 45 µl of Tris-EDTA buffer at +55°C for 1.5-2 hours.

25 Example 3: DNA extraction and qPCR

- DNA extraction for cells isolated from the fibrous material was essentially carried out as described by Rinttilä *et al* (2004). In brief, lysis reagents were added to the tube with glass beads and FastPrep bead beater was used three times at the speed of 6.5 m/s for 1 minute. The tubes were incubated at 65°C
30 for 20 min, vortexing with Thermomixer every 2 minutes. 800 µl of phenol-chloroform-isoamylalcohol (24:23:1) was added, mixed and centrifuged at 10000g for 5 min. 600 µl of liquid phase was transferred into a new tube and

extracted with chloroform:isoamylalcohol (24:1). 270µl 100% isopropanol was used to precipitate the DNA and the liquid was removed after centrifugation of 20000g at +4°C for 15 min. The pellet was washed twice with 1 ml (-20°C) 70% ethanol and centrifuged with 20000g at +4°C for 5 minutes. After
5 centrifugation, the pellet was dried in vacuum desiccator at +45°C for 20 minutes and dissolved into 45 µl of Tris-EDTA buffer at +55°C for 1.5-2 hours.

DNA samples were diluted 1:2, 1:4 and 1:8 for qPCR analysis. Broad-range primers as described in Nadkarni *et al.* 2002 were used for 40 cycles with an annealing temperature of 60°C. Sample results were calculated according to a
10 standard curve based on 10-fold diluted standards.

Example 4: Compression filtration

Packaging board inoculated with two levels of strains of Bacillus D group. Low and Mid-Low) as described in Example 1 was homogenized with a Waring blender into buffer and pressed with compression filtrate to separate the solid
15 and the liquid phase. qPCR result from filtrate obtained from a compression filtration represented only 21 and 11% of the cultivation result for Low and Mid-Low inoculation level, respectively, see **Figure 1**. Much lower result from the compression filtrate than cultivation result indicates that compression filtration is not an adequate separation method for microbes from packaging board.

20 Example 5: Filtration bag centrifugation is an inadequate separation method for microbes

Packaging board (10 g) was cut into small pieces with sterile scissors excluding the edges of the board sample. The resulting pieces were mixed with 300 ml of citric acid buffer (0.05 M, pH 5) and homogenized in a Waring
25 blender. Known gravimetric amount of homogenized, soaking wet packaging board (35±0.1 g) was transferred into 50 ml Falcon tubing containing a filter bag (F57 filter bag for fiber and *in vitro* studies, Ankom Technology) and centrifugally filtered. Ideally, indissoluble matrix would be trapped in the filter bag while the bacteria would sediment into the pellet. Nevertheless, the qPCR
30 result represented 27% of cultivation result, indicating that sole filtration bag centrifugation is not an adequate separation method for microbes from

packaging board suspended into a solution. Result using qPCR and cultivation is shown as **Figure 2**.

Example 6: Enzyme treatment

- Four enzyme preparations containing cellulolytic activities (so called “whole cellulases” or “mixed cellulases”) were tested in relation to their capability to break down board samples suspended in a buffer. The tested enzymes were Ecostone L900 (AB Enzymes), Optimase™ CX40L, Optimase™ CX60L and Multifect (Genencor). The main activities according to manufacturers’ announcements and other publicly available information are as follows:
- 10 Ecostone L900 is a cel5 enriched *Trichoderma* cellulase preparation. OPTIMASE™ CX 40L enzyme preparation contains cellulase and hemicellulases as main enzyme activities. Optimase CX60L contains multiple enzyme activities but is standardized on the basis of its activity on carboxymethylcellulose (CMC). Xylanase is the main activity for Multifect.
- 15 To ensure that enzyme activities would not limit the break-down of board substrate, the following series of test settings (see **Table 1**) where the ratio of enzyme volume to board mass ranged from 5 to 15 x 10⁻⁵ was carried out. The efficiency did not change even though more enzyme per board was provided indicating that already the smallest volume contained so much enzyme that the
- 20 enzymatic activity did not limit the break-down of board.

Table 1.

Test setting	Board (g)	Buffer (ml)	Enzyme volume (μl)
1	10	300	500
2	10	300	700
3	10	250	850
4	10	150	1500

5	15	150	2200
6	20	250	1700
7	15	250	1300
8	10	250	800

All enzymes were applied as maximal volume so that enzyme activity would not limit the break-down of board substrate. Incubation temperature, pH and other conditions was adjusted to optimal range for each enzyme according to recommendations of the manufacturers. The capability of the enzymes to break-down the board substrate was estimated visually and the two enzymes with cellulose as main activity were superior to the other two, indicating that celluloses are preferred over hemicellulases.

Example 7: Analysis of spiked packaging boards with qPCR.

Two different packaging boards were inoculated with four levels of strains of *Bacillus* D group (Low, Mid-Low, Mid-High and High) as described in **Example 1**. Culturing was carried out according to FDA standard protocol (Standard Methods for the Examination of Dairy Products, 17th edition, APHA publications). For qPCR analysis, packaging boards (10 g) were cut into small pieces with sterile scissors excluding the edges of the board sample. The resulting pieces were mixed with 300 ml of citric acid buffer (0.05 M, pH 5) and homogenized in a Waring blender. A known gravimetric amount of homogenized, soaking wet packaging board (35 ± 0.1 g) was transferred into a storage bottle and incubated in an incubator shaker at 40°C with a cellulase enzyme (AB Enzymes, Ecostone L900). The sample was transferred into 50 ml Falcon tubing containing a filter bag and centrifugally filtered. Indissoluble matrix was trapped in the filter bag while the bacteria sedimented into the pellet. The samples for which viable cells were to be measured were treated with propidium monoazide at the pellet stage before the lysis reagents were added. qPCR results shown in **Figures 3a** and **3b** showed excellent linearity and low standard deviation between replicate samples. They were also in line with cultivation results i.e. showed good recovery and accuracy (90 and 97 %,

respectively). The experiment was repeated several times using various boards without inoculation. The results obtained using enzyme treatment and qPCR were in line with the respective cultivation results. Due to a very low level of endogenous microbes no statistically meaningful differences could be
5 observed. Data is not shown.

Example 8: Comparison of determination by cultivation and qPCR with and without cellulase pretreatment

Cultivation after suspending the board sample showed higher microbial level than the quantitative polymerase chain reaction when no enzyme pretreatment
10 is applied prior to DNA extraction and qPCR analysis. Pretreatment with cellulose prior to the DNA extraction and qPCR analysis, however, shows good accordance to cultivation result. Filtration bag centrifugation was used to the separation of microbes before qPCR, Results are shown as **Figure 4**.

Example 9: Enzyme pretreatment as compared to other pretreatments

15 Culturing of the packaging board samples was carried out according to the FDA standard protocol (Standard Methods for the Examination of Dairy Products, 17th edition, APHA publications). Compression filtration was carried out as described in **Example 1** and filtration bag centrifugation as described in **Example 5**. Enzyme treatment using commercial Ecostone L900 was carried
20 out as described in Example 7.

The cellulase pretreated samples showed a level respective to cultivation (similar or slightly higher) while other pretreatments (compression filtration and filtration bag centrifugation) and samples without pretreatment showed lower level as compared to cultivation. Results are shown as **Figure 5**.

References

Nadkarni, M.A., F.E. Martin, N.A. Jacques, and N. Hunter. 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 148:257-266.

Rinttilä T, Kassinen A, Malinen E, Krogus L, Palva A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J. Appl. Microbiol. 2004; 97(6):1166-1177.

Standard Methods for the Examination of Dairy Products, 17th edition, APHA publications. 2004

Claims

1. A method for determination of a microorganism content in material comprising cellulose within the pulp and paper industry comprising the steps of:
 - 5 (a) providing a suspension of a sample of the material comprising cellulose; and
 - (b) treating the suspension with one or more enzyme preparation having cellulase activity; and
 - (c) separating the microorganisms from the enzyme treated suspension; and
 - 10 (d) isolating nucleic acids from the separated microorganisms; and
 - (e) conducting a PCR analysis on the isolated nucleic acids, wherein the PCR result indicates the microorganism content.
- 15 2. The method according to claim 1, wherein the suspension is provided by suspending a sample in a solution.
3. The method according to claim 1 or 2, wherein the cellulase activity is a mixture of cellulase activities.
4. The method according to any of the preceding claims, wherein the cellulase activity is at least one endoglucanase activity.
- 20 5. The method according to any of the preceding claims, wherein the enzyme preparation further contains hemicellulase activity or activities.
6. The method according to any of the preceding claims, wherein the microorganisms are separated from the suspension using filter bag centrifugation.
- 25 7. The method according to any of the preceding claims, wherein the primers for PCR are broad-range primers for bacteria or other microorganism.
- 30 8. The method according to any of the preceding claims, wherein the primers for PCR are specific to certain species or genus of microorganism.
9. The method according to any of the preceding claims, wherein material comprising cellulose is consumer board, preferably board for food industry.
- 35

10. The method according to any of the preceding claims, wherein the microorganism belongs to bacteria.
11. The method according to claim 10, wherein said bacteria represents a *Bacillus* species.
- 5 12. The method according to any of claims 1 to 9 wherein the microorganism is a fungi or mold.
13. The method according to any of the preceding claims wherein the cells are treated with propidium monoazide.
- 10 14. Use of cellulase in pretreatment of a material comprising cellulose within the pulp and paper industry before determination of microorganisms using PCR.
15. The use according to claim 14, wherein paper or board material is pretreated with endoglucanase enzyme before a quantitative determination of microorganisms.

1/3

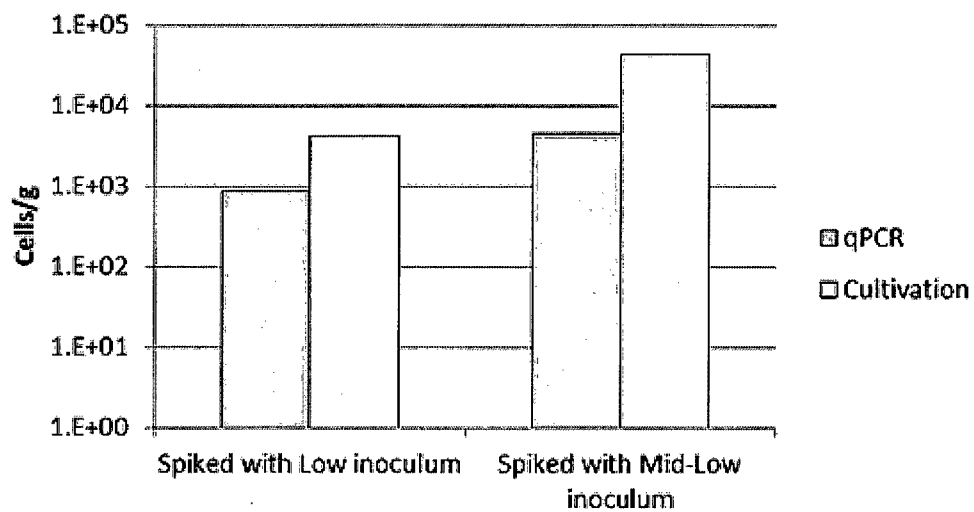


Fig. 1

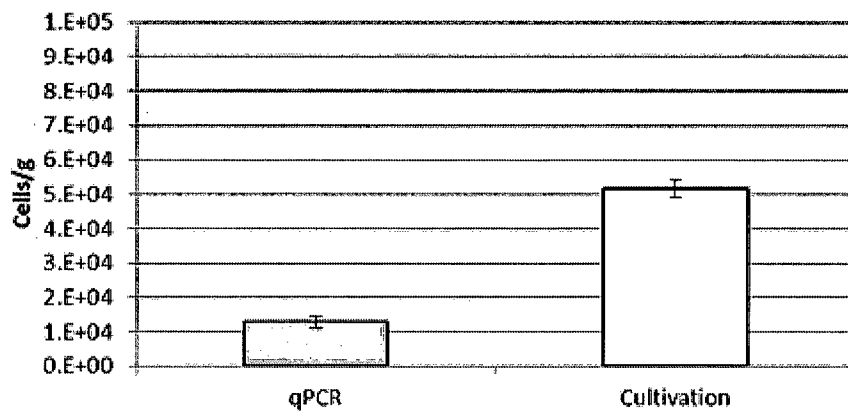


Fig. 2

2/3

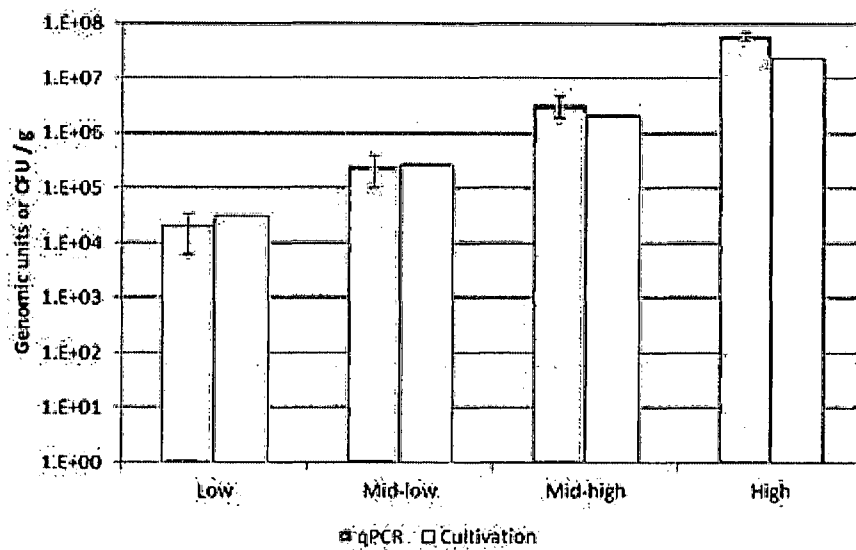


Fig. 3a

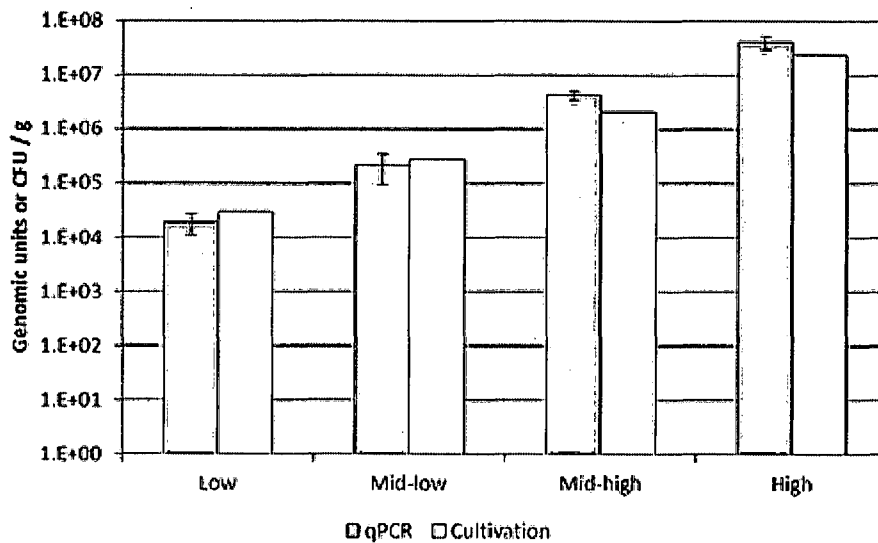


Fig. 3b

3/3

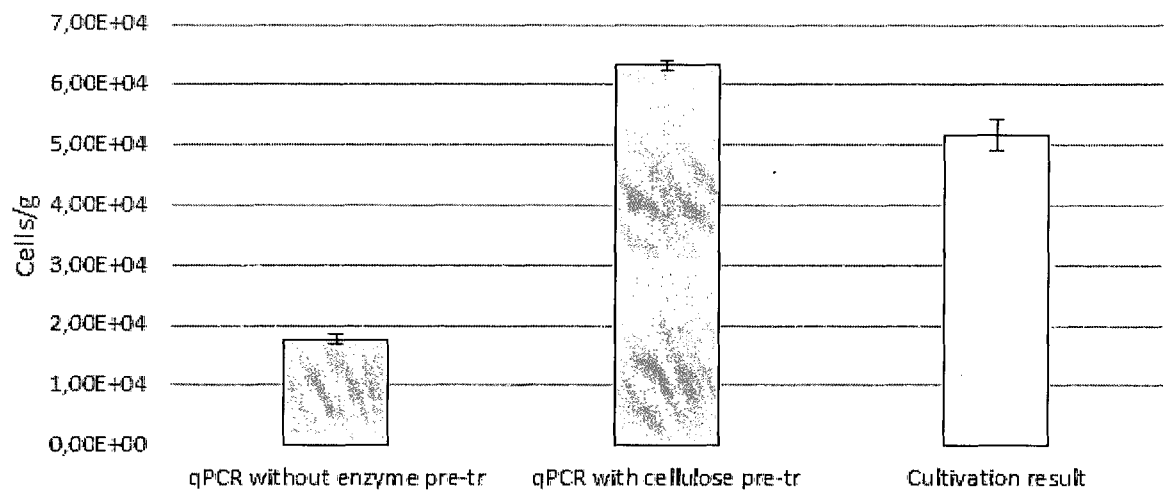


Fig. 4

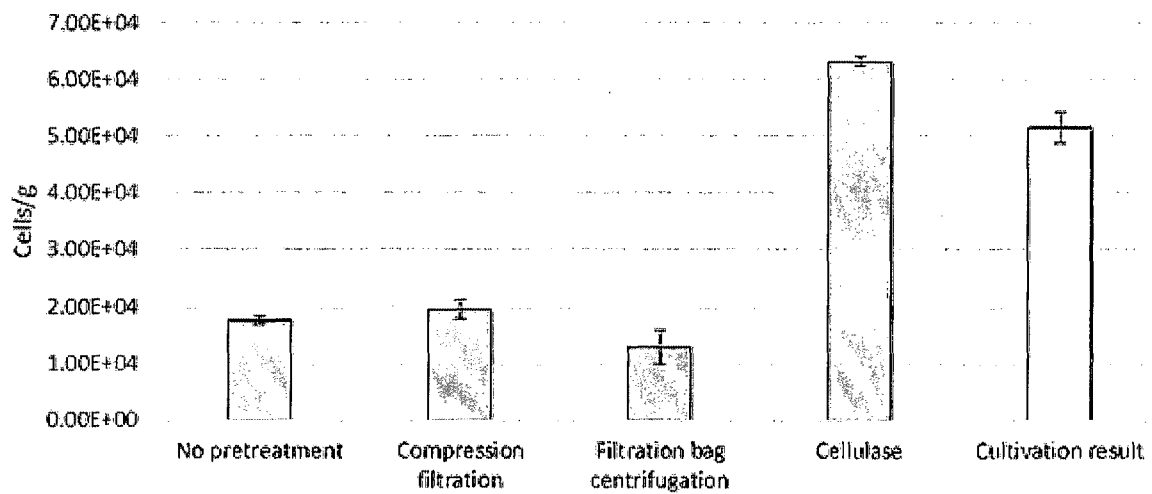


Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2015/058278

A. CLASSIFICATION OF SUBJECT MATTER

IPC: see extra sheet

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C12Q, D06M, G01N

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

SE, DK, FI, NO classes as above

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

EPO-Internal, PAJ, WPI data, BIOSIS, COMPENDEX, EMBASE, INSPEC, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 20040014122 A1 (BREEN ALEXANDER W ET AL), 22 January 2004 (2004-01-22); paragraphs [0003]-[0005], [0016]-[0017], [0023], [0030], [0051]-[0054] --	1-15
A	WO 9747764 A1 (VALTION TEKNILLINEN ET AL), 18 December 1997 (1997-12-18); page 2, line 16 - page 2, line 31; page 3, line 32 - page 4, line 8; page 4, line 12 - page 4, line 14; page 4, line 21 - page 4, line 24; page 5, line 20 - page 5, line 27; page 6, line 1 - page 6, line 2; page 6, line 12 - page 6, line 15; page 8, line 18 - page 8, line 22 --	1-15



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
"E" earlier application or patent but published on or after the international filing date	"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
"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)	"&" document member of the same patent family
"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	

Date of the actual completion of the international search

19-02-2016

Date of mailing of the international search report

19-02-2016

Name and mailing address of the ISA/SE
Patent- och registreringsverket
Box 5055
S-102 42 STOCKHOLM
Facsimile No. + 46 8 666 02 86

Authorized officer
Linus Plym Forshell
Telephone No. + 46 8 782 28 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2015/058278

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5928875 A (BREEN ALEXANDER W ET AL), 27 July 1999 (1999-07-27); column 1, paragraph [0001]; column 1, paragraph [0003]; column 2, paragraph [0003]; abstract --	1-15
A	WO 2004042082 A1 (KEMIRA OYJ ET AL), 21 May 2004 (2004-05-21); page 3, line 17 - page 3, line 25; page 4, line 17 - page 5, line 16 --	1-15
A	US 20130189152 A1 (RICE LAURA E ET AL), 25 July 2013 (2013-07-25); paragraphs [0003], [0013]-[0014]; abstract -- -----	1-15

Continuation of: second sheet

International Patent Classification (IPC)

C12Q 1/04 (2006.01)

C12Q 1/22 (2006.01)

C12Q 1/34 (2006.01)

C12Q 1/68 (2006.01)

D06M 16/00 (2006.01)

G01N 33/34 (2006.01)

D06M 101/06 (2006.01)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/IB2015/058278

US	20040014122 A1	22/01/2004	NONE		
WO	9747764 A1	18/12/1997	FI	962408 A0	11/06/1996
			FI	101809 B	31/08/1998
US	5928875 A	27/07/1999	AU	3572499 A	13/12/1999
			BR	9911593 A	02/10/2001
			CA	2331553 A1	02/12/1999
			CN	1307644 A	08/08/2001
			EP	1082455 A1	14/03/2001
			ID	28105 A	03/05/2001
			JP	2002516668 A	11/06/2002
			WO	9961667 A1	02/12/1999
WO	2004042082 A1	21/05/2004	AU	2003276306 A1	07/06/2004
US	20130189152 A1	25/07/2013	NONE		