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(54) INGREDIENTS FOR ANIMAL FEED COMPOSITIONS

ZUTATEN FÜR TIERFUTTERZUSAMMENSETZUNGEN

INGRÉDIENTS POUR COMPOSITIONS D'ALIMENTATION D'ANIMAUX

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(56) References cited:

EP-A1- 2 218 773 WO-A1-97/10352
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- **WEON HANG-YEON ET AL: "Deinococcus cellulosityticus sp nov., isolated from air", INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY, SOCIETY FOR GENERAL MICROBIOLOGY, READING, GB, vol. 57, no. Part 8, 1 August 2007 (2007-08-01), pages 1685-1688, XP002534882, ISSN: 1466-5026, DOI: 10.1099/IJS.0.64951-0**
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EP 2 793 607 B9

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DescriptionFIELD OF THE INVENTION

5 [0001] The present application discloses ingredients for animal feed compositions which are not part of the invention for enhancing animal growth and/or animal health. The present invention relates to methods for producing ingredients. The methods disclosed herein further allow improving the palatableness and/or digestibility of feed compositions. More specifically, the invention describes the use of a mix of *Deinococcus* bacteria and biomass as a supply of organic constituents in feed compositions.

BACKGROUND OF THE INVENTION

10 [0002] The feeding of livestock, such as pets, cattle, ovine, porcine, poultry, fish, -including commercial and ornamental fish-, schrimp and other crustaceans, is a constant problem in the field of animal breeding and agro-food industry. Because of the limited production facilities and lack of improvement in production technology, animal breeding is both costly and time-consuming. To improve the production and the profitability of animal breeding, the most common solution is to provide industrial feed compositions, at least to complete the diet of the animals.

15 [0003] While feedstuffs are not of constant composition, several constituents are required to fulfil the nutritional goal. Indeed, even if the biological response to the feed depends on the ability of the animal to derive useful nutrients from said feed, the feedstuffs used in the feeding generally comprise digestible proteins, lipids, carbohydrates and fiber.

20 [0004] Animal feed compositions are usually formulated with low cost formulations. Of particular importance is the limited supply of digestible energy and the associated cost. The most costly nutrients are proteins and amino acids, including the essential amino acids. However, the production of animals that contain a significant rate of proteins would be necessary to proportionate the increase in world population.

25 [0005] The use of fish meal or meat and bone meal as protein raw material has been developed because of its low cost. Indeed, meat and bone meal is a product of the rendering industry that comprises about 50% protein, and 8-12% fat. It is primarily used in the formulation of animal feed to improve the amino acid profile of the feed. However, the feeding of bone meal as protein to cattle is thought to have been responsible for the spread of mad cow disease. Consequently, in most parts of the world, the use of bone meal as protein supply is no longer allowed in feed for ruminant animals.

30 [0006] Another raw material used for preparing feed compositions for ruminant animals is cellulosic material, which is a cheap and non direct valuable material. However, even if enzymes in ruminants allow the degradation of cellulose, several pre-treatments are performed to partially hydrolyze the cellulose and hemicellulose in order to help the further degradation by ruminant animals and enhance the digestibility of this raw material. For example, the addition of various exogenous enzymes, such as cellulases and hemicellulases, to the feedstuff comprising cellulosic material is useful to decrease polymer crystallinity and to increase access to the polymer backbone. In the same way, the thermo-chemical pretreatment of the cellulosic material is also possible to partially hydrolyse the hemicellulose and cellulose portions.

35 [0007] Although these treatments improve carbohydrate digestibility and more generally the bioavailability of nutriments of the cellulosic material, they can be expensive and economically prohibitive. In addition, even if pretreated, the cellulosic material is still indigestible and is therefore incapable of providing nourishment for the non ruminant animals, such as pigs and poultry. Furthermore, these treatments do not provide proteins, which are the most expensive constituent in animal feed compositions.

40 [0008] Consequently, there is a need of less expensive, safer and more nutritious food to commercial farm and battery animals, including both ruminant and non ruminant animals, commercial fish and schrimp aquaculture, and pets.

45 [0009] It is noted that patent applications EP 2 218 773, WO 2011/107506 and WO 2009/063079 disclose bacterial strains which are able to digest lignocellulosic biomass.

SUMMARY OF THE INVENTION

50 [0010] The present invention concerns the subject-matter of the claims.

[0011] The present invention discloses mixes of *Deinococcus* bacteria and biomass which are not part of the invention, such as animal and/or vegetal and/or algal biomass, and relates to the use thereof in animal feeding, e.g., as part of animal feed compositions. The invention also relates to a method of preparing feedstuff using biomass and *Deinococcus* bacteria as feedstock. The application also discloses methods which are not part of the invention for increasing the nutritive value, palatableness, or digestibility of biomass. The compositions and methods of the invention are suitable for nourishment of any animal, including preferably livestock (including ruminants and non-ruminants), breeding animals, avian animals, fishes or companion animals.

55 [0012] The inventors have discovered that *Deinococcus* bacteria in combination with non usable raw materials, such

as lignocellulosic biomass, can be used as a safe and low cost nutriment for animals. Both ingredients cooperate to produce high value feedstuff. Both the biomass and the *Deinococcus* bacteria are transformed, by the invention, from low nutritive value products into useful feedstuff ingredients. The present application shows that *Deinococcus* bacteria may be used in feedstuff, are safe and contain valuable nutritive agents. The present application also shows that biomass serves as a growing medium for *Deinococcus* bacteria, thereby allowing their expansion and enrichment, while the *Deinococcus* bacteria are able to digest the biomass, thereby converting the biomass into high nutritive value products with better digestibility. The feedstuff ingredients as disclosed herein therefore includes organic (e.g., proteins, amino acids, sugars) and inorganic compounds (e.g., minerals) which are utilized in the normal metabolism of animals and fish and provide essential nutrients that are not synthesized in sufficient amount or not synthesized such as essential amino acids.

[0013] Therefore, the present application discloses an ingredient for feedstuff which is not part of the invention comprising a mix of biomass and a *Deinococcus* bacterium, said biomass being at least partially digested by said *Deinococcus* bacterium.

[0014] The invention relates to the use of a mix of biomass and a *Deinococcus* bacterium to prepare a feedstuff.

[0015] The present application also discloses a feedstuff composition which is not part of the invention comprising an ingredient as defined above. Typically, the feedstuff composition comprises at least 10% by weight of said ingredient. The feedstuff composition may further comprise additional ingredients such as, without limitation, alfalfa, barley, blood meal/meat meal, fat animal poultry, potato wastes, or tomato wastes .

[0016] An object of this invention relates to the use of a composition comprising a biomass and *Deinococcus* bacteria, for animal feeding.

[0017] A further object of this invention relates to the use of a mix of biomass and *Deinococcus* bacteria, said biomass being at least partially digested by said *Deinococcus* bacteria, as an ingredient for feedstuff.

[0018] A further object of this invention relates to the use of a mix of biomass and *Deinococcus* bacteria, said biomass being at least partially digested by said *Deinococcus* bacteria, as a protein supply for feedstuff. As further illustrated in the examples, *Deinococcus* bacteria are themselves high source of proteins and, in addition, they hydrolyse proteins of biomass providing therefore available amino acids.

[0019] The invention also relates to the use of *Deinococcus* bacteria as a source of amino acids for animal feeding.

[0020] The invention also relates to a method for preparing a feedstuff comprising:

- submitting a biomass to at least partial digestion by *Deinococcus* bacteria, and
- formulating a mix of at least partially digested biomass and bacteria, as ingredient for feedstuff.

[0021] In a particular embodiment, the method comprises a further step of adding to the mix other ingredients commonly used for feedstuff.

[0022] In an alternative embodiment, the method comprises:

- (a) combining a biomass and *Deinococcus* bacteria to obtain a mix,
- (b) maintaining the mix under conditions allowing digestion of the biomass by said *Deinococcus* bacteria,
- (c) collecting the mix of (a) or (b), and
- (d) optionally mixing the mix of (c) to one or several further ingredients.

[0023] The feedstuff may be packaged in any suitable form or container.

[0024] The biomass for use in the invention may comprise vegetal biomass, animal biomass and/or algal biomass. In a particular embodiment, the biomass is a lignocellulosic biomass. In another embodiment, the biomass is a protein-containing biomass. The digestion of the biomass may be performed in aerobic or anaerobic conditions, preferably in a reactor, for managing easily the reaction parameters (e.g., time of reaction, temperature, or feedstock quantities).

[0025] The present application also discloses a method for improving the nutritive value of a biomass which is not part of the invention, comprising subjecting said biomass to at least partial digestion by *Deinococcus* bacteria. Said method may further comprise a step wherein the nutritive value of said at least partially digested/hydrolyzed biomass is analysed or controlled.

[0026] The present application also discloses a method for improving the palatableness and/or digestibility of a biomass, which is not part of the invention comprising subjecting said biomass to at least partial digestion by *Deinococcus* bacteria, in order to at least partially degrade complex sugars of said biomass. Said method may further comprise a step wherein the degradation of the complex sugars is controlled.

[0027] The compositions and methods of the invention may be used as nutrient or nutrient additive for any animal, especially non-human mammals such as livestock (including ruminants and non-ruminants), breeding animals, or companion animals; avian animals, or fishes.

LEGEND TO THE FIGURES**[0028]**

5 Figure 1: Growth of strain DG01 (*D. Geothermalis* 01) on 1% pre-treated rapeseed straw with or without addition of cellulase and β -glucosidase.

10 Figure 2: TLC analysis showing consumption of xylose and glucose removed from 1% pre-treated rapeseed straw inoculated with DG01. Control consists on the pre-treated rapeseed straw without bacteria. Enzymes are added at T0 in the culture (0.11g of cellulase/g of cellulose and 0.05g of β -glucosidase/g of cellulose). The total enzymatic hydrolysis is performed using 1.1g of cellulase/g of cellulose, 0.5g of β -glucosidase/g of cellulose and 5.5g of xylanase/g hemicelluloses.

15 Figure 3: Growth of strain DG01 on whole wheat (1%, with or without Termamyl, and 6%) or fermentation residues (6%) showing consumption of gluten (%) contained in the medium. Strain DG01 is able to hydrolyze gluten from whole wheat or fermentation residues.

DETAILED DESCRIPTION OF THE INVENTION

20 **[0029]** The invention relates, generally, to the use of *Deinococcus* bacterial strains to produce animal feed or additives with biomass. More particularly, the invention provides a low cost and high level supply for proteins and amino acids, based on the growth of *Deinococcus* bacteria and degradation/transformation of the biomass. The mix of *Deinococcus* bacteria and a biomass as described herein may be used to provide animals low cost and/or high level source of valuable mono and di-acids or any metabolites derived from these mono or di-acids, derived from sugars such as xylan or hemicellulose hydrolysate contained in the biomass. According to the invention, unprocessed material of biological origin, including vegetal, algal or animal biomass, or secondary biomass, which essentially contains hydrolyzed pre-treated biomass products, may be used in combination with *Deinococcus* bacteria as nourishing/nutritive feedstock, animal feed or additive.

25 **[0030]** It is therefore herein described, an ingredient for feedstuff comprising a mix of biomass and *Deinococcus* bacteria, which is not part of the invention said biomass being at least partially digested by *Deinococcus*.

30 **[0031]** An object of this invention relates to the use of a composition comprising a biomass and *Deinococcus* bacteria, for animal feeding.

Definitions

35 **[0032]** The term "*biomass*" according to the invention typically designates any biological material. In particular, the term biomass includes organic material of biological origin, including vegetal, algal or animal origin, which may be unprocessed or pretreated. Examples of biomass include, without limitation, forestry products, including mature trees unsuitable for lumber or paper production, pulp, recycled paper, organic waste, agricultural products, such as grasses, straw, crops and animal manure, and aquatic products, such as algae and seaweed. Examples of biomass include wood or vegetal material derived from numerous types of plants, including miscanthus, hemp, switchgrass, sugarbeet, wheat, barley, corn, rice, soy, rapeseed (including canola), sorghum, sugarcane, peanut, cotton, lupine, and a variety of tree species, ranging from eucalyptus to oil palm, poplar, willow. Specific sources of biomass include, without limitation, plant residues, hardwood or softwood stems, cobs, straw, grass, leaves, seeds, paper, etc. (see for instance Sun et al., Bioresource Technology 83 (2002) 1-11). The term biomass also encompasses transformed biomass or secondary biomass, which essentially contains hydrolysed pre-treated biomass products. In a preferred embodiment, biomass according to the invention comprises any lignocellulosic material, for example, cellulose, hemicelluloses and/or xylan.

40 **[0033]** The biomass according to the invention may comprise raw biomass and/or secondary biomass. The "*raw biomass*" is unprocessed material from biological matter. Examples include, without limitation, forestry products, such as mature trees unsuitable for lumber or paper production, agricultural products, such as grasses, crops and animal manure, and aquatic products, such as algae and seaweed. The "*secondary biomass*" is any material initially derived from raw biomass, which has undergone significant chemical and physical changes. Examples include, without limitation, paper, leather, cotton, hemp, natural rubber products, food processing by-products, fish and animal meals, and used cooking oils.

45 **[0034]** The term "*lignocellulosic biomass*" according to the invention designates an organic biological material containing lignin, cellulose, hemicellulose and/or xylan. The term lignocellulosic biomass generally designates unprocessed material of biological origin, e.g., raw biomass. Examples of lignocellulosic biomass include, without limitation, wood or vegetal material derived from numerous types of plants, including miscanthus, rapeseed, switch grass, hemp, sugarbeet,

wheat, wheat straw, corn, poplar, willow, sorghum, sugarcane, and a variety of tree species, ranging from eucalyptus to oil palm.

[0035] As used herein, the term "*biomass derivatives*" designates all molecules derived from raw biomass and/or from secondary biomass, as defined above.

[0036] In the context of the present application, the term "*Deinococcus* bacterium" includes wild type bacterium, or natural variant strains of *Deinococcus*, e.g., strains obtained through accelerated evolution, by DNA-shuffling technologies, as well as recombinant strains obtained by insertion of eukaryotic, prokaryotic and/or synthetic nucleic acid(s).

[0037] In the context of the invention, "*proteins*" mean all biochemical compounds comprising one or more polymer chain(s) of amino acids residues bonded together. The protein supply of the invention includes proteins, peptides, polypeptides, amino acids and related derivatives.

[0038] In the context of the invention, an "exogenous component", such as an "exogenous protein" refers to a component originating from the biomass or a culture medium and, conversely, an "endogenous component" refers to a component originating from the bacterium.

[0039] In the context of the invention, "*starch*" refers to a carbohydrate consisting of a large number of glucose units joined together by 1-4 and 1-6 glycosidic bonds. Starch is an energy storage molecule accumulated by many plants and bacteria, and starch molecules arrange themselves in the plant in semi-crystalline granules.

Digestion of the biomass by the bacteria to produce high value feedstuff

[0040] The present invention is based, inter alia, on the combination of biomass and *Deinococcus* bacteria which cooperate to generate high nutritive value, and high digestibility products suitable for feedstuff. The invention shows biomass supports the growth and expansion of the bacteria, increasing the nutritive value of the mix, while the bacteria are able to digest the biomass, further increasing the nutritive value, digestibility and palatableness of the mix.

[0041] According to the invention, the biomass may be at least partially digested by the *Deinococcus* bacteria.

[0042] The term digested or digestion includes all biological modification or transformation of the biomass, such as degradation or hydrolysis of components of the biomass such as raw material, cell walls, polymers (e.g., sugars, proteins), etc. Digestion may be partial, meaning that only a portion of some of the components of the biomass is digested, typically 5%, 10%, or more. Partial digestion indicates that at least part of the biomass has been modified or transformed, which typically results in an increased nutritive value, digestibility or palatableness.

[0043] *Deinococcus* bacteria may advantageously catalyze (or contribute to the catalysis of) the degradation of various components of a biomass, such as sugar polymers like starch, xylan or cellulose into oligosaccharides of smaller degree of polymerization and monosaccharides. Indeed, *Deinococcus* bacteria which express particular enzymes and/or have the ability to transform raw biomass have been disclosed in the art by Applicant. In this regard, preferred *Deinococcus* bacteria for use in the invention synthesize xylanases and/or cellulases and/or amylases. Xylanases are enzymes that catalyze the hydrolysis of xylan, a major component of hardwood and softwood hemicelluloses. Amylases are involved in the hydrolysis of starch polymers. Cellulases are enzymes that catalyze the hydrolysis of cellulose or hemicellulose, a major component of hardwood and softwood.

[0044] In a particular embodiment, the digestion of the biomass designates a reduction in the level of cellulose or hemicellulose of the biomass, preferably a reduction by at least 5%. Such a decrease facilitates digestibility of the biomass by all kinds of animals, including non ruminant animals.

[0045] In a preferred embodiment, the digestion of the biomass designates the conversion of hemicellulose from said biomass into smaller oligosaccharides and/or xylose, mannose, arabinose or galactose, and/or the conversion of cellulose and/or starch from said biomass into glucose. Preferably, the digestion encompasses conversion of at least 5% hemicellulose, cellulose, or starch from said biomass.

[0046] Such at least partial digestion of the sugars improves the digestibility and/or palatableness of the biomass, since C5 sugars like xylose, which cause the non appetite of the substrate, are digested by *Deinococcus* (Br J Nutr. 2010 May; 103(10):1507-13).

[0047] In another embodiment, digestion comprises the cellular sugar oxidation, particularly including DP3-DP7 sugars (DP: degree of polymerization) e.g.; triose, tetrose, pentose, hexose, and heptose, for example selected from xylose, arabinose, glucose, galactose, or fucose residues as monomers.

[0048] Simultaneously to the degradation/digestion of the biomass, the growth of the bacteria increases. Consequently, because the level of bacteria increases inside the mix, the level of proteins and lipids increases too. Then, according to the invention, the digestion of the biomass by *Deinococcus* bacteria, even indigestible biomass such as wheat bean or canola seed cattle cakes, provides a high level of directly nourishing constituents for animals. The invention allows making the most of several vegetal biomasses that were not used for animal feeding because of their indigestibility and/or few levels of nutriment. It is for example the case for rapeseed cattle cakes which contain a few level of proteins compared to soy bean cattle cakes. Such biomass comprises 1 to 6 g/l of vegetal proteins and no significant amount of lipids. After a partial digestion of said biomass by *Deinococcus* bacteria according to the invention, one may expect

about 12 to 25 g/l of proteins and about 1 to 2.5 g/l of lipids. In the same way, several animal biomasses without industrial interest, such as poultry feathers, may be advantageously used as biomass to be mixed with *Deinococcus* for providing animal feedstuff ingredient.

[0049] In addition, since *Deinococcus* sp. produce naturally carotenoids, the mix biomass /*Deinococcus* of the invention may contain carotenoids which have a positive impact on both the visual and gustative quality and the health of the animals fed with said mix. For example, based on a vegetal biomass without any carotenoid, one may expect about 15 mg/kg of carotenoids in the mix after the partial digestion of the vegetal biomass and the growth of the bacteria.

[0050] The final amount of proteins, amino acids, lipids, vitamins, carotenoids etc. produced or synthesized by *Deinococcus* and recovered in the mix will depend on several industrial parameters, such as the nature and the conditions of the reaction, the time of reaction, the initial amount of bacteria and the kind of biomass used. It will be apparent to one skilled in the art that various adaptations can be made to adapt the method of the invention to the need.

[0051] For example, the digestion level of the biomass may vary depending on the industrial/economic and/or nutritive requirements. If a high level of nutritive constituents is preferred to a low cost production, the time of reaction may be increased for allowing a complete digestion of the biomass. In the final mix, one may expect a lack of vegetal fraction, the bacteria having almost totally digested the biomass. Conversely a low cost ingredient, with a minimum level of proteins, can be desired. The balance between the time of reaction and the level of nutritive constituents may be easily adapted.

[0052] The mix as disclosed herein, or final mix, comprises the remaining biomass (the part of the biomass which has not been hydrolyzed/digested by the bacteria) and *Deinococcus* bacteria, whose number has increased.

[0053] In an embodiment of the invention, the *Deinococcus* bacterium used presents a proteolytic activity, particularly useful to at least partially hydrolyse proteins contained in the biomass. Proteases, also known as proteinases or proteolytic enzymes, are enzymes that begin protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain.

The inventors have discovered that *Deinococcus* bacteria may present a high proteolytic activity. According to the invention, *Deinococcus* bacteria having a proteolytic activity may be advantageously used in combination with several vegetal, algal or animal biomasses containing proteins to provide highly digestible amino acids. The bacteria hydrolyze the proteins, providing amino acids and peptides that are easily absorbed by animals. The resulting mix may be used as an ingredient for feed composition that provides both exogenous amino acids (and/or peptides) and endogenous nutriment (from the bacteria themselves) including proteins and amino acids.

For example, *Deinococcus* bacteria may be contacted with algae such as *Micratinium pusillum* and/or *Chlorella sp* so that the resulting mix may be used instead of fish meal as ingredient for feedstuff containing useful amino acids.

Other examples of biomasses containing proteins include beet pulp, soybeans, alfalfa and chicken feathers.

The use of *Deinococcus* bacteria can also reduce the viscosity of biomass (vegetal, animal, or algal), which represents a further advantage. Indeed, the proteolytic activity of the *Deinococcus* bacteria reduces the viscosity of gelatin-containing biomass. Also, the pectinolytic activity of *Deinococcus* bacteria reduces the viscosity of pectin-containing biomass.

The inventors have discovered that *Deinococcus* bacteria may present a high pectinolytic activity. According to the invention, *Deinococcus* bacteria synthesizing pectinases may be advantageously used in combination with a biomass containing pectin to decrease its viscosity and so increase its palatableness. The bacteria hydrolyze the pectin, eliminating the viscous properties of the biomass. Such viscous biomass may be used as growing medium for *Deinococcus* bacteria, and the resulting mix may be used as ingredient for feedstuff.

In a particular embodiment of the invention, the *Deinococcus* bacterium used in the mix for feedstuff therefore presents a pectinolytic activity, particularly useful to at least partially hydrolyse pectin contained in some viscous biomass.

It is the case for example of beet pulp that contains a high level of pectin (15 to 20% by weight). The beet pulp is a feedstock that is not easily exploitable because of the pectin making this biomass viscous. It is not possible to feed animals directly with such viscous biomass and its set into pleasant feed requires costly treatments.

In one embodiment, the viscous biomass is only part of the biomass of the mix. For example, the used biomass comprises 60% of lignocellulosic biomass, such as canola seed cattle cake, and 40% of viscous biomass, such as beet pulp.

Process for preparing the mix

[0054] The digestion of the biomass by *Deinococcus* bacteria may be advantageously conducted in a reactor, for managing the parameters of the reaction easily. Depending on the strain(s) of bacteria and/or on the biomass used, the reaction may be conducted in an aerobic or anaerobic reactor.

[0055] In one embodiment, the biomass is introduced in one time, together with the bacteria. In another embodiment, the reactor may be reloaded with biomass or bacteria during the process. In such case, the nature and quantity of biomass may vary to improve the final content in nutrients in the final mix. The final mix comprises the remaining biomass, which has not been hydrolyzed/digested yet, the products resulting from digestion/hydrolysis (unless consumed by the bacteria), and the bacteria which have been expanded.

The amount of liquid added inside the reactor may depend on the form of the final composition, i.e. liquid or solid form, and on the moisture content of the biomass.

[0056] In another embodiment, the preparation of the mix of the invention is conducted in open country. For example, the biomass and *Deinococcus* bacteria are first mixed together before to be spread on the soil. This initial mix can be covered with a canvas sheet or conversely the mix can be put in the fresh air.

[0057] According to the invention, the mix can be used directly as part of an animal feed composition. Otherwise, the mix can be processed (for example dehydrated, filtered, dried, milled, etc.) before to be used. In another embodiment, the mix may be treated to kill or inactivate the bacteria, or to eliminate remaining raw biomass. The resulting product comprises essentially bacteria and digested biomass components and may be used as straight protein and lipid raw material.

[0058] The mix of biomass and *Deinococcus* bacteria according to the invention may be used as an ingredient in an animal feed composition. An animal feed composition as used therein is a composition for animal nutrition, in solid or liquid form. The feed may be defined as substance with sufficient nutritional value to allow growth and maintenance of adequate body conditions on an animal. For example, an animal feed composition may be on the form of pellets, meal, grains, extruded or expanded grains, tablets powder, bolus form or mix thereof.

[0059] Preferentially, the animal feed composition as disclosed herein, including the mix of bacteria and biomass, is in a form and/or a composition approved by a governmental institution such as National Food Administration (for example AFSSA in France, ACIA in Canada, or FAD in the US).

[0060] Preferably, the animal feed composition as disclosed herein comprises at least 10% by weight of the mix of *Deinococcus* bacteria and at least partially digested/hydrolyzed biomass. Depending on the other ingredients used to form the animal feed composition, and their impact on both the physical quality of the feeds after forming (such as pelleting) and the nutritional quality required, the level of the mix of the invention may be increased or decreased. For example, to obtain pellets with a good quality standard in terms of hardness and durability an amount of about 40% of pre-gelatinized starch may be added. If native starch is used, the required amount may be higher. In the same way, the amount of dispersible protein may also positively affect hardness and durability properties of pelleted feeds.

[0061] In this regard, the invention also relates to a method for preparing a feedstuff comprising:

- submitting a biomass to at least partial digestion by *Deinococcus* bacteria, and
- formulating a mix of at least partially digested biomass and bacteria, as ingredient for feedstuff.

[0062] In a particular embodiment, the method comprises a further step of adding to the mix other ingredients commonly used for feedstuff.

[0063] In an alternative embodiment, the method comprises:

- (a) combining a biomass and *Deinococcus* bacteria to obtain a mix,
- (b) maintaining the mix under conditions allowing digestion of the biomass by said *Deinococcus* bacteria,
- (c) collecting the mix of (a) or (b), and
- (d) optionally mixing the mix of (c) to one or several further ingredients.

[0064] The feedstuff may be packaged in any suitable form or container.

[0065] In the mix, it is possible to use one species or strain of a *Deinococcus* bacterium, or to combine various strains, or the same or distinct species of *Deinococcus* bacteria. Also, in addition to *Deinococcus* bacteria, the mix or feedstuff product may comprise further bacteria or yeast cells, if appropriate. Furthermore, additional agents such as enzymes may be added to the biomass.

[0066] As indicated above, the invention may be used to produce feedstuff or feed additives suitable for use in any non-human animals. It is particularly suitable for livestock (including ruminants and non-ruminants), breeding animals, avian animals, fishes or companion animals. Specific examples include cattle, ovine, porcine, poultry, fish, shrimp and crustaceans.

[0067] Further aspects and advantages of the invention will be disclosed in the following experimental section, which should be considered as illustrative of the invention.

EXAMPLES

Example 1: Identification of mesophilic and thermophilic *Deinococcus* strains with biomass degrading activities

[0068] This example discloses tests suitable to determine whether a genus, a species and/or a bacterial strain is able to function in a method for preparing feedstuff according to the invention. Non limitative examples of tests that may be performed to identify bacteria exhibiting particular enzymatic activities are described below.

Materials and Methods

Complex Medium Glucose (CMG) 1% composition

5 **[0069]**

Peptone : 2 g/L
 Yeast extract: 5 g/L
 Autoclavation 121 °C, 15 min.
 10 Glucose : 10 g/L - Filter sterilized (0.22 μm)

Then addition of MOPS, micronutrients, vitamins, FeCl₃, K₂HPO₄ (see below)

Media composition (MM)

15 **[0070]** 14g/L of agar are added to 704 ml of ultrapure water. Then an autoclavation is performed. Then, after medium cooling, 80 ml of MOPS 10X, 8 ml of FeC13 100X, 8 ml of K₂HPO₄ 100X, 80μL of micronutrients 10 000X, and 80μL of vitamins 10 000X are added.

20 Media composition for solid screening of cellulolytic activity (5% AZO-Cellulose)

[0071] 14g/L of agar are added to 704 ml of ultrapure water. Then an autoclavation is performed. Then, after medium cooling, 80 ml of MOPS 10X, 8 ml of FeC13 100X, 8 ml of K₂HPO₄ 100X, 80μL of micronutrients 10 000X, and 80μL of vitamins 10 000X are added. Finally, AZO-Cellulose solution at 5% is added.

25 Media composition for solid screening of proteolytic activity (1% milk)

[0072] 10g/L of milk powder and 14g/L of agar are added to 704 ml of ultrapure water. Then an autoclavation is performed. Then, after medium cooling, 80 ml of MOPS 10X, 8 ml of FeC13 100X, 8 ml of K₂HPO₄ 100X, 80μL of micronutrients 10 000X, and 80μL of vitamins 10 000X are added.

Media composition for solid screening of amylolytic activity (0.5% starch)

35 **[0073]** 5g/L of starch and 14g/L of agar are added to 704 ml of ultrapure water. Then an autoclavation is performed. Then, after medium cooling, 80 ml of MOPS 10X, 8 ml of FeC13 100X, 8 ml of K₂HPO₄ 100X, 80μL of micronutrients 10 000X, and 80μL of vitamins 10 000X are added.

Media composition for solid screening of xylanolytic activity (5% AZO-xylan)

40 **[0074]** 14g/L of agar are added to 704 ml of ultrapure water. Then an autoclavation is performed. Then, after medium cooling, 80 ml of MOPS 10X, 8 ml of FeC13 100X, 8 ml of K₂HPO₄ 100X, 80μL of micronutrients 10 000X, and 80μL of vitamins 10 000X are added.

[0075] Finally, AZO-Xylan solution at 5% is added.

45 MOPS-Buffer mixture 10X, pH7.0

[0076]

50	MOPS acid	400 mM
	NH ₄ Cl	200 mM
	NaOH	100 mM
	KOH	100 mM
	CaCl ₂	5μM
55	Na ₂ SO ₄	2.76 mM
	MgCl ₂	5.28 mM

EP 2 793 607 B9

Filter sterilized (0.22 μ m)

Micronutrients 10 000X

5 **[0077]**

	(NH ₄) ₆ (Mo ₇)O ₂₄	30 μ M
	H ₃ BO ₃	4 mM
10	CoCl ₂	300 μ M
	CuSO ₄	100 μ M
	MnCl ₂	2.5 mM
	ZnSO ₄	100 μ M

15 Adjusted to pH 5 with HCl.

Filter sterilized (0.22 μ m)

Vitamins 10 000X

20 **[0078]** 10 mg/L of each : D-biotin, Niacin (nicotinic acid), Pyridoxin (pyroxidal hydrochloride) B6, Thiamin (vitamin B1 hydrochloride) - Stock pH4 - filter sterilized (0.22 μ m).

FeC13 100X

25 **[0079]** 2 mM FeC13 in 2mM sodium citrate, filter sterilized (0.22 μ m)

K₂HPO₄ 100X

30 100 g/L, autoclaved.

Detection of enzymatic activities

Detection of the cellulolytic activity

35 *Solid screening (test on agar plate)*

[0080] A preculture was carried out in CMG 1% medium in microplate using isolated clones (5 colonies in 200 μ L of CMG 1% medium).

40 **[0081]** From a stationary phase, 5 μ L of preculture were spotted on agar plates containing MM and 5% AZO-cellulose.

[0082] The cellulolytic enzymatic activity was followed by measuring the hydrolysis halo diameter after 1, 2 and 5 days (1 plate/ day was required).

Liquid screening

45 **[0083]** A preculture was carried out in CMG 1 %medium in microplate using isolated clones (5 colonies in 200 μ L of CMG 1% medium).

[0084] From a stationary phase, 5 μ L of preculture were added to 200 μ l MM + 1% CMC or 1% CMC4M or 1% cellobiose in microplates.

50 **[0085]** The cellulolytic enzymatic activity was estimated by following the growth at OD_{600nm} (reading twice a day for 5 days).

Detection of the proteolytic activity

55 *Solid screening (test on agar plate)*

[0086] A preculture was carried out in CMG 1% medium in microplate using isolated clones (5 colonies in 200 μ L of CMG 1% medium).

EP 2 793 607 B9

[0087] From a stationary phase, 5 μ L of preculture were spotted on agar plates containing MM and 1% milk.

[0088] The proteolytic enzymatic activity was followed by measuring the hydrolysis halo diameter after 1, 2 and 5 days (1 plate/ day was required).

5 *Liquid screening*

[0089] A preculture was carried out in CMG 1 %medium in microplate using isolated clones (5 colonies in 200 μ L of CMG 1% medium).

[0090] From a stationary phase, 5 μ L of preculture were added to 200 μ l MM + 1% pepton or 1% casein in microplates.

10 [0091] The proteolytic enzymatic activity was estimated by following the growth at OD_{600nm} (reading twice a day for 5 days).

Detection of the amylolytic activity

15 *Solid screening (test on agar plate)*

[0092] A preculture was carried out in CMG 1% medium in microplate using isolated clones (5 colonies in 200 μ L of CMG 1% medium).

[0093] From a stationary phase, 5 μ L of preculture were spotted on agar plates containing MM and 0.5% starch.

20 [0094] The amylolytic enzymatic activity was followed by measuring the hydrolysis halo diameter after 1, 2 and 5 days (1 plate/ day was required).

[0095] On starch containing-agar plates, the hydrolysis halo revelation was done by addition Gram's iodine reagent (1 plate/day was also required).

25 *Liquid screening*

[0096] A preculture was carried out in CMG 1 %medium in microplate using isolated clones (5 colonies in 200 μ L of CMG 1% medium).

[0097] From a stationary phase, 5 μ L of preculture were added to 200 μ l MM + 0.5% starch in microplates.

30 [0098] The amylolytic enzymatic activity was estimated by following the growth at OD_{600nm} (reading twice a day for 5 days).

Detection of the xylanolytic activity

35 *Solid screening (test on agar plate)*

[0099] A preculture was carried out in CMG 1% medium in microplate using isolated clones (5 colonies in 200 μ L of CMG 1% medium).

[0100] From a stationary phase, 5 μ L of preculture were spotted on agar plates containing MM and 5% AZO-xylan.

40 [0101] The xylanolytic enzymatic activity was followed by measuring the hydrolysis halo diameter after 1, 2 and 5 days (1 plate/ day was required).

Liquid screening

45 [0102] A preculture was carried out in CMG 1 %medium in microplate using isolated clones (5 colonies in 200 μ L of CMG 1% medium).

[0103] From a stationary phase, 5 μ L of preculture were added to 200 μ l MM + 0.5% xylan in microplates.

[0104] The xylanolytic enzymatic activity was estimated by following the growth at OD_{600nm} (reading twice a day for 5 days).

50

Results

[0105] Table 1 (below) lists examples of bacteria identified with solid screening tests and having suitable biomass-digestion activity for use in feedstuff production.

55 [0106] The hydrolysis halo diameter has been measured after 2 days for the proteolytic and amylolytic activities, and after 5 days for the xylanolytic and cellulolytic activities.

[0107] More precisely, for proteolytic activity, a high activity corresponds to a hydrolysis halo diameter higher than 2.4 cm, a medium activity corresponds to a hydrolysis halo diameter between 2 cm and 2.35 cm, and a low activity corresponds

EP 2 793 607 B9

to a hydrolysis halo diameter lower than 1.95 cm.

[0108] For amylolytic activity a high activity corresponds to a hydrolysis halo diameter higher than 2.4 cm, a medium activity corresponds to a hydrolysis halo diameter between 2.1 cm and 2.35 cm, and a low activity corresponds to a hydrolysis halo diameter lower than 1.9 cm.

5 **[0109]** For xylanolytic activity a high activity corresponds to a hydrolysis halo diameter higher than 2.8 cm, a medium activity corresponds to a hydrolysis halo diameter between 2.1 cm and 2.7 cm, and a low activity corresponds to a hydrolysis halo diameter lower than 2.05 cm.

10 **[0110]** For cellulolytic activity a high activity corresponds to a hydrolysis halo diameter higher than 1.6 cm, a medium activity corresponds to a hydrolysis halo diameter between 1.1 cm and 1.35 cm, and a low activity corresponds to a hydrolysis halo diameter lower than 0.9 cm.

Table 1: List of *Deinococcus* strains having cellulolytic, proteolytic, amylolytic and/or xylanolytic activities (solid screening)

	2 days	2 days	5 days	5 days
	Proteolytic activity	Amylolytic activity	Xylanolytic activity	Cellulolytic activity
15 DRH01	+++	+++	-	-
DRH02	++	-	-	-
20 DRH03	++	+	-	-
DRH46	++	+	-	+++
M1-5A	+++	+++	-	+
25 M2-8F	++	++	-	-
M2-9H	++	+	-	-
M3-5A	+	++	-	+++
M3-6B	+	++	-	+++
30 M3-6G	+	++	-	++
M3-7C	++	+	-	+
DRH05	+	+	-	-
35 DRH06	+	++	-	-
DRH07	+	+	+	
DRH38	+	+	+++	-
DRH39	+	-	+	-
40 M11-12B	+	+	-	-
M13-1A	+	-	-	-
M13-8D	+	+	-	-
45 M23-1G	++	++	-	-
M23-2A	+++	++	-	-
M23-2E	+++	++	-	-
M23-2F	++	++	-	-
50 M23-3A	++	+++	+++	-
M31-1H	++	++	-	-
M31-2A	+++	+++	-	-
55 M31-2B	+++	++	-	-
M31-8F	++	++	-	-
M35-1F	++	-	+	-

EP 2 793 607 B9

(continued)

	2 days	2 days	5 days	5 days
	Proteolytic activity	Amylolytic activity	Xylanolytic activity	Cellulolytic activity
5 DG01	++	-	++	-
MC2-2A	++	-	+++	-
MC3-4A	++	++	-	-
10 MC3-4B	+++	-	+++	-
MD2-3B	++	-	++	-
MD2-3B bis	+	-	+	-
15 MX4-2B	+	+	-	-
MX4-2D	+	+	-	-
MX4-4A	++	-	+	-
20 DG01_04	++	++	++	-
MC5-12E	+++	++	-	-
(+++) : high activity (++) : medium activity (+) : low activity (-) no activity				

[0111] The Table 2 (below) lists examples of bacteria identified with liquid screening tests and having variable enzymatic activities for production of feedstuff. Growth of the bacteria is followed by measuring the OD at 600 nm.

30 Table 2: List of *Deinococcus* strains having cellulolytic, proteolytic, amylolytic and/or xylanolytic activities (liquid screening)

	1% Casein	0.5% starch	0.5% xylan	1% CMC4M
35 DRH01	+	+	+	-
DRH22	+	-	-	-
DRH25	+	-	-	-
DRH46	+	+	+	+
40 M3-5A	+	+	-	+
M3-6B	+	+	-	+
M3-6G	+	+	-	+
45 M4-9B	+	+	-	+
M5-1D	+	+	-	-
M5-5A	+	+	-	+
DRH05	+	+	-	-
50 DRH06	+	+	-	-
DRH37	+	+	+	-
DRH38	+	+	+	-
DRH39	+	+	+	-
55 M11-12B	+	+	+	-
M13-1A	+	+	-	-

EP 2 793 607 B9

(continued)

	1% Casein	0.5% starch	0.5% xylan	1% CMC4M	
5	M23-2A	+	+	+	-
	M23-3A	+	-	+	-
	M31-3C	+	+	+	-
	M31-3D	+	+	+	-
10	M31-8F	+	+	+	-
	M35-1F	+	+	-	-
	MC2-2A	+	-	+	-
15	MC2-2C	+	-	+	-
	MC3-4A	+	+	+	-
	MX4-2B	+	+	-	-
20	MX4-2D	+	+	+	-
(+):good growth ($OD_{600nm} \geq 0.5$) (-):low or no growth ($OD_{600nm} < 0.5$).					

[0112] This example shows that Deinococcus bacteria having suitable biological activity may be selected from public collections, and used in the invention to produce feedstuff.

Example 2: Production of a Rapeseed straw-Deinococcus Mix

[0113] A mix of rapeseed straw and Deinococcus bacteria has been prepared. More particularly, 1% pretreated rapeseed straw has been contacted with a Deinococcus bacterium (e.g., strain DG01). The capacity of the mixture to produce a valuable mix for feedstuff has been determined by verifying the capacity of Deinococcus to expand and digest rapeseed straw, either alone or after enzymatic supplementation, and to produce high nutritive content.

Materials and Methods

Rapeseed straw

[0114] Rapeseed straw was obtained from Sofiproteol and was ground in a blender and then passed through a sieve to obtain fine sections, less than 1mm in length.

Cellulase and β -glucosidase

[0115] The commercial enzymes used were a cellulase from Trichoderma reesei (SIGMA ref.C8546-5KU) and a beta-glucosidase from almonds (SIGMA ref.49290-1G).

H₂SO₄ hydrothermal pretreatment

[0116] Pretreatment was performed into an erlen flask with 20% w/v rapeseed straw and 0.5% w/w H₂SO₄ in tap water. This mixture was autoclaved 10 min at 120°C (time of cycle : 1.5h) and then diluted with sterile tap water to obtain the final rapeseed straw concentration. pH was adjusted to 7 with 20M NaOH solution (checked with pH paper). Mineral solutions: 20mM NH₄Cl and 5.7mM K₂HPO₄ was added before inoculation.

Counting protocol

[0117]

- Take 1mL of homogeneous culture (CMG, technical substrate) in Ependorff 2 ml
- Vortex 10 s and then apply ultrason in an ultrasonic bath for 10 minutes and vortex again 10 s.

EP 2 793 607 B9

[0118] In 96-wells microplate :

- Dispense 180 μ L of sterile MilliQ water in 9 wells in triplicate
- In triplicate: Perform serial dilutions of 1/10th from the well No. 1 (corresponding to pure sample) to the well No. 10 (corresponding to dilution 10⁻⁹): take 20 μ L and put it in 180 μ L sterile milliQ water from the following well, mix & reverse pipetting three times. Change cone between each well.
- With the multi-channel pipette, spot on PGY-agar plate, 5 μ L of each dilution in duplicate.
- Incubate 2 days at 45 ° C (for thermophile Deinococcus)
- Count the number of colonies on the first countable dilution:
Average the six spots corrected by the dilution factor and multiply by 200 to get the number of CFU / mL

TLC protocol

[0119]

- Spot 5 μ l of sample in TLC silica gel.
- Dry spotted samples with hot air gun.
- Migration of TLC in solvent Butanol/ acetone/ H₂O - 4/5/1 solvent.
- At the end of migration, dry TLC with Hot air gun.
- Reveal TLC using a solution containing 12g ammonium molybdate + 0.5g ammonium cerium nitrate in 80ml of H₂SO₄ 10%.

Enzymatic hydrolysis and culture

- [0120] For DG01, enzymes were added to the culture medium to hydrolyze polymers into sugar monomers.
- [0121] Rapeseed straw contains 40% cellulose. The enzyme loading is 0.11g cellulase/g cellulose and 0.05g beta-glucosidase/g cellulose. The enzyme solutions were filtered through 0.22 microns before introduction into the culture.
- [0122] Preculture was done for 3 days in CMG 1%. Cell pellet was washed three times in sterile water and then used to inoculate culture medium at DOi of 0.2 ie $\approx 10^7$ CFU/mL.
- [0123] Growth was performed for 9 days at 30°C for DRH46 and 45°C for DG01.
- [0124] Growth is controlled by a counting of bacteria according to the protocol below.

Results

- [0125] The growth of DG01 was followed by counting (UFC/ml) (figure 1) and the consumption of sugars was estimated using TLC analysis (figure 2).
After 9 days, all free glucose and xylose were consumed by DG01wt. When this consumption was reported to total sugars, it appeared that a major part of glucose and xylose was consumed. These results therefore show that the mix comprises partially digested biomass, with reduced xylose content.
- [0126] Similar results are obtained with other Deinococcus bacteria as listed in Table 1, such as DRH01, DRH02, DRH03, or DRH46.
- [0127] Cultures were lyophilized and amino-acid composition, soluble nitrogen and NDF/ADF/ADL fibers determination was carried out on dry mass.
Table 3 below compares the amino-acid composition of rapeseed straw (g/kg dry mass) to the amino-acid composition of mix as disclosed herein (g/kg dry mass).
The results show that the mix as disclosed herein comprises at least 20 times more aminoacids than the initial rapeseed straw.

Table 3: Amino acids composition of the mix and rapeseed straw

	Mix of the invention (g/kg dry mass)	Rapeseed straw (g/kg dry mass)
aspartic acid	51,3	2,6
Threonine	31,9	1,4
Serine	18,9	1,4
glutamic acid	65,1	3,1
Proline	26	1,3
Glycine	37,1	1,8
Alanine	46,8	1,8

EP 2 793 607 B9

(continued)

	Mix of the invention (g/kg dry mass)	Rapeseed straw (g/kg dry mass)	
5	Valine	36,2	1,8
	Cysteine	1,6	0,6
	Methionine	10,4	0,4
	Isoleucine	20,6	1,2
	Leucine	42,5	2,1
10	Thyrosine	16,1	0,7
	Phenylalanine	19,5	1,4
	Lysine	29,4	1,2
	Histidine	9,7	0,4
15	arginine	40	1,2

[0128] The mix as disclosed herein therefore transforms a poor biological material into a rich and digestible mix for feedstuff.

Example 3: Production of a Wheat-Deinococcus mix.

[0129] A mix of *Deinococcus* and Wheat biomass was prepared by inoculation of *Deinococcus* strain DG01 on a medium made of whole wheat (1% with or without Termamyl addition, and 6%) or fermentation residues (1% or 6 %) supplemented with NH₄Cl 20 mM and K₂HPO₄ 5.7 mM.

[0130] The capacity of the mixture to produce a valuable mix for feedstuff has been determined by verifying the capacity of *Deinococcus* to expand on wheat and to reduce the gluten content of wheat.

[0131] Gluten consumption was evaluated by using Enzyme Immunoassay for the quantitative determination of Gliadin (soluble fraction of gluten)/Gluten. This test is based on the principle of the enzyme-linked immunoabsorbent assay.

Protocol

[0132] Protein consumption is measured using a commercial kit from Libios (Ref GLI-E02).

[0133] Samples preparation: 100 mg of finely ground lyophilized technical substrate is resuspended in 1 ml EtOH40%, mixed during 5min and finally centrifuge for 10 min at 3800 rpm. The supernatant is diluted (1/500000) in sample dilution buffer1X.

Elisa TEST:

[0134] Samples and standards are tested in duplicate.

- 100µl of standards and samples are added in 96-wells microplate coated with antigliadin antibody.
- Incubate 20 min at room temperature.
- Wash wells three times with 300µl washing solution 1X.
- Add 100µl of secondary conjugated antibody (Antigliadin peroxidase) in empty wells.
- Wash wells three times with 300µl washing solution 1X.
- Add 100µl of TMB solution (substrate).
- Incubate 20min at room temperature, in the dark.
- Add 100µl of stop solution (color shift from blue to yellow)
- Homogenize and read with a spectrophotometer at OD_{450nm}.

Result

[0135] The concentration of gliadin is directly proportional to the colour intensity of the test sample. Because of the equal amount of gliadin and gluten in wheat, the gluten concentration of the sample is calculated by multiplication with the factor 2.

As shown figure 3, *Deinococcus* is able to hydrolyze gluten from whole wheat or fermentation residues thereof, and to consume it after 2 days of growth at 45°C. After 48h, all proteins contained in the vegetal biomasses are consumed by the *Deinococcus* strain.

[0136] Similar results are obtained with other *Deinococcus* bacteria as listed in Table 1, such as DRH01, DRH03, or

DRH46.

[0137] These results therefore clearly demonstrate *Deinococcus* strains can exhibit a strong proteolytic activity resulting in a more digestible mix from wheat suitable for use in animal consumption.

5 Example 4: Analysis of the amino acid composition of *Deinococcus geothermalis*

[0138] The amino acid composition of *Deinococcus geothermalis* (strain M36-7D_21) has been determined after growing the cells in a medium containing peptone and glucose as sole carbon sources. The composition was then compared to the amino acid composition of yeasts that are commonly used as a complementary protein source in fish diet and/or as a supplement in animals feed to compensate amino acids and/or vitamin deficiencies of cereals.

Materials and Methods

Complex Medium Glucose (CMG) 1% composition

[0139]

Peptone 2 g/L ;
 Yeast Extract 5 g/L ;
 20 Glucose 55 mM (10 g/L) ;
 MOPS acid 40 mM ;
 NH₄Cl 20 mM ;
 NaOH 10 mM ;
 KOH 10 mM ;
 25 CaCl₂·2H₂O 0,5 μM ;
 Na₂SO₄·10H₂O 0,276 mM ;
 MgCl₂·6H₂O 0,528 mM ;
 (NH₄)₆(Mo₇)O₂₄·4H₂O 3 nM ;
 H₃BO₃ 0,4 μM ;
 30 CoCl₂·6H₂O 30 nM ;
 CuSO₄·5H₂O 10 nM ;
 ZnSO₄·7H₂O 10 nM ;
 D-Biotin 1 μg/L ;
 Niacin (nicotinic acid) 1 μg/L ;
 35 Pyridoxin (pyridoxal HCl ou vitamine B6) 1 μg/L ;
 Thiamin HCl (vitamine B1) ;
 FeCl₃ 20 μM ;
 Sodium Citrate. 2H₂O 20 μM ;
 K₂HPO₄ 5,7 mM.

40 The final concentration of MnCl₂ was 5,25 μM.

[0140] Cells were grown in CMG 1% medium containing 6μg/ml of Bleomycin in a fermentor of 3,5 L at 45°C and under agitation of 400 rpm.

45 About 100 to 200 ml of the culture medium were harvested by centrifugation (4000 rpm, 20 min at 4°C) at exponential and stationary phase of growth and washed prior to lyophilisation. The cultures were then lyophilized and amino acid composition was carried out on dry mass.

[0141] The amino acids composition and quantification have been determined by HPLC.

[0142] The values of the amino acid composition of yeast extract is derived from S. Cortassa *et al*, 2002 (S. Cortassa *et al*, 2002, "An introduction to metabolic and cellular engineering" World scientific Publishing).

Results

55 **[0143]** Table 4 below compares the amino acid composition of *Deinococcus geothermalis* to the amino acid composition of Yeast extract. Quantities are given in g/100g of dried matter.

Table 4: Amino acid compositions of *Deinococcus* compared to yeast extract.

	Yeast extract ¹	D. geothermalis Exponential Growth phase	D. geothermalis Stationary growth phase
5	Amino acids :		
	Aspartic acid	5,10	4,65
	Glutamic acid	6,50	5,98
	Alanine	nd	4,67
10	Arginine§	0,78	3,92
	Cysteine	nd	0,31
	Glycine	2,40	3,05
	Histidine*§	0,94	0,93
15	Isoleucine*§	2,90	1,94
	Leucine*§	3,60	4,38
	Lysine*§	4,00	2,34
	Methionine*§	0,79	1,51
	Phenylalanine*§	2,20	1,88
20	Proline	nd	2,22
	Serine	nd	1,75
	Threonine*§	nd	2,82
	Tryptophane*§	0,88	0,76
25	Tyrosine	0,60	1,55
	Valine*§	3,40	3,31
	Total amino acids :	34,00	48,00
	Proteins :	29,45	41,05
			46,19

"§": Amino acids whose carbon skeletons are not synthesized by animal cells and which are essential for fish growth (H. George Ketola 1982, Comp biochem physiol 73B, N°1, p17-24);

"*" : Amino acids whose carbon skeletons are not synthesized by animal cells and which are essential for monogastric mammals;

"nd": not determined

[0144] These data show that the total amino acids content of *Deinococcus* cells reaches 48% and 54% (g/100 g of dried matter) in the exponential and stationary growth phase, respectively. These values are higher than those obtained with yeast extract (34%) whatever the growth phase. The total *Deinococcus* protein content is also higher than that of the yeast extract. In addition, compared to the yeast extract, *Deinococcus* provides significant amount of arginine, which is an essential amino acid for fish (H. George Ketola 1982, Comp biochem physiol 73B, N°1, p17-24). *Deinococcus* biomass provides also more tyrosine and leucine compared to yeast extract, leucine being an essential amino acid for both monogastric mammals and fish.

[0145] Therefore, *Deinococcus* bacteria may be used as a source of highly valuable proteins and amino acids for feedstuff, and may even replace yeast extracts in feedstuff compositions.

Example 5: Analysis of particular culture conditions

Culture conditions allowing carotenoid production

[0146] *Deinococcus geothermalis* strain MX6-1E was grown in 1 L medium containing 20 g/L peptone and 10 g/L yeast extract in 1 L fermentor at 45°C under 0.35 L/min air.

[0147] DO regulated at 20 % by cascade control on aeration and agitation rate.

[0148] After 20 hours, the culture medium displayed a strong red color, indicating presence of carotenoids in said culture medium.

[0149] This experimentation confirms that *Deinococcus* bacteria may produce significant amount of carotenoids. In addition, *Deinococcus* biomass provides more methionine than yeast extract when the cells are in exponential phase of growth and methionine is an essential amino acid for animal cells and is also required for fish growth.

Culture conditions allowing substantial expansion

[0150] *Deinococcus geothermalis* strain MX6-1E-14 was grown in 1 L CMG 10% medium (as described in example 4) containing 20 g/L glucose in 1 L fermentor at 45°C.

5 **[0151]** In that culture condition, the optical density at 600 nm (OD_{600nm}) reaches the value 20 in 40 hours (specific growth rate being 0,5 h⁻¹) indicating a strong expansion of the cells.

SEQUENCE LISTING

10 **[0152]**

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15

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45 **Claims**

1. Use of a mix of biomass and *Deinococcus* bacteria, said biomass being at least partially digested by said *Deinococcus* bacteria, as protein supply for feedstuff.

50

2. Use of claim 1, wherein the biomass comprises lignocellulosic biomass.

3. Use of claim 1 or 2, wherein the biomass comprises proteins.

4. A method for preparing a feedstuff comprising submitting a biomass to at least partial digestion by *Deinococcus* bacteria to obtain a mix, and formulating said mix as ingredient for feedstuff.

55

5. The method of claim 4, wherein the biomass comprises lignocellulosic biomass selected from the group comprising cereal bean, such as wheat bean, vegetal cattle cake, such as rapeseed cattle cake and soybean cattle cake, sugar

cane and derivatives thereof, corn, sugarbeet, miscanthus, switch grass, hemp, poplar, willow, sorghum, tree species, ranging from eucalyptus to oil palm.

- 5 6. The method of claim 4 or 5, wherein the biomass comprises proteins and is selected preferably from beet pulp, soybeans, alfalfa and chicken feathers.
7. The use of a *Deinococcus* bacterium for animal feeding.
- 10 8. The use of claim 7, wherein the *Deinococcus* bacterium is used as a source of amino acids.
9. The use of claim 7, wherein the *Deinococcus* bacterium is used as a mix of *Deinococcus* bacteria and a biomass.

Patentansprüche

- 15 1. Verwendung einer Mischung aus Biomasse und *Deinococcus*-Bakterien, wobei die Biomasse zumindest teilweise von den *Deinococcus*-Bakterien verdaut wird, als Proteinlieferant für Futtermittel.
- 20 2. Verwendung nach Anspruch 1, wobei die Biomasse Lignocellulose-Biomasse umfasst.
3. Verwendung nach Anspruch 1 oder 2, wobei die Biomasse Proteine umfasst.
- 25 4. Verfahren zur Herstellung eines Futtermittels, umfassend das Unterwerfen einer Biomasse einer zumindest teilweisen Verdauung durch *Deinococcus*-Bakterien, um eine Mischung zu erhalten, und das Formulieren der Mischung als Bestandteil für Futtermittel.
- 30 5. Verfahren nach Anspruch 4, wobei die Biomasse Lignocellulose-Biomasse umfasst, ausgewählt aus der Gruppe umfassend Getreidebohnen wie Weizenbohnen, pflanzlichen Rinderkuchen wie Raps-Rinder-Kuchen und Sojabohnen-Rinder-Kuchen, Zuckerrohr und Derivate davon, Mais, Zuckerrüben, Elefantengras (*Miscanthus*), Rutenhirse, Hanf, Pappel, Weide, Sorghum-Hirse, Baumarten reichend von Eukalyptus bis Ölpalme.
- 35 6. Verfahren nach Anspruch 4 oder 5, wobei die Biomasse Proteine umfasst und vorzugsweise aus Rübenschnitzel, Sojabohnen, Luzerne und Hühnerfedern ausgewählt ist.
7. Die Verwendung eines *Deinococcus*-Bakteriums zur Tierfütterung.
8. Verwendung nach Anspruch 7, wobei das *Deinococcus*-Bakterium als Quelle für Aminosäuren verwendet wird.
- 40 9. Verwendung nach Anspruch 7, wobei das *Deinococcus*-Bakterium als eine Mischung von *Deinococcus*-Bakterien und einer Biomasse verwendet wird.

Revendications

- 45 1. Utilisation d'un mélange de biomasse et de bactéries *Deinococcus*, ladite biomasse étant au moins partiellement digérée par lesdites bactéries *Deinococcus*, en tant que source de protéines pour l'alimentation animale.
2. Utilisation selon la revendication 1, dans laquelle la biomasse comprend une biomasse lignocellulosique.
- 50 3. Utilisation selon la revendication 1 ou 2, dans laquelle la biomasse comprend des protéines.
4. Procédé pour préparer une alimentation animale comprenant la soumission d'une biomasse à au moins une digestion partielle par des bactéries *Deinococcus* pour obtenir un mélange, et la formulation dudit mélange en tant qu'ingrédient pour l'alimentation animale.
- 55 5. Procédé selon la revendication 4, dans lequel la biomasse comprend une biomasse lignocellulosique choisie parmi le groupe comprenant la graine de céréale, telle que le grain de blé, le tourteau de bovin végétal, tel que le tourteau de colza et le tourteau de soja, la canne à sucre et ses dérivés, le maïs, la betterave à sucre, le miscanthus, le panic

EP 2 793 607 B9

raide, le chanvre, le peuplier, le saule, le sorgho, des espèces d'arbres, allant de l'eucalyptus au palmier à huile.

6. Procédé selon la revendication 4 ou 5, dans lequel la biomasse comprend des protéines et est choisie de préférence parmi la pulpe de betterave, les graines de soja, la luzerne et les plumes de poulet.

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7. Utilisation d'une bactérie *Deinococcus* pour l'alimentation animale.

8. Utilisation selon la revendication 7, dans laquelle la bactérie *Deinococcus* est utilisée en tant que source d'acides aminés.

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9. Utilisation selon la revendication 7, dans laquelle la bactérie *Deinococcus* est utilisée en tant que mélange d'une bactérie *Deinococcus* et d'une biomasse.

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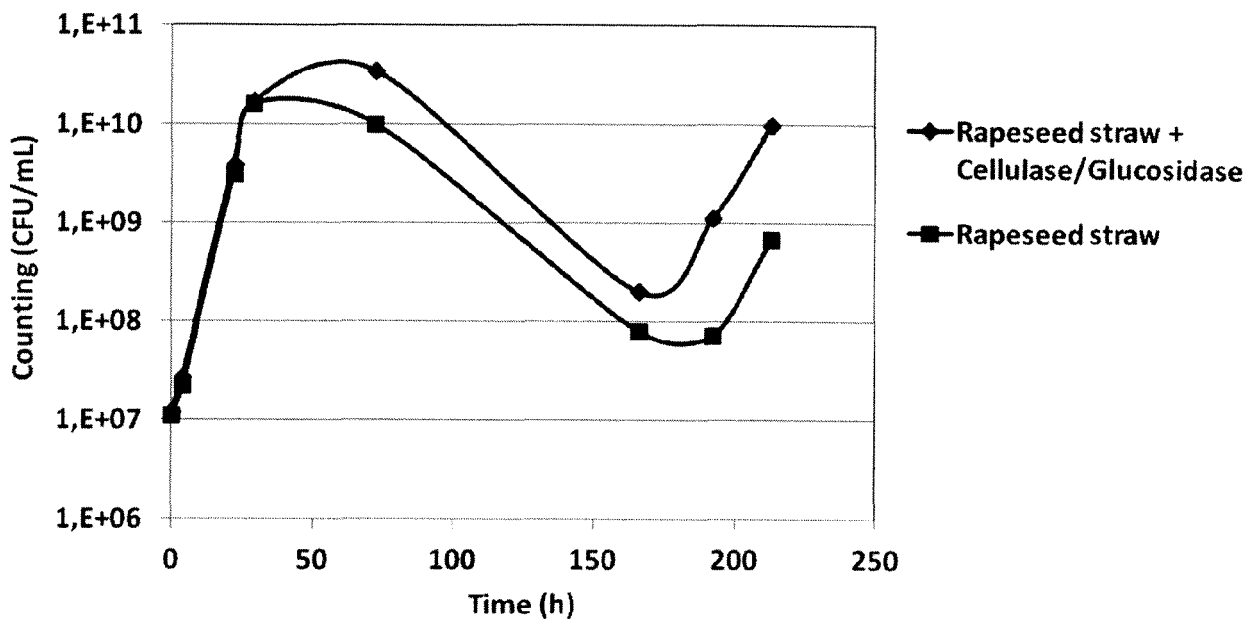
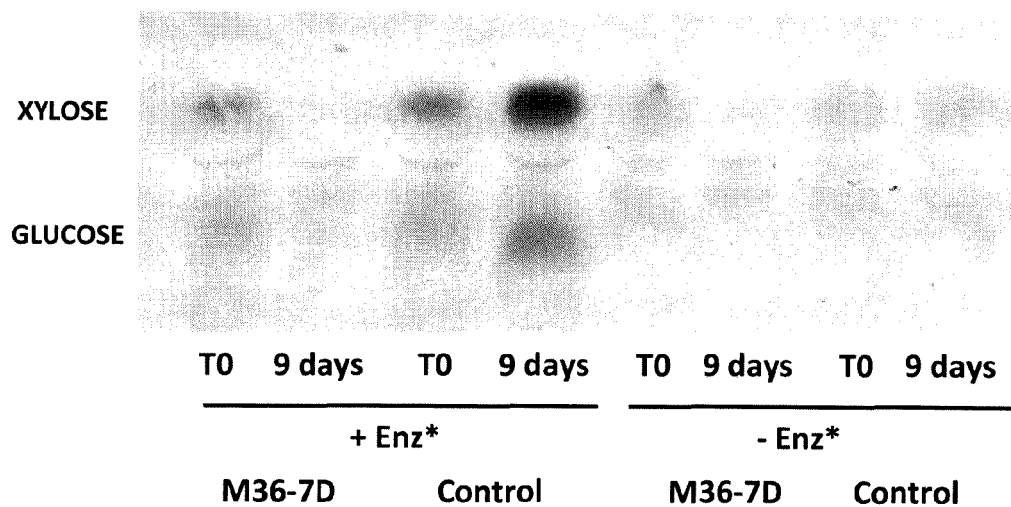


Figure 1



*Enz = Cellulase+ β -Glucosidase added in the culture at T0
 0,11g of cellulase/g of cellulose
 0,05g of β -Glucosidase/g of cellulose

Figure 2

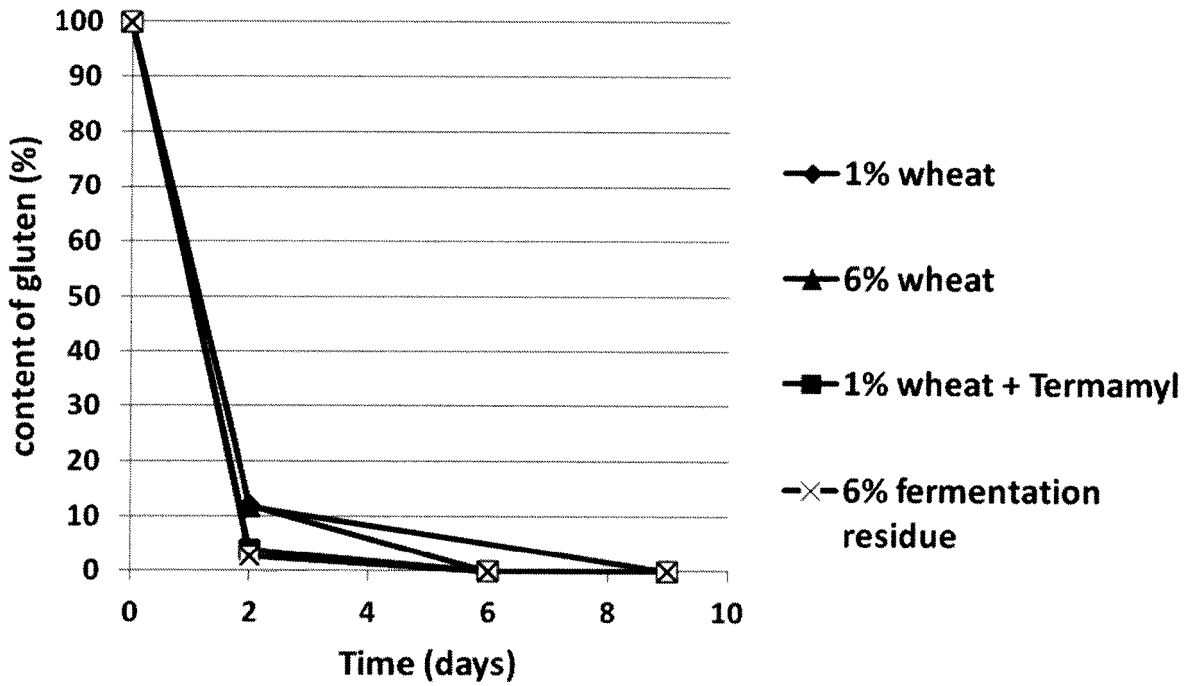
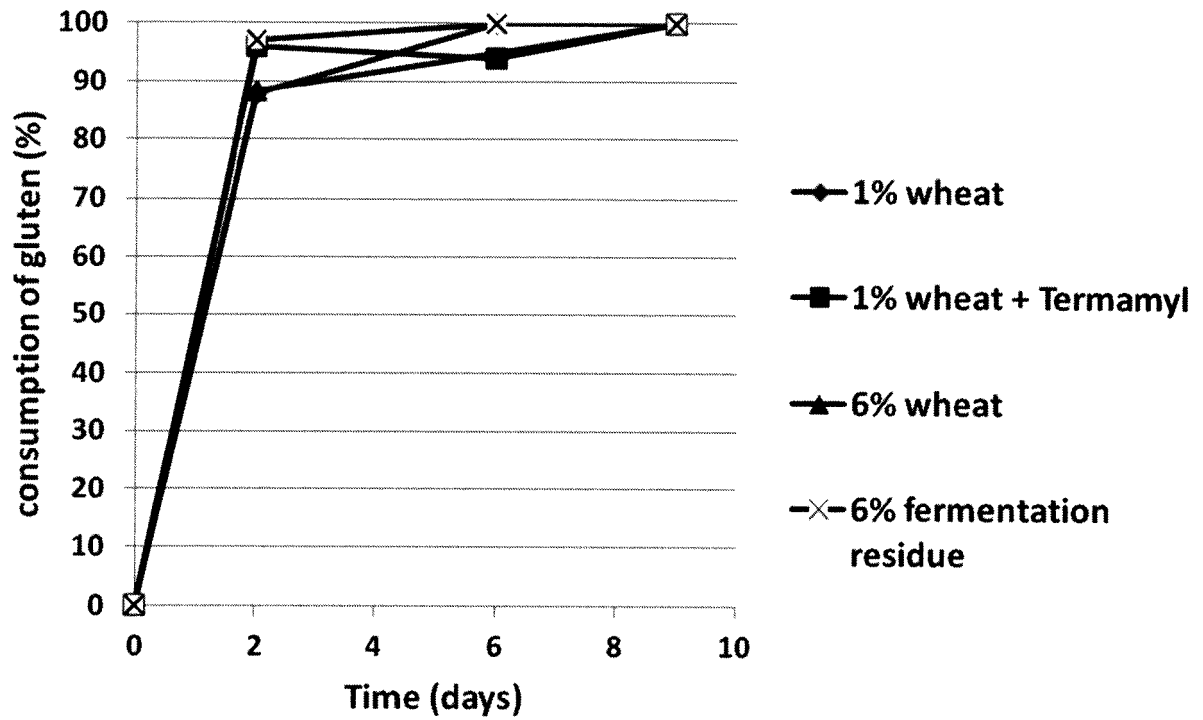


Figure 3

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- EP 2218773 A [0009]
- WO 2011107506 A [0009]
- WO 2009063079 A [0009]

Non-patent literature cited in the description

- **SUN et al.** *Bioresource Technology*, 2002, vol. 83, 1-11 [0032]
- **DEINOCOCCUS.** *Br J Nutr.*, May 2010, vol. 103 (10), 1507-13 [0046]
- **S. CORTASSA et al.** An introduction to metabolic and cellular engineering. World scientific Publishing, 2002 [0142]
- **H. GEORGE KETOLA.** *Comp biochem physiol*, 1982, vol. 73B (1), 17-24 [0143] [0144]