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(54) Title: MICROBIOLOGICAL KERATIN PROCESSING

(57) Abstract: The invention relates to processing keratinous material by treating with keratinolytic microbes. The invention also relates to compositions and feed comprising keratinolytic microbes and at least partly hydroiyzed keratinous material.

MICROBIOLOGICAL KERATIN PROCESSING

FIELD OF THE INVENTION

The present invention relates to a novel way of processing keratin containing material by using keratinolytic microbes. The present invention also relates to manufacturing feed material from keratin containing raw material by said microbes. The present invention also relates to processing keratin containing material, such as feathers, into soluble and digestible protein rich material. The present invention also relates to novel bacteria that are useful in e.g. manufacturing feed from keratin containing material, such as feathers, hair, hooves and horns.

BACKGROUND

Feathers are composed of nearly pure keratin which is one of the most common proteins of the animal body. In birds, about 6% of the total weight of the animal is from feathers and feather waste is an important side stream of poultry industry. It is estimated that millions of tons of feathers is produced annually.

Currently, feathers produced in poultry industry are not utilized properly. Feathers are generally regarded as waste and processed by burning. Some of the feathers containing side streams are processed into feather meal by heating in high temperature and high pressure, followed by treatment with a strong base, such as NaOH.

Despite their high protein content, feathers are not as such suitable for feed production. The difficulty in processing feathers into feed is that keratin has a very stable structure which makes it poorly digestible. Previous processing methods involve hydrolysis in high temperature and pressure, as well as chemical treatment with strong acids or bases. Said methods require large amounts of energy, are expensive, and still leave the feather meal in a poorly digestible form which cannot be properly digested by animals.

Keratins are fibrous water-insoluble proteins that form the majority of the epidermis in vertebrates. Keratin is found e.g. in hair, nails, feathers, scales, wool and horns. Keratins are characterized by their high sulfur content caused by the sulfur containing amino acids: cysteine, methionine and cystine. Based on their sulfur content keratins are classified into hard keratins (such as hair, nail, feather, and wool having a lot of disulfide bonds) and soft keratins (such as skin containing fewer disulfide bonds).

Structurally keratin found in feathers is type β-keratin. In this structure the β-sheets are stacked tightly into superhelical polypeptide chains linked with numerous disulfide bonds, hydrogen bonds and hydrophobic interactions which makes the structure rigid and poorly soluble. As a result, β-keratin cannot be efficiently hydrolyzed by proteases such as trypsin, pepsin, papain, subtilisin and chymotrypsin.

Previous methods for the utilization of keratin for developing new and durable biotechnologies for the degradation of keratinous residues, especially feathers, into soluble and digestible protein rich material have applied methods with purified keratinolytic proteases. However, keratin is deficient in some nutritionally essential amino acids such as methionine and phenylalanine, and the keratin protein hydrolysates have to be enriched by adding essential amino acids for e.g. use as feed.

Vasileva-Tonkova et al. 2009 have suggested supplementing keratin hydrolysate with essential amino acids.

Bortsch and Coello 2005 have suggested using *R. rosea* LPB-3 for fermenting sterilized and heat treated feathers that are hammer milled before fermentation.

US 5,772,968 describes a system, including equipment and methods, to hydrolyze keratin based materials. The hydrolysis system makes use of an apparatus that promotes the breakdown of keratin by heat, expansion, stirring, mixing and drying. The hydrolysis system is used to promote keratin hydrolysis to increase its digestibility and that it can be used as animal feed supplement.

US 4,172,073 discloses keratin hydrolysis obtained of animals structures by using high-pressure saturated steam to get a meal soluble in water and suitable as animal feed.

WO 2010102362 discloses a method for manufacturing enzymatic preparations obtained from bird feathers. The method includes washing, drying and delipidation of chicken feathers before cultivation with *Bacillus* sp. in yeast extract containing medium and recovery of the resulting enzyme preparation.

Despite some progress in the field, the prior art has failed to provide an effective and environmentally sustainable method for processing keratinous material into soluble, more easily digestible protein rich biomass that can be directly utilized as a protein source in animal feed. Currently available methods rely on harsh processing conditions involving extensive use of energy and chemicals, including enzymes. Thus, there still exists a need to provide an economically feasible and effective

processing method to convert keratinous material into more easily digestible biomass that can be utilized in various applications including animal feed.

SUMMARY

An object of the invention was to at least partially solve or alleviate the aforementioned problems of prior art in processing keratinous material, such as feathers, into soluble protein rich material containing microbial biomass. The objects of the invention are achieved by using a microbiological process which utilizes the ability of certain microbes, identified by the present inventors, to solubilize keratinous material, according to what is stated in the independent claims. The preferred embodiments of the invention are discloses in the dependent claims.

The present invention is based on the identification and utilization of several keratin degrading microbes isolated from bird nest samples from Southern Finland. The present invention makes use of the unique ability of said microbes to degrade keratin containing material, such as feathers. The present inventors have found that the ability of the identified microbes to degrade keratin makes it possible to utilize said microbes in processing keratinous material even without pre-processing of the keratin containing material.

The present inventors describe for the first time several microbes isolated from bird nests that are shown to be able to degrade keratinous material. In particular, the inventive microbes make it possible to ferment keratinous material without chemical pre-treatment, optionally using only a mild thermal treatment for the keratinous material to reduce the amount of contaminating microbes. The inventive process is useful for producing e.g. feed material or other proteinaceous products from keratin containing material.

Another object of the present invention was to identify novel microbes which are able to degrade keratin and which can be used to improve the digestibility of keratin containing material by partially or completely transforming it into microbial biomass, which can then be used as feed material. In other words, the keratin containing material is not simply degraded, instead the product will contain both bacterial proteins and partially hydrolyzed keratin, because in the process part of the proteins from the keratin containing material will be converted into bacterial proteins. Thus, the amino acid composition of the proteins in the final product is different than that of (hydrolyzed) keratin alone. One advantage of this is that the final product has improved digestibility compared to keratin based proteins alone.

Accordingly, aspects of the present invention relate to a process of hydrolyzing keratinous material wherein biological material comprising keratin is treated in aqueous medium with microbes that are able to degrade keratin.

A significant advantage of the present invention is that the novel microbiological process abolishes the need to use pre-processing steps requiring large amounts of energy and harmful chemicals, and it also enables the valorization of a poorly utilizable feather side stream into more valuable products.

Further advantages of the present invention include: feather material can be converted into bacterial biomass which can as such be utilized as a protein source in animal nutrition. The use of microbial biomass as feed material is allowed in EU (Commission regulation 68/2013) and currently (August 2013) feather/microbial biomass could be used in fish, fur and pet animal feeds as a protein source. Especially in fish feed new protein sources are needed, since fish production relies largely on the use fish meal and imported soy, which is not sustainable.

In another aspect the invention relates to a composition comprising microbially treated biomass obtained by treating keratinous material with keratin degrading microbes according to the invention.

In another aspect the invention relates to protein rich feed material comprising keratinous material and keratinolytic microbes according to the invention.

In another aspect the invention relates to use of microbially treated mass manufactured using the process in which keratinous material is treated with keratin degrading microbes for manufacturing feed material, or fertilizer.

The characterizing features of the invention are presented in the appended claims.

DEFINITIONS

Unless otherwise specified, the terms, which are used in the specification and claims, have the meanings commonly used in the field. Specifically, the following terms have the meanings indicated below.

The terms "keratinolytic microbes" refers to particulate microbes that are able to degrade keratin. Examples of such microbes include gram-positive bacteria such as *Bacillus* and *Streptomyces*, yeast and fungi.

The term "protein rich fermented mass" refers to a composition comprising keratinous fermentation residue, which includes keratin-based proteins, keratinolytic bacteria and bacterial proteins.

The term "microbial biomass" or "bacterial biomass" refers to a composition containing both bacterial proteins and partially hydrolyzed keratin. Bacterial cells

are utilized as part of the biomass composition. The proteins from the keratin containing starting material have at least partly been converted into bacterial proteins. Thus, the amino acid composition of the proteins in the final product is different than that of (hydrolyzed) keratin alone.

The term feed material refers to materials that can be used as feed, as defined by the EU regulation 575/2011.

The term keratinous or keratin-containing material refers to all biological materials or biomass containing keratin proteins. Non-limiting examples of keratinous materials are feathers, hair, hooves and horns.

Unless otherwise noted, all percentages are by weight.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows the change in the weight of the feathers after bacterial fermentation. The larger the negative value, the more abundant degradation. The ability of the isolated strains to utilize feather material is shown measured as a reduction of feather material weight during incubation. The loss of weight (%) of the feather material during incubation is shown compared to the negative control without added bacteria. Each bar represents the mean of three repetitions. Asterisk (*) indicates isolates whose test results significantly differed from those of the negative control (t-test).

Figure 2 shows the correlation between feather mass loss and visual estimation of feather degradation, performed in triplicate. The following scale was used in visual estimation: 0: No visible degradation; 1: Only little degradation; a lot of intact feather fragments present; 2: Abundant degradation; some intact feather fragments present; 3: Abundant degradation; no intact feather fragments present

Figure 3 shows the feather degrading ability of various strains: Examples of feather degrading potential of some strains: **A)** visual estimation 0, strain Jh k2; **B)** visual estimation 1, strain Ep ek1; **C)** visual estimation 2, strain Sp k6; **D)** visual estimation 3, strain Mp k22.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have surprisingly found that keratin containing material can be effectively processed by treating said material with keratinolytic bacteria isolated from bird nests. The keratinolytic bacteria can be used to convert keratin containing material into soluble protein rich material (largely consisting of bacterial biomass) which is useful e.g. as a feed component.

Keratinolytic microbes according to the invention

Keratinolytic microbes were isolated from bird nest samples from Southern Finland. As is understood by a person skilled in the art, the particular species identified in this disclosure to possess keratinolytic activity are merely examples of suitable microbes to be used according to the invention. A skilled person is able to identify from bird nest samples alternative microbes of same or related species and/or genus by following the teaching provided in the present application.

The keratinolytic microbes are selected from gram-positive spore forming aerobic strains of bacilli. Suitably, the keratinolytic microbe according to the invention is non-pathogenic in humans and animals.

In one embodiment the keratinolytic microbes according to the invention comprise mixtures of aerobic keratinolytic bacteria.

The bacterial cultures may optionally comprise other additives generally used in the fermentation technology and in the manufacture of feed and feed additives, such as salts and additional nutrients.

In one embodiment the inventive microbes are selected from microbes that are able to grow on keratin substrate as the sole nitrogen and carbon source.

In one embodiment the gram-positive aerobic strains are selected from the bacterial group consisting of gram-positive genera *Bacillus*, *Paenibacillus*, *Rummeliibacillus*, and *Sporosarcina*; or combinations thereof.

In one embodiment the microbe according to the invention is selected from the bacteria belonging to the genus *Bacillus*.

In one embodiment the microbial strains belong to the gram-positive genera *Bacillus* spp. and related genera (Bacillales), preferably *B. methylotrophicus, B. subtilis, B. amyloliquefaciens, B. atrophaeus* and *B. licheniformis/sonorensis, Paenibacillus, Rummeliibacillus,* and *Sporosarcina,* or combinations thereof. In one non-limiting aspect the microbe is not *B. licheniformis* PWD-1.

In one embodiment, the gram-positive aerobic strains are selected from the group consisting of gram-positive aerobic strains of *Bacillus spp.*, preferably *B. methylotrophicus, B. subtilis, B. amyloliquefaciens, B. atrophaeus* and *B. sonorensis*, or combinations thereof.

In one embodiment the microbe according to the invention is selected from the group consisting of *B. methylotrophicus,* strain Ks k28*; B. methylotrophicus,* strain Ks k14*; B. subtilis,* strain Ep k11*; B. methylotrophicus,* strain Sp k8*; B. subtilis,* strain Sp k14*; B. methylotrophicus,* strain Ks k23*; B. methylotrophicus, strain* Sp k9*; B. methylotrophicus,* strain Pp k18*; B. methylotrophicus,* strain Sp k4*; B. amyloliquefaciens,* strain Ks k21*; B. atrophaeus,* strain Up k24*; B.*

amyloliquefaciens, strain Up k2*; B. methylotrophicus,* strain Tp itik2b*; B. licheniformis/sonorensis,* strain Mp k13*; B. subtilis,* strain Mp k19*; B. subtilis,* strain Ep k17*; B. plakortidis,* strain Sp k7*; B. methylotrophicus,* strain Sp k2*; B. aerophilus/altitudinis/stratophericus* strain Sp k30*; B. megaterium* strain Mp k22*; B. methylotrophicus,* strain Tmp k23*; B. licheniformis/sonorensis,* strain Sp k6*; B. licheniformis/sonorensis,* strain Up k23*; Rummeliibacillus stabekisii,* strain Ep k4*; Rummeliibacillus stabekisii,* strain Ap itik15*; B. pumilus/ safensis,* strain Sp k11*; and Rummeliibacillus stabekisii,* strain Pp k7.

A subset of all isolated strains has been deposited according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, 1977, in VTT culture collection, P.O. Box 1000, FI-02044 VTT, FINLAND (http://culturecollection.vtt.fi/), with the following codes (E-number):

B. methylotrophicus, Ks k28; E-133292
B. methylotrophicus, Ks k14; E-133293
B. subtilis, Ep k11; E-133294
B. methylotrophicus, Sp k8; E-133295
B. subtilis, Sp k14; E-133296
B. methylotrophicus Ks k23; E-133297
B. methylotrophicus, Sp k9; E-133298
B. methylotrophicus, Pp k18; E-133299
B. methylotrophicus, Sp k4; E-133300
B. amyloliquefaciens, Ks k21; E-133301
B. atrophaeus, Up k24; E-133302
B. amyloliquefaciens, Up k2; E-133303
B. methylotrophicus, Tp itik2b; E-133304
B. licheniformis/sonorensis Mp k13; E-133305
B. subtilis, Mp k19; E-133306
B. subtilis, Ep k17; E-133307
B. plakortidis, Sp k7; E-133308
B. methylotrophicus, Sp k2; E-133309
*B. aerophilus/ altitudinis/ stratosphericus*Sp k30; E-133310
B. megaterium Mp k22; E-133311
B. methylotrophicus, Tmp k23; E-133312
B. licheniformis/sonorensis, Sp k6; E-133314
B. licheniformis/sonorensis, Up k23; E-133315
Rummeliibacillus stabekisii, Ep k4; E-133316
Rummeliibacillus stabekisii, Ap itik15; E-133317
B. pumilus/ safensis, Sp k11; E-133318
Rummeliibacillus stabekisii, pp k7. E-133319

The keratinolytic microbes according to the invention can be used to process keratinous material into easily digestible proteinaceous material, i.e. microbial biomass.

Process of treating keratin containing material with microbes

In one embodiment the keratin containing material is treated by using a process comprising

1. Providing biological material comprising keratin in an aqueous medium
2. Adding keratinolytic microbes and, optionally, additional nutrients into the keratinous material to provide a mixture; and
3. Incubating the mixture in conditions promoting keratinolysis and the growth of the keratinolytic microbes to provide a protein rich biomass.

In the first step the keratinous material serves as the raw material of the process. The keratinous material may comprise e.g. feather, hair, wool, horn, skin or combinations thereof and it may optionally be pre-treated before processing. Suitably, the pre-treating comprises mechanical grinding and/or sterilization. Suitably, the keratin containing material is provided as a suspension, slurry or mixture of keratin containing material and water. Optionally, a suitable culture medium supporting growth of the keratinolytic microbes may be used instead of water. Due to heavy microbial contamination in certain types of keratinous raw material, such as feather, some kind of heat or other treatment may be needed to bring the levels of contaminating microbes down. This may be necessary for the protection of the process workers from potential animal pathogens and also to render the fermentation process more easily controllable as well as the final product safer for users (such as farmers) and the target animal species. Heat treatment is not necessary to facilitate keratinolysis and any suitable heat sterilization or other sterilization method can be used as long as it destroys potential pathogens. With fairly pure feather material mild heat-treatment at 60°C is enough. When keratinolysis is done with thermo-mechanical pretreatment the temperature applied is higher (133°C, 3 bar, 20 min). According to the invention both mild pasteurization at 60°C, as well as sterilization at 121°C, 15 min may be used.

In the second step keratinolytic microbes are added to the raw material comprising keratin. The raw material is inoculated by adding a pre-cultured microbial culture into the fermentation mixture. Suitably, microbial cultures may comprise one or more microbial strains, such as bacteria, preferably aerobic bacteria.

In the third step the fermenting conditions are selected so that they promote both keratinolysis and microbial growth, i.e. microbial biomass production. The exact conditions (e.g. growth temperature, time, nutrients added) of treatment depend on several parameters of the process and are strain-specific, as is understood by persons skilled in the art.

Optionally, the resulting biomass comprising microbes and keratinous material is dried by using a suitable method such as filtering, drying or centrifugation to remove excess water and optionally further dried and extruded into pellets, granules or dried into powder.

Suitably normal atmospheric pressure is used in the method.

Suitably, fermentation is carried out in aerobic conditions.

If desired, in order to improve keratinolysis, other additives may be used in the process to enhance keratinolysis and/or biomass growth, such as salts and other microbial nutrients.

In embodiment 1 the present invention provides a process of hydrolyzing keratinous material characterized in that the process comprises

a. Providing biological material comprising keratin in an aqueous medium;

b. Adding keratinolytic microbes into the keratinous material while mixing to provide a mixture; and

c. Fermenting the mixture at least partly in conditions promoting keratinolysis and/or growth of the keratinolytic microbes to provide a protein rich fermented mass,

wherein the keratinolytic microbe is selected from the group consisting of

B. methylotrophicus, Ks k28; deposited strain E-133292

B. methylotrophicus, Ks k14; deposited strain E-133293

B. subtilis, Ep k11; deposited strain E-133294

B. methylotrophicus, Sp k8; deposited strain E-133295

B. subtilis, Sp k14; deposited strain E-133296

B. methylotrophicus Ks k23; deposited strain E-133297

B. methylotrophicus, Sp k9; deposited strain E-133298

B. methylotrophicus, Pp k18; deposited strain E-133299

B. methylotrophicus, Sp k4; deposited strain E-133300

B. amyloliquefaciens, Ks k21; deposited strain E-133301

B. atrophaeus, Up k24; deposited strain E-133302

B. amyloliquefaciens, Up k2; deposited strain E-133303

B. methylotrophicus, Tp itik2b; deposited strain E-133304

B. licheniformis/sonorensis Mp k13; deposited strain E-133305

B. subtilis, Mp k19; deposited strain E-133306

B. subtilis, Ep k17; deposited strain E-133307

B. plakortidis, Sp k7; deposited strain E-133308

B. methylotrophicus, Sp k2; deposited strain E-133309

B. aerophilus/ altitudinis/ stratosphericus Sp k30; deposited strain E-133310

B. megaterium Mp k22; deposited strain E-133311

B. methylotrophicus, Tmp k23; deposited strain E-133312

B. licheniformis/sonorensis, Sp k6; deposited strain E-133314

B. licheniformis/sonorensis, Up k23; deposited strain E-133315

Rummeliibacillus stabekisii, Ep k4; deposited strain E-133316

Rummeliibacillus stabekisii, Ap itik15; deposited strain E-133317

B. pumilus/ safensis, Sp k11; deposited strain E-133318

Rummeliibacillus stabekisii, Pp k7: deposited strain E-133319;

or combinations thereof.

In embodiment 2 is provided the process according to embodiment 1 wherein the keratinous material is selected from the group consisting of feathers, hair, hooves, nails, horns and/or combinations thereof.

In embodiment 3 is provided the process according to embodiment 2 wherein the feathers are selected from major species (chickens or turkeys for fattening, laying hens), minor species (other poultry for fattening and other laying birds) as defined by EU regulation 1831/2003 and 429/2008, and/or combinations thereof.

In embodiment 4 is provided the process according to any one of embodiments 1-3 wherein the biological material is pre-treated by milling, thermal treatment, chemical treatment, enzymatic treatment and/or combinations thereof.

In embodiment 5 is provided the process according to any one of embodiments 1-4 wherein the keratinous material serves as the sole carbon and nitrogen source of the keratinolytic microbe.

In embodiment 6 is provided the process according to any one of embodiments 1-5 wherein fermentation is carried out in aerobic conditions.

In embodiment 7 is provided the process according to any one of embodiments 1-6 wherein after the fermenting step the process comprises a filtering step, a

centrifugation step or a drying step to increase the solids content of the fermented mass.

In embodiment 8 is provided the process according to any one of embodiments 1-7 wherein a component selected from protease, keratinase, polysaccharide, microbe, keratin hydrolysate or a combination thereof is recovered from the at least partly fermented mixture.

In embodiment 9 is provided composition comprising protein rich fermented mass produced according to any one of embodiments 1-8.

In embodiment 10 is provided protein rich feed material characterized in that the protein rich feed comprises keratinous material and keratinolytic bacteria selected from the group consisting of

a. gram-positive aerobic strains of bacilli, preferably *B. methylotrophicus, B. subtilis, B. amyloliquefaciens, B. atrophaeus, B. sonorensis, B. plakortidis, B. aerophilus/ altitudinis/stratosphericus, B. megaterium, B. pumilus/safensis, Rummeliibacillus stabekisii;* or

b. *B. methylotrophicus,* strain Ks k28*; B. methylotrophicus,* strain Ks k14*; B. subtilis,* strain Ep k11*; B. methylotrophicus,* strain Sp k8*; B. subtilis,* strain Sp k14*; B. methylotrophicus,* strain Ks k23*; B. methylotrophicus,* strain Sp k9*; B. methylotrophicus,* strain Pp k18*; B. methylotrophicus, strain* Sp k4*; B. amyloliquefaciens,* strain Ks k21*; B. atrophaeus,* strain Up k24*; B. amyloliquefaciens,* strain Up k2*; B. methylotrophicus,* strain Tp itik2b*; B. licheniformis/sonorensis,* strain Mp k13*; B. subtilis,* strain Mp k19*; B. subtilis,* strain Ep k17*; B. plakortidis,* strain Sp k7*; B. methylotrophicus,* strain Sp k2*; B. aerophilus/altitudinis/stratosphericus* strain Sp k30*; B. megaterium* strain Mp k22*; B. methylotrophicus,* strain Tmp k23*; B. licheniformis/sonorensis,* strain Sp k6*; B. licheniformis/sonorensis,* strain Up k23*; Rummeliibacillus stabekisii,* strain Ep k4*; Rummeliibacillus stabekisii,* strain Ap itik15*; B. pumilus/ safensis,* strain Sp k11*;* and *Rummeliibacillus stabekisii,* strain Pp k7, or combinations thereof.

In a specific embodiment of embodiment 10, the gram-positive aerobic strains in alternative a. are selected from the group consisting of gram-positive aerobic strains of *B. methylotrophicus, B. subtilis, B. amyloliquefaciens, B. atrophaeus* and *B. sonorensis*, or combinations thereof.

In embodiment 11 is provided protein rich feed material characterized in that the feed material is manufactured from keratin containing material by

c. hydrolyzing keratinous material according to the process of any one of embodiments 1-8;
d. optionally supplementing the protein rich fermented mass with other feed materials and additives to produce a nutritionally balanced feed for the target animal; and
e. optionally drying the provided mass to provide a slurry, paste, pellets, granules, or powder; and wherein

the protein rich feed comprises keratinolytic microbes used in fermenting.

In embodiment 12 is provided protein rich feed according to embodiment 11 wherein the protein rich feed comprises composition according to embodiment 9.

In embodiment 13 is provided the protein rich feed according to embodiment 11 or 12 wherein the keratinolytic microbe is selected from the group consisting of

B. methylotrophicus, Ks k28; deposited strain E-133292

B. methylotrophicus, Ks k14; deposited strain E-133293

B. subtilis, Ep k11; deposited strain E-133294

B. methylotrophicus, Sp k8; deposited strain E-133295

B. subtilis, Sp k14; deposited strain E-133296

B. methylotrophicus Ks k23; deposited strain E-133297

B. methylotrophicus, Sp k9; deposited strain E-133298

B. methylotrophicus, Pp k18; deposited strain E-133299

B. methylotrophicus, Sp k4; deposited strain E-133300

B. amyloliquefaciens, Ks k21; deposited strain E-133301

B. atrophaeus, Up k24; deposited strain E-133302

B. amyloliquefaciens, Up k2; deposited strain E-133303

B. methylotrophicus, Tp itik2b; deposited strain E-133304

B. licheniformis/sonorensis Mp k13; deposited strain E-133305

B. subtilis, Mp k19; deposited strain E-133306

B. subtilis, Ep k17; deposited strain E-133307

B. plakortidis, Sp k7; deposited strain E-133308

B. methylotrophicus, Sp k2; deposited strain E-133309

B. aerophilus/ altitudinis/ stratosphericus Sp k30; deposited strain E-133310

B. megaterium Mp k22; deposited strain E-133311

B. methylotrophicus, Tmp k23; deposited strain E-133312

B. licheniformis/sonorensis, Sp k6; deposited strain E-133314

B. licheniformis/sonorensis, Up k23; deposited strain E-133315

Rummeliibacillus stabekisii, Ep k4; deposited strain E-133316

Rummeliibacillus stabekisii, Ap itik15; deposited strain E-133317

B. pumilus/ safensis, Sp k11; deposited strain E-133318

Rummeliibacillus stabekisii, pp k7; deposited strain E-133319;

or combinations thereof.

In embodiment 17 is provided use of the fermented mass manufactured using the process according to any one of embodiments 1-11 for manufacturing feed, feed supplement, food product, food additive, dietary supplement, fertilizer, cosmetic compound, material component for production of paper, cardboard, moldable packaging, nonwovens, textiles, filters, coatings, films, foams or composites.

The following examples are illustrative embodiments of the present invention and they are not meant to limit the invention in any way.

EXAMPLES

EXAMPLE 1 - Bird nest and feather samples

Bird nest samples (containing mainly feather but also moss, hay, bark, etc.) were obtained from Finland, from various little birds' nests.

EXAMPLE 2 - Isolation of keratinolytic microbes

Feather containing sample material was mixed with peptone buffer (1.25 g sample in 50 ml buffer) and thoroughly mixed with Stomacher for 4 times 5 min. Part of the sample was heat treated (20 min at 75°C) to destroy the vegetative cells. Both heat treated and non-heat treated samples were serially diluted and inoculated onto milk and *Bacillus* agar and aerobically incubated for 3 d at 30°C.

An optional way to isolate keratinolytic bacteria was first to mix the sample (1 g) with sporulation medium (40 min) as above. The mixture was then transferred into Schott bottle and incubated for 3 d at 30°C under agitation (100 rpm/min) in aerobic conditions. Thereafter the heat treatment and the culture were performed as above (for heat treated and non-heat treated samples).

Individual bacterial isolates were picked from agar plates and cultured on *Bacillus* agar plates and finally stored at -80°C in beads. Pure cultures were the inoculated on milk plates to check for caseinolytic activity. All caseinolytic isolates were then inoculated on feather agar plates or feather broth to identify the keratinolytic isolates among the caseinolytic ones. Feather agar plates contained only pre-treated feathers (washed, heat-treated to reduce the contaminating microbial load and mechanically ground to reduce the particle size), water and some salts (NaCl, Na_2HPO_4 x 2 H_20, NaH_2PO_4 x 2 H_20) (and agar) to improve the buffering capacity of the medium. For the feather broth the feathers were not ground, only heat-treated. Keratinolytic isolates were identified based on the clear halo that formed around the bacterial colonies.

At this point the following keratinolytic bacteria were identified from the following phyla, class, order, family, and genera:

phylum "Proteobacteria"
class Gammaproteobacteria
order Pseudomonadales
family Pseudomonadaceae
genus *Pseudomonas*
order Xanthomonadales
family Xanthomonadaceae
phylum Firmicutes
class Bacilli
order Bacillales
family Planococcaceae
genus *Rummeliibacillus*
genus Sporosarcina
family Bacillaceae 1
genus *Bacillus*
family Bacillales Incertae Sedis XII
genus Exiguobacterium
family Paenibacillaceae
genus *Paenibacillus*

In case microbes other than bacteria are isolated, the above protocol can easily be modified by replacing the *Bacillus* agar plates with another selective plate (in the case of yeast and molds e.g. PDA (potato dextrose agar) supplemented with antibiotics that kill bacteria (e.g. chloramphenicol)).

EXAMPLE 3 – Analysis of keratinolytic activity

Keratinolytic activity of the isolates was confirmed by re-inoculation the isolates into feather broth containing washed, sterilized (heat-treatment at 121°C for 15 min) and whole feathers, water and salts (NaCl, Na_2HPO_4 x 2 H_20, NaH_2PO_4 x 2 H_20). No other nitrogen or carbon source than feathers was necessary. Aerobic incubation was performed at 30°C and the bacterial growth was measured at several time points by culturing.

EXAMPLE 4 – Identification and characterization of keratinolytic bacteria

The identification of the keratinolytic isolates was based on partial 16S rRNA gene sequencing. Sequences were analyzed with RDP 10.31 (Ribosomal Database Project, https://rdp.cme.msu.edu/index.jsp), (EMBL-EBI) ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and DNAMAN (Lynnon Corporation, versio 4.1). Different isolates representing the same species were initially fingerprinted with RAPD (random amplified polymorphic DNA) using different primers for Bacillales, Xanthomonadales, and Pseudomonadales to exclude the incorporation of the same strain twice. All isolates with similar RAPD type within a single sample were considered to represent the same strain.

Table 1. Primers used in identification and sequencing.

Task	Primer	Sequence	Reference
Universal bacterial PCR and sequencing, Forward	7-f SEQ ID NO: 1	AGAGTTTGAT(C/T)(A/C)TGGCTCAG	(Satokari et al. 2001)
Reverse	1510-r SEQ ID NO: 2	ACGG(C/T)TACCTTGTTACGACTT	
RADP-PCR, Bacillales	OPL-01 SEQ ID NO: 3	GGCATGACCT	
RAPD-PCR, Xanthomonadales	OPA-2 SEQ ID NO: 4	TGCCGAGCTG	(Mättö et al. 2004)
RAPD-PCR, Pseudomonadales	OPA-3 SEQ ID NO: 5	AGTCAGCCAC	(Mättö et al. 2004)

Table 2. PCR programs used.

	Temp, °C	Time	Temp, °C	Time	Temp, °C	Time

	Univ. bacterial PCR		Sequencing PCR		RAPD	
Initial denaturation	94	5 min	96	1 min	95	5 min
Denaturation	94	45 s	96	10 s	95	1 min
Primer attachment	56	45 s	50	5 s	32	1 min
Elongation	72	2 min	60	4 min	72	2 min
Final elongation	72	7 min			72	10 min
	35 cycles		25 cycles		35 cycles	

EXAMPLE 5. Keratinolytic activity of selected strains on feather

The ability of the isolated strains to utilize feather material – measured as a reduction of feather material weight during incubation – is demonstrated in Figure 1. For the study bacterial isolates (taken from frozen stock) were grown in *Bacillus* broth overnight, cell density adjusted to McFarland 1.5 and feather broth bottles (70 ml; composition as above) inoculated with 1% bacterial inoculum in triplicate. Bottles were aerobically incubated at 30 °C for 2 days under agitation (170 rpm/min). After incubation the broth was filtered (Whatman 41; pore size 20 µm), washed and then filters were dried at 60 °C and weighed.

Feather degradation was also visually evaluated and recorded. Visual inspection of the cultures proved that reduction in feather material weight was a good indicator of feather utilization (comparison shown in Figure 2).

Figure 3 also shows examples of test bottles and the various performances of the bacterial strains studied.

Table 3. The most efficient strains in the utilization of feather material.

Species	Strain	Reduction in the weight of the feathers (%)
B. methylotrophicus	Ks k28	30
B. methylotrophicus	Ks k14	28
B. subtilis	Ep k11	26
B. methylotrophicus	Sp k8	25
B. subtilis	Sp k14	25
B. methylotrophicus	Ks k23	25
B. methylotrophicus	Sp k9	24
B. methylotrophicus	Pp k18	24
B. methylotrophicus	Sp k4	23
B. amyloliquefaciens	Ks k21	23
B. atrophaeus	Up k24	23
B. amyloliquefaciens	Up k2	22
B. methylotrophicus	Tp itik2b	20
B. licheniformis/sonorensis	Mp k13	20
B. subtilis	Mp k19	19
B. subtilis	Ep k17	18
B. plakortidis	Sp k7	18
B. methylotrophicus	Sp k2	17
B. aerophilus/ altitudinis/ stratosphericus	Sp k30	17
B. megaterium	Mp k22	16
B. methylotrophicus	Tmp k23	16
B. licheniformis/sonorensis	Sp k6	14
B. licheniformis/sonorensis	Up k23	14
Rummeliibacillus stabekisii	Ep k4	13
Rummeliibacillus stabekisii	Ap itik15	12
B. pumilus/ safensis	Sp k11	11
Rummeliibacillus stabekisii	pp k7	10

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Mättö J, Malinen E, Suihko M-, Alander P, Palva A & Saarela M. 2004. Genetic heterogeneity and functional properties of intestinal bifidobacteria. Journal of Applied Microbiology 97, 459-470.

Satokari RM, Vaughan EE, Akkermans AD, Saarela M & de Vos WM. 2001. Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 67, 504-513.

Vasileva-Tonkova, E., Gousterova, A. & Neshev, G. 2009. Ecologically safe method for improved feather wastes biodegradation. International Biodeterioration & Biodegradation 63: 1008–1012.

CLAIMS

1. A process of hydrolyzing keratinous material **characterized** in that the process comprises

   a. providing biological material comprising keratin in an aqueous medium;

   b. adding keratinolytic microbes into the keratinous material while mixing to provide a mixture; and

   c. fermenting the mixture at least partly in conditions promoting keratinolysis and/or growth of the keratinolytic microbes to provide a protein rich fermented mass,

   wherein the keratinolytic microbe is selected from the group consisting of

   B. methylotrophicus, Ks k28; deposited strain E-133292

   B. methylotrophicus, Ks k14; deposited strain E-133293

   B. subtilis, Ep k11; deposited strain E-133294

   B. methylotrophicus, Sp k8; deposited strain E-133295

   B. subtilis, Sp k14; deposited strain E-133296

   B. methylotrophicus Ks k23; deposited strain E-133297

   B. methylotrophicus, Sp k9; deposited strain E-133298

   B. methylotrophicus, Pp k18; deposited strain E-133299

   B. methylotrophicus, Sp k4; deposited strain E-133300

   B. amyloliquefaciens, Ks k21; deposited strain E-133301

   B. atrophaeus, Up k24; deposited strain E-133302

   B. amyloliquefaciens, Up k2; deposited strain E-133303

   B. methylotrophicus, Tp itik2b; deposited strain E-133304

   B. licheniformis/sonorensis Mp k13; deposited strain E-133305

   B. subtilis, Mp k19; deposited strain E-133306

   B. subtilis, Ep k17; deposited strain E-133307

   B. plakortidis, Sp k7; deposited strain E-133308

   B. methylotrophicus, Sp k2; deposited strain E-133309

   B. aerophilus/ altitudinis/ stratosphericus Sp k30; deposited strain E-133310

B. megaterium Mp k22; deposited strain E-133311

B. methylotrophicus, Tmp k23; deposited strain E-133312

B. licheniformis/sonorensis, Sp k6; deposited strain E-133314

B. licheniformis/sonorensis, Up k23; deposited strain E-133315

Rummeliibacillus stabekisii, Ep k4; deposited strain E-133316

Rummeliibacillus stabekisii, Ap itik15; deposited strain E-133317

B. pumilus/ safensis, Sp k11; deposited strain E-133318

Rummeliibacillus stabekisii, Pp k7: deposited strain E-133319;

or combinations thereof.

2. The process according to claim 1, wherein the keratinous material is selected from the group consisting of feathers, hair, hooves, nails, horns and/or combinations thereof.
3. The process according to claim 2, wherein the feathers are selected from the major species (chickens or turkeys for fattening, laying hens), minor species (other poultry for fattening and other laying birds) as defined by EU regulations 1831/2003; 429/2008, and/or combinations thereof.
4. The process according to any one of claims 1–3, wherein the biological material is pre-treated by milling, thermal treatment, chemical treatment, enzymatic treatment and/or combinations thereof.
5. The process according to any one of claims 1–4, wherein the keratinous material serves as the sole carbon and nitrogen source of the keratinolytic microbe.
6. The process according to any one of claims 1–5, wherein fermentation is carried out in aerobic conditions.
7. The process according to any one of claims 1-6, wherein after the fermenting step the process comprises a filtering step, a centrifugation step or a drying step to increase the solids content of the fermented mass.
8. The process according to any one of claims 1-7, wherein a component selected from protease, keratinase, polysaccharide, microbe, keratin hydrolysate or a combination thereof is recovered from the at least partly fermented mixture.
9. Composition comprising protein rich fermented mass produced according to any one of claims 1-8.
10. Protein rich feed material, **characterized** in that the protein rich feed material comprises keratinous material and keratinolytic bacteria selected from the group consisting of

a. gram-positive aerobic strains of bacilli, selected from *B. methylotrophicus, B. subtilis, B. amyloliquefaciens, B. atrophaeus, B. sonorensis, B. plakortidis, B. aerophilus/altitudinis/stratosphericus, B. megaterium, B. pumilus/safensis, or Rummeliibacillus stabekisii;* or

b. *B. methylotrophicus,* strain Ks k28*; B. methylotrophicus,* strain Ks k14*; B. subtilis,* strain Ep k11*; B. methylotrophicus,* strain Sp k8*; B. subtilis,* strain Sp k14*; B. methylotrophicus,* strain Ks k23*; B. methylotrophicus,* strain Sp k9*; B. methylotrophicus,* strain Pp k18*; B. methylotrophicus, strain* Sp k4*; B. amyloliquefaciens,* strain Ks k21*; B. atrophaeus,* strain Up k24*; B. amyloliquefaciens,* strain Up k2*; B. methylotrophicus,* strain Tp itik2b*; B. licheniformis/sonorensis,* strain Mp k13*; B. subtilis,* strain Mp k19*; B. subtilis,* strain Ep k17*; B. plakortidis,* strain Sp k7*; B. methylotrophicus,* strain Sp k2*; B. aerophilus/altitudinis/stratosphericus* strain Sp k30*; B. megaterium* strain Mp k22*; B. methylotrophicus,* strain Tmp k23*; B. licheniformis/sonorensis,* strain Sp k6*; B. licheniformis/sonorensis,* strain Up k23*; Rummeliibacillus stabekisii,* strain Ep k4*; Rummeliibacillus stabekisii,* strain Ap itik15*; B. pumilus/ safensis,* strain Sp k11*;* and *Rummeliibacillus stabekisii,* strain Pp k7, or combinations thereof.

11. Protein rich feed material, **characterized** in that the feed material is manufactured from keratin containing material by

a. hydrolyzing keratinous material according to the process of any one of claims 1-8;

b. optionally supplementing the protein rich fermented mass with other feed materials and additives to produce a nutritionally balanced feed for the target animal; and

c. optionally drying the provided mass to provide a slurry, paste, pellets, granules, or powder; and wherein the protein rich feed material comprises keratinolytic microbes used in fermenting.

12. The protein rich feed material according to claim 11 wherein the protein rich feed material comprises composition according to claim 9.

13. The protein rich feed material according to claim 11 or 12 wherein the keratinolytic microbe is selected from the group consisting of

B. methylotrophicus, Ks k28; deposited strain E-133292

B. methylotrophicus, Ks k14; deposited strain E-133293

B. subtilis, Ep k11; deposited strain E-133294

B. methylotrophicus, Sp k8; deposited strain E-133295

B. subtilis, Sp k14; deposited strain E-133296

B. methylotrophicus Ks k23; deposited strain E-133297

B. methylotrophicus, Sp k9; deposited strain E-133298

B. methylotrophicus, Pp k18; deposited strain E-133299

B. methylotrophicus, Sp k4; deposited strain E-133300

B. amyloliquefaciens, Ks k21; deposited strain E-133301

B. atrophaeus, Up k24; deposited strain E-133302

B. amyloliquefaciens, Up k2; deposited strain E-133303

B. methylotrophicus, Tp itik2b; deposited strain E-133304

B. licheniformis/sonorensis Mp k13; deposited strain E-133305

B. subtilis, Mp k19; deposited strain E-133306

B. subtilis, Ep k17; deposited strain E-133307

B. plakortidis, Sp k7; deposited strain E-133308

B. methylotrophicus, Sp k2; deposited strain E-133309

B. aerophilus/ altitudinis/ stratosphericus Sp k30; deposited strain E-133310

B. megaterium Mp k22; deposited strain E-133311

B. methylotrophicus, Tmp k23; deposited strain E-133312

B. licheniformis/sonorensis, Sp k6; deposited strain E-133314

B. licheniformis/sonorensis, Up k23; deposited strain E-133315

Rummeliibacillus stabekisii, Ep k4; deposited strain E-133316

Rummeliibacillus stabekisii, Ap itik15; deposited strain E-133317

B. pumilus/ safensis, Sp k11; deposited strain E-133318

Rummeliibacillus stabekisii, pp k7; deposited strain E-133319;

or combinations thereof.

14. Use of the fermented mass manufactured using the process according to any one of claims 1-8 for manufacturing feed material, feed additive, food product, food additive, dietary supplement, fertilizer, cosmetic compound, material component for production of paper, cardboard, moldable packaging, nonwovens, textiles, filters, coatings, films, foams or composites.

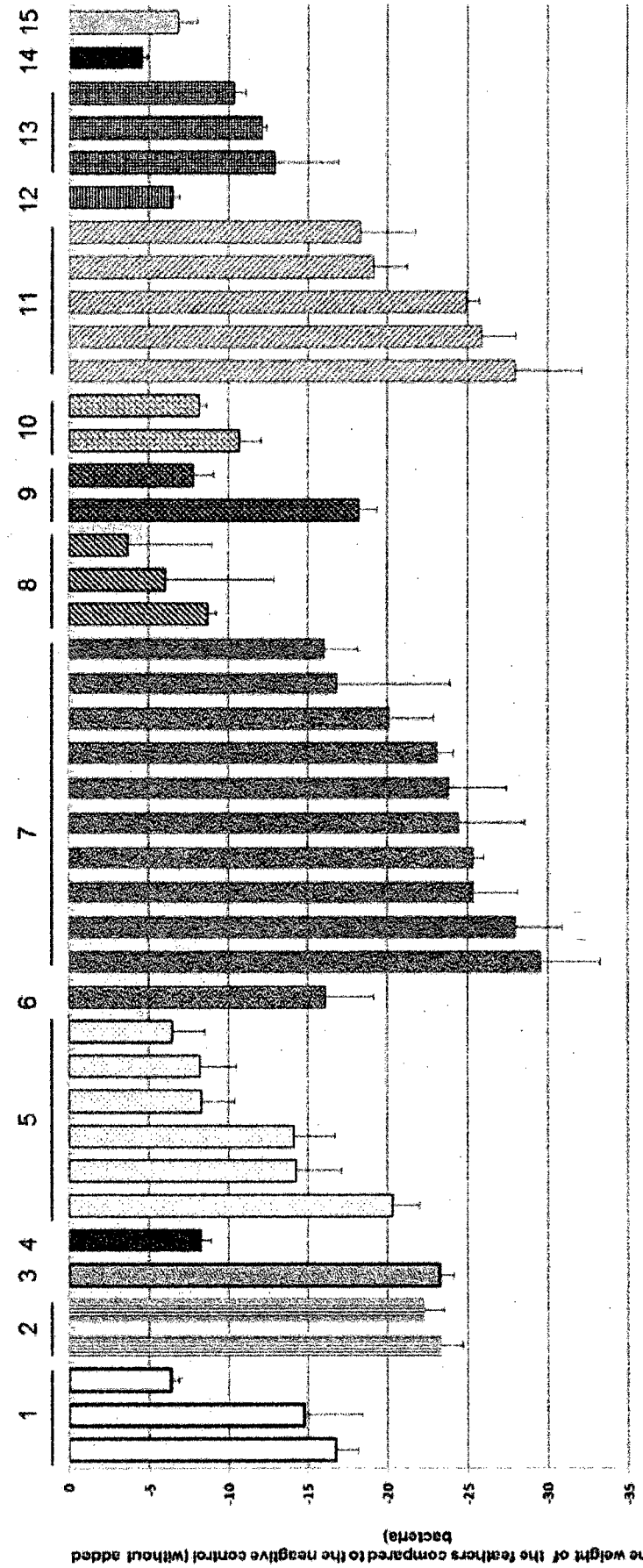
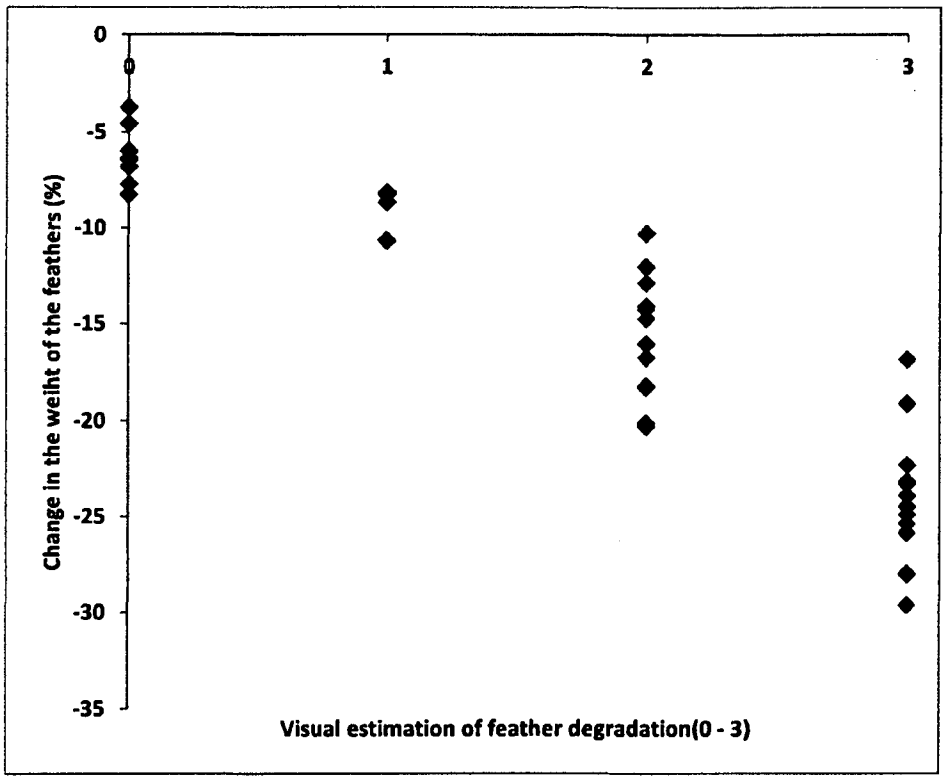

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
0 -5 -10 -15 -20 -25 -30 -35
Change (%) in the weight of the feathers compared to the negative control (without added bacteria)
1) B. aerophilus/ altitudins/ stratosphericus sp k30, up k3, sp k 13
2) B. amyloliquefaciens ks k21, up k2
3) B. atrophaeus up k24
4) B. hwajinpoensis ks k25
5) B. licheniformis/ sonorensis mp k13, up.k23, sp k6, sp, k1, sp k21, sp k28
6) B. megaterium mp k22
7) B. methylotrophicus ks k28, ks k14, ks k23, sp k8, sp k9, pp k16, sp k4, sp k2, tp itik2b, tmp k23
8) B. mycoides/ weihenstephanensis ep ek1, ek k22, jh k2
9) B. plakortidis sp k7, up k4
10) B. pumilus/ safensis sp k11, mp k11
11) B. subtilis tmp k17, ep k11, sp k14, mp k19, ep k 17
12) P. amylolyticus/ xylanexedens mp k25
13) R. stabekisii ep k4, ap itik15, pp k7
14) S. koreensis ks y1 k8
15) S. urease up k6

FIG. 1

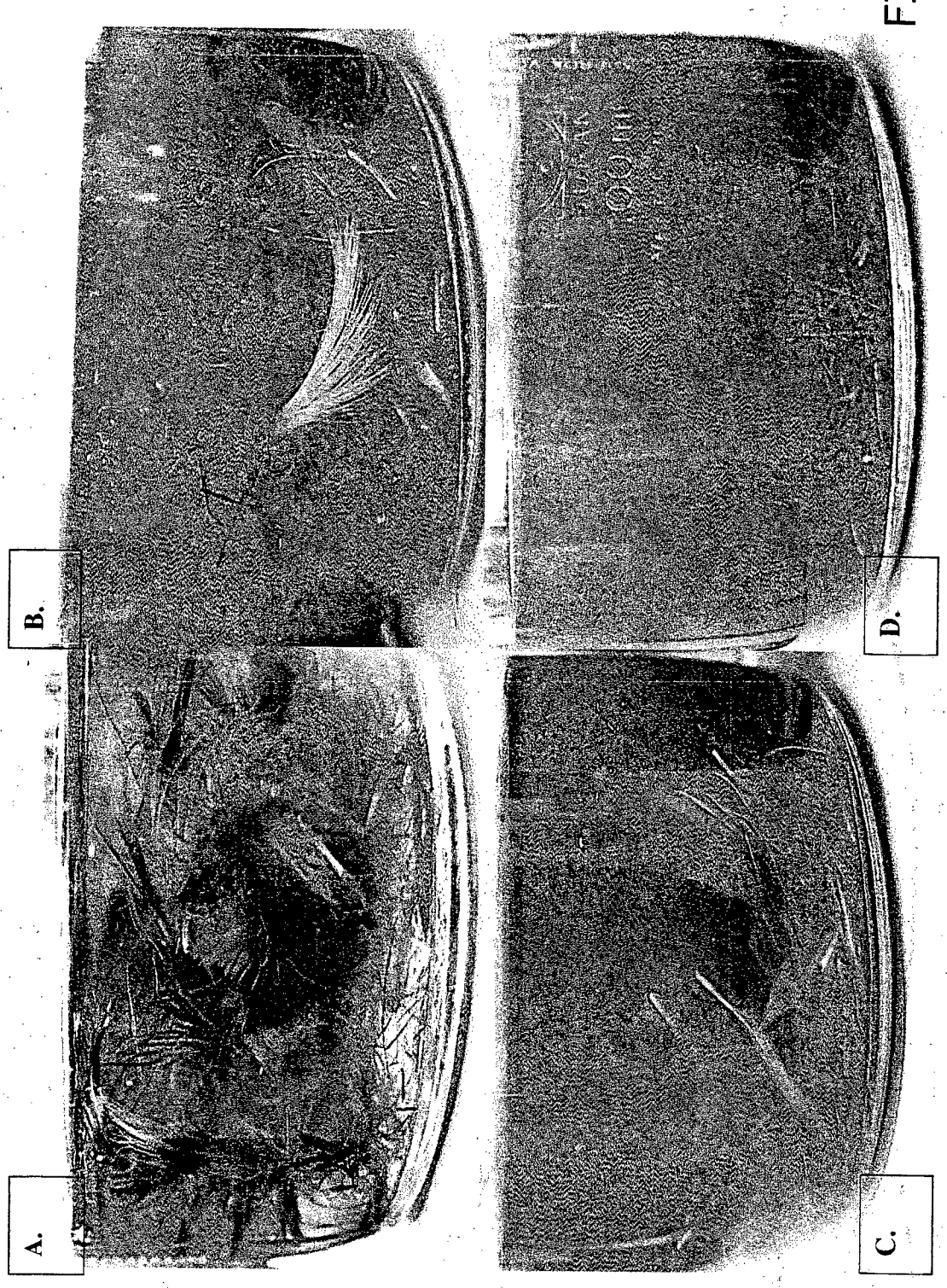


Visual values:

0	**No visible degradation**
1	Only little degradation; a lot of intact feather fragments present
2	Abundant degradation; some intact feather fragments present
3	Abundant degradation; no intact feather fragments present

FIG. 2

FIG. 3



INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI2014/000019

A. **CLASSIFICATION OF SUBJECT MATTER**

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: C12P, A23K

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

FI, SE, NO, DK

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

EPO-Internal, WPI, BIOSIS, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 101869184 B (HIGH TECH RES CT OF SHANDONG ACADEMY OF AGRICULTURAL SCIENCES) 27 June 2012 (27.06.2012)	9-14
A	& machine translation into English by Questel on STN [online], STN CNFULL [retrieved 24.11.2014] machine translation into English, page 1, Abstract; pages 3-5, Invention content	1-8
X	CN 102517235 B (UNIV HUNAN AGRICULTURAL) 16 January 2013 (16.01.2013) & machine translation into English by Questel on STN [online] , STN CNFULL [retrieved 25.11.2014] machine translation into English, page 1, Abstract; page 4, Invention content, paragraph 4; page 5, paragraph 3; pages 7-8, Implementation example 4; claims 1-6	9, 14

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 November 2014 (26.11.2014)	03 December 2014 (03.12.2014)

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INTERNATIONAL SEARCH REPORT

International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Fakhfakh Nahed et al. Total solubilisation of the chicken feathers by fermentation with a keratinolytic bacterium, Bacillus pumilus A1, and the production of protein hydrolysate with high antioxidative activity. Process Biochemistry, September 2011. Vol. 46, No. 9, p. 1731-1737 Abstract; page 1732, chapter 3.1.1 Effect of feather concentration, page 1736, paragraphs 1 and 2	9, 14
X	Fakhfakh N. et al. Wool-waste valorization: production of protein hydrolysate with high antioxidative potential by fermentation with a new keratinolytic bacterium, Bacillus pumilus A1. Journal of Applied Microbiology, August 2013, Vol. 115, No. 2, p. 424-433 (Article first published online: 3 June 2013) Abstract	9, 14
X	Kumar, E. V. et al. Biodegradation of poultry feathers by a novel bacterial isolate Bacillus altitudinis GVC11. Indian Journal of Biotechnology, October 2011, Vol. 10, No. 4, p. 502-507 the whole document	9, 14
X	Agrahari S. et al. Isolation and characterization of feather degrading enzymes from Bacillus megaterium SN1 isolated from Ghazipur poultry waste site. Applied Biochemistry and Microbiology, March 2012, Vol. 48, No. 2, p. 175-181 Abstract; page 176, paragraph 3; page 177, paragraph 6; page 180, paragraph 4	9, 14

INTERNATIONAL SEARCH REPORT
Information on Patent Family Members

International application No.
PCT/FI2014/000019

Patent document cited in search report	Publication date	Patent family members(s)	Publication date
CN 101869184 B	27/06/2012	CN 101869184 B	27/06/2012
CN 102517235 B	16/01/2013	CN 102517235 B	16/01/2013

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CLASSIFICATION OF SUBJECT MATTER

IPC
C12P 21/06 (2006.01)
A23K 1/00 (2006.01)
A23K 1/10 (2006.01)
C12N 1/20 (2006.01)
C12R 1/07 (2006.01)