

METHOD OF EXTRACTION AND FERMENTATION

FIELD OF THE INVENTION

[0001] The present invention relates generally to the field of biotechnology. In particular, the present invention relates to methods of extraction and fermentation.

BACKGROUND OF THE INVENTION

[0002] Bran is a major side stream product from flour milling industry. It is commonly used as feed ingredient. However, due to high content of non-starch polysaccharides, its inclusion in feed is limited, especially in monogastric animals. Wheat bran, for example, also contains high amount of ferulic acid and there are studies focusing on exploring ways to extract the phenolic acid from wheat bran. However, there is a dearth of technical exploration of combining both processes for wheat bran treatment. Thus, there is a need for a combined process to better exploit bran obtained as a side product.

SUMMARY

[0003] In one aspect, the present disclosure refers to a method of obtaining ferulic acid from bran followed by subsequent fermentation of the bran, the method comprising: a. combining the bran, water, and an enzyme to obtain a mixture, wherein the enzyme hydrolyses hemicellulose; b. incubating the mixture of step a in an incubation step; c. separating the mixture of step b into liquid and solid phases; d. isolating the liquid phase obtained in step c and obtaining ferulic acid from the same; e. inoculating the obtained solid phase of step c with a microorganism which ferments bran; f. incubating the inoculated solid phase of step e in a fermentation step; thereby obtaining fermented bran.

BRIEF DESCRIPTION OF THE DRAWINGS

[0004] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0005] **FIG. 1** shows a schematic diagram summarizing the “Integrated Process” of ferulic acid extraction and fermentation of bran.

[0006] **FIG. 2** shows the experimental setup and outcomes of three processes for treating bran, in this example, wheat bran. The “Integrated Process” includes enzymatic treatment followed by solid-state fermentation using DSM33837. The “Control Process” refers to wheat bran in water (1:12.5 ratio, w/w) without addition of any exogenous enzymes or DSM33837. “Fermentation” involves only DSM33837 in a solid-state fermentation.

[0007] **FIG. 3** shows the characterization of dried, and modified products of wheat bran. The products were obtained either through “Integrated Process”, “Fermentation” or “Control Process”. FIG. 3a shows live colony-forming units of DSM33837 were recovered in products from both the “Integrated

Process” and “Fermentation” but not from the “Control Process”. FIG. 3b shows increased reducing sugar content in both products from the “Integrated Process” and “Fermentation”, but not from the “Control Process”. These results showed that wheat bran incubated with DSM33837, with or without incorporating ferulic acid extraction step, leads to an increased functionality of wheat bran.

5 [0008] FIG. 4 shows the experimental setup and outcome of an “Alternative Process” for treating bran, as shown by way of the example of wheat bran. “Alternative Process” includes both enzymatic treatment and *Bacillus* fermentation in a single step. Quantifiable amounts of ferulic acid could not be detected from using the “Alternative Process”.

[0009] FIG. 5 shows a schematic diagram summarizing the claimed method applied to the example
10 of ferulic acid extraction and fermentation of wheat bran.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0010] Ferulic acid has antioxidant properties, giving it many potential commercial applications in health, cosmetic and food. Extraction of ferulic acid and other phenolic compounds from products such
15 as, but not limited to, bran, is a topic of interest to valorise this lignocellulosic biomass.

[0011] In the present disclosure, an enzymatic method was used to extract ferulic acid in an environmentally friendly method. Also, the bran resulting from the methods disclosed herein is solvent-free and can be used in other applications, such as, but not limited to, animal feed ingredients.

[0012] Extraction of ferulic acid can be performed on various types of biomasses, such as, but not
20 limited to, corn stoves, rice bran, wheat bran. Thus, in one example, the ferulic acid is extracted from bran.

[0013] The extraction, as disclosed herein, is performed enzymatically. Thus, in one example, the enzyme is obtained from a microorganism. In another example, the enzyme can be a synthetic or recombinant enzyme, for example, one that is obtained in the laboratory using genetic methods known in
25 the art. So long as the required enzyme is produced by a microorganism, a person skilled in the art would be able to obtain said enzyme using methods known in the art. For example, such an enzyme can be isolated or obtained from a microorganism of the genus of *Aspergillus sp.*

[0014] Ferulic acid esterase (FAE), also known as feruloyl esterase is an enzyme capable of hydrolysing the ester bond which is present between polysaccharide forming the plant cell wall and
30 phenolic acid. Ferulic acid esterase has been shown to work synergistically with other hemicellulases to breakdown of lignocellulosic biomass. In other words, enzymes that hydrolyse (hemi-)cellulose can be used in the methods and processes disclosed herein.

[0015] The methods described herein can be performed using one or more enzymes as disclosed herein. In one example, the enzymes are, but are not limited to, ferulic acid esterase, xylanase, and
35 combinations thereof. In one example, the ferulic acid esterase can be obtained from any one or more of the following exemplary microorganisms: *Aspergillus niger*, *Aspergillus tubingensis*, *Aspergillus terreus*,

Aspergillus clavatus; *Lactobacillus amylovorus*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, and/or *Lactobacillus helveticus*. In another example, the enzyme is *Aspergillus niger* ferulic acid esterase (AnFAE).

[0016] Bran, as used herein, also known as miller's bran, refers to the grain husk or the hard outer layers of a grain, such as, but not limited to, cereal grains. It consists of the combined aleurone and pericarp. Along with germ, it is an integral part of whole grains, and is often produced as a by-product of milling in the production of refined grains. Bran makes up about 10% to 15% of the total wheat kernel weight. Specifically, wheat bran is an abundant side product generated during the milling process. In addition, wheat bran contains 10% to 25% starch due to incomplete separation of the endosperm and contains about roughly 35% non-starch carbohydrate mainly, made up of arabinoxylan, a major fibre component in bran. Bran is present in cereal grain, including but not limited to rice, corn (maize), wheat, oats, barley, rye and millet. Bran is not the same as chaff, which is a coarser scaly material surrounding the grain but not forming part of the grain itself, and which is indigestible to humans.

[0017] The term "meal" refers to the coarse-ground, edible part of various grains. Meal is often used in animal feed and can also be characterised as being a coarser blend than flour, whereby flour refers to a powder made by grinding raw grains, roots, beans, nuts, or seeds. It is noted that this does not refer only to the wheat flour commonly used, but refers to the ground product of raw grains, roots, beans, nuts, or seeds. "Meal" also refers to products that remain after oil extraction of grains, brans or oilseeds has been completed. A meal may or may not contain further additives and agents for, for example, enriching the meal, or preventing caking after grinding.

[0018] As used herein, the term "defatted" refers to the removal of fat from the product in question. When used in conjunction with meal, for example, defatted meal refers to meal which has a lower fat content than otherwise untreated (non-defatted) meal.

[0019] As a person skilled in the art will appreciate, the presently disclosed methods can be performed on brans that are naturally enriched in ferulic acids. Thus, in one example, the bran is bran which contains ferulic acid. In another example, the bran is obtained from grains such as, but not limited to, wheat, maize, rice, defatted rice, sorghum, barley, rye and oat. In another example, the bran disclosed herein is wheat bran.

[0020] Arabinoxylan is further associated with varying levels of phenolic acids such as ferulic acid. Thus, disclosed herein is a method enables the extraction of ferulic acid from bran. The method disclosed herein also allows for subsequent fermentation of the product left over after ferulic acid extraction in the form of a sequential or integrated method or process.

[0021] High amounts of non-starch carbohydrate such as arabinoxylan, can bring about digestion issues in, for example, livestock. In addition, xylan can act as an antinutritional components by trapping energy and nutrients in meal that contains xylan or arabinoxylan. Thus, microorganisms disclosed herein function to ferment biomass in order to improve the biochemical and nutritional composition of the

biomass. In other words, fermentation of the biomass results in a form of “pre-digestion”, resulting in improved bioavailability of the nutrients and energy contained in the meal and bran treated using the method disclosed herein.

[0022] A person skilled in the art will appreciate that the methods and processes disclosed herein can be performed using microorganisms capable of fermentation. Specifically, the microorganism used herein must be capable of fermenting the starting material of the fermentation process. Thus, in one example, fermentation is performed using microorganisms such as, but not limited to, a microorganism of the genus of *Bacillus* spp., *Lactobacillus* spp., *Aspergillus* spp., *Streptococcus* spp., and combinations thereof. In another example, the microorganism can be, but is not limited to, *Bacillus* spp. strains DSM33837, DSM33839 and DSM33838, and combinations thereof. In a further example, the microorganism is DSM33837.

[0023] The present disclosure describes a method of bran pre-treatment and extraction of ferulic acid in a preceding method. In one example, the bran obtained herein can be use as, for example, an animal feed ingredient. Thus, in one example, there is disclosed a method of obtaining ferulic acid from bran (also referred to as the extraction step), followed by subsequent fermentation of the bran, after extraction, the method comprising: combining the bran, water, and an enzyme to obtain a mixture, wherein the enzyme hydrolyses (hemi-) cellulose; incubating the mixture in an incubation step; separating the mixture into liquid and solid phases; isolating the obtained liquid phase and obtaining ferulic acid from the same; inoculating the obtained solid phase with a microorganism which ferments bran; incubating the inoculated solid phase in a fermentation step; thereby obtaining fermented bran. In another example, after the ferulic acid has been obtained from the liquid phase, the remaining liquid phase is returned to the starting material of the extraction step. In another example, the fermented bran obtained herein is used in animal feed, as an animal feed ingredient.

[0024] In one example, the incubation step, for example step b as disclosed herein, is carried out for at least 15 minutes, at least 30 minutes, at least 1 hour, at least 2 hours, at least 3 hours, at least 4 hours, at least 5 hours, at least 6 hours, at least 7 hours, at least 8 hours, at least 9 hours, at least 10 hours, at least 11 hours, at least 12 hours, at least 13 hours, at least 14 hours, at least 15 hours, at least 16 hours, at least 17 hours, at least 18 hours, at least 19 hours, at least 20 hours, at least 21 hours, at least 22 hours, at least 23 hours, or at least 24 hours, or about 15 minutes, about 30 minutes, about 1 hour, about 2 hours, about 3 hours, about 4 hours, about 5 hours, about 6 hours, about 7 hours, about 8 hours, about 9 hours, about 10 hours, about 11 hours, about 12 hours, about 13 hours, about 14 hours, about 15 hours, about 16 hours, about 17 hours, about 18 hours, about 19 hours, about 20 hours, about 21 hours, about 22 hours, about 23 hours, or about 24 hours.

[0025] As will be appreciated by a person skilled in the art, the duration of the incubation step will depend on, for example, the quality of the enzyme used (and thereby the efficacy of the enzyme). Thus,

the more efficient the enzyme, the shorter the incubation duration can be. Such a duration can also depend on the type and quality of the starting material used.

[0026] As will be appreciated by a person skilled in the art, whether an incubation step as disclosed herein, or an enzyme digestion as disclosed herein, has been carried out for a sufficient length of time will depend on, for example, if release of the desired product can be detected and/or quantified. In one example, the detection and/or quantification of ferulic acid indicates that the incubation step has been performed successfully.

[0027] In another example, the combining of bran, water and enzymes, as disclosed herein, is done according to a ratio of bran to water. It is of note that when wheat bran is mixed with equal amount of water (i.e., a 1:1 ratio of bran to water), the bran will absorb all the liquid and it will not be possible to separate liquid from the bran. Thus, as disclosed here, bran and water are combined in a ratio of, but not limited to, at least 1:5 (bran to water), or about 1:6, about 1:7, about 1:8, about 1:9, about 1:10, about 1:11, about 1:12, about 1:13, about 1:14, about 1:15, about 1:16, about 1:17, about 1:18, about 1:19, about 1:20, about 1:21, about 1:22, about 1:23, about 1:24, about 1:25, about 1:26, about 1:27, about 1:28, about 1:29, about 1:30, about 1:31, about 1:32, about 1:33, about 1:34, about 1:35, about 1:36, about 1:37, about 1:38, about 1:39, about 1:40, about 1:41, about 1:42, about 1:43, about 1:44, about 1:45, about 1:46, about 1:47, about 1:48, about 1:49, or about 1:50.

[0028] In one example, the amount of ferulic acid esterase is, but is not limited to, at least 0.10 μ g per gram of bran, or about 0.11 μ g, about 0.12 μ g, about 0.13 μ g, about 0.14 μ g, about 0.15 μ g, about 0.16 μ g, about 0.17 μ g, about 0.18 μ g, about 0.19 μ g, or about 0.20 μ g per gram of bran.

[0029] In another example, the amount of xylanase is, but is not limited to, at least 0.4% per gram of bran, or about 0.5% per gram of bran, about 0.6% per gram of bran, about 0.7% per gram of bran, about 0.8% per gram of bran, about 0.9% per gram of bran, about 1% per gram of bran, about 1.5% per gram of bran, or about 2% per gram of bran.

[0030] As shown in the "alternative process" as disclosed herein, the order in which the sub-processes of the claimed process are performed has an impact on the resulting product. For example, if the fermentation step is performed prior to the enzyme extraction/digestion, the following issues can occur: proteases secreted by bacteria during fermentation can inhibit feruloyl esterase activity, thereby negatively affecting the yield of the method; carry-over of bacteria from fermentation into the liquid phase of ferulic acid extraction can hamper and complicate purification of ferulic acid and the reuse of water, thereby making the resulting method less economical; without being bound by theory, enzyme digestion (in order to release ferulic acid into the liquid phase) requires large amounts of water. Therefore, if enzymatic digestion takes place simultaneously with bacterial fermentation, the release of ferulic acid may not be efficient; reducing sugars generated by bacterial fermentation will be washed away from the fermented bran if the process was performed simultaneously or in a reversed sequence, thereby diminishing the nutrient content and nutritional value of the resulting fermented bran.

[0031] In order to obtain the ferulic acid from the products of the methods disclosed herein, the ferulic acid needs to be separated from its (carrier) liquid phase. A person skilled in the art will appreciate that this can be done using methods known in the art. These methods can be, but are not limited to, distillation, vacuum distillation, batch distillation, continuous distillation, solvent extraction, and solid-phase extraction.

[0032] In one example, the ferulic acid obtained from the methods disclosed herein is obtained at a yield of between 0.4mg ferulic acid per gram bran to 6 mg ferulic acid per gram bran. In another example, the yield of ferulic acid is between 0.4mg to 0.8mg, between 0.5mg to 1mg, between 0.75mg to 1.25mg, between 0.8mg to 1.3mg, between 0.9mg to 1.4mg, between 1.3mg to 1.8mg, between 1.5mg to 2mg, between 1.75mg to 2.25mg, between 1.8mg to 2.3mg, between 1.9mg to 2.4mg, between 2.3mg to 2.8mg, between 2.5mg to 3mg, between 2.75mg to 3.25mg, between 2.8mg to 3.3mg, between 2.9mg to 3.4 mg, between 3.3 mg to 3.8 mg, between 3.5 mg to 4 mg, between 3.75 mg to 4.25 mg, between 3.8mg to 4.3mg, between 3.9mg to 4.4mg, between 4.3mg to 4.8mg, between 4.5mg to 5mg, between 4.75mg to 5.25mg, between 4.8mg to 5.3mg, between 4.9mg to 5.4mg, between 5.3mg to 5.8mg, or between 5.5mg to 6mg. In another example, the yield of ferulic acid is between 1.5mg to 1.75mg ferulic acid per gram bran. In yet another example, the yield of ferulic acid is about 1.65 mg ferulic acid per gram bran.

[0033] In addition, separation of a solid phase or component from a liquid phase can be performed using methods such as, but not limited to centrifugation, filtration, and sedimentation.

[0034] In one example, the fermentation step as disclosed herein is a solid-state fermentation (SSF) step.

[0035] Although alkaline and enzymatic treatments have been proposed in literatures for the extraction of ferulic acid, these methods present several issues, such as environmental issues and sustainability, and costs. These issues have been addressed in the present application by, for example, the recycling of water used in extraction of ferulic acid, as well as using the residual wheat bran in a fermentation process for the production of animal feed ingredient. Examples of animal feed ingredients include, but are not limited to, animal feed ingredients which contain probiotic bacillus, as a result of the fermentation process of the bran disclosed herein. Such animal feed ingredients obtained by the process disclosed here have increased reducing sugar content, compared to bran that had not undergone the claimed process.

[0036] To valorise bran, for example wheat bran, the present disclosure shows an exemplary workflow wherein ferulic acid is first extracted from bran, followed by subsequent solid-state fermentation (SSF) of the residual bran, which can then be further processed to be used in the production of animal feed ingredients. (See, for example, FIG. 1).

[0037] Also, the enzymatic treatment as disclosed herein allows for the extraction of ferulic acid without compromising the growth of the microorganisms required for subsequent fermentation. The water used for ferulic acid extraction containing residual enzymes can be reused for treating subsequent batches

of bran. This will also have a positive economic impact on the method disclosed herein, which is then considered to be environmentally friendly and cost-effective compared to known methods.

[0038] In addition, it is shown that microbially fermented bran contains higher amount of reducing sugar than non-fermented bran, thereby showing and underlining that the bran obtained from the methods disclosed herein has improved bioavailability of the nutrients it contains.

[0039] To illustrate the above, experiments were performed as described in the experimental section disclosed herein.

[0040] After 16 hours, the liquid fraction is separated from the wheat bran. Ferulic acid from this liquid phase was analysed by high performance liquid chromatography (HPLC). The ferulic acid yield was 1.65g/kg wheat bran, as shown in the results provided in Table 1.

[0041] Table 1 shows the results of quantification of extracted ferulic acid from the three processes disclosed herein, the "Integrated Process", the "Control Process" and "Fermentation". After 16 hours, the liquid fraction is separated from the wheat bran. Ferulic acid from the liquid phase was analysed by HPLC. The amount of ferulic acid was quantified using ferulic acid standard curve. Values that fall below the range covered by the standard curve is considered not quantifiable (N.Q.). The ferulic acid yield from the "Integrated Process" is 1.65 mg/g of wheat bran. Wheat bran treated with the other processes did not result in a quantifiable yield.

Process for wheat bran	Ferulic acid yield (mg/g wheat bran)
Integrated Process	1.65
Control Process	Not quantifiable
Fermentation	Not quantifiable

[0042] The residual wheat bran undergoes solid state fermentation with DSM33837 and then dried. The dried solid-state fermentation (SSF) product was rehydrated and analysed for viable microbes, reducing sugar content.

[0043] Results from the "Alternative Process" setup (see FIG. 4), showed that when enzyme extraction and bacterial fermentation were done simultaneously, there was no quantifiable ferulic acid recovered in the process (data not shown), highlighting the challenge of combining two processes in a single process flow.

[0044] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various

modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0045] As used in this application, the singular form “a,” “an,” and “the” include plural references unless the context clearly dictates otherwise. For example, the term “a genetic marker” includes a plurality of genetic markers, including mixtures and combinations thereof.

[0046] As used herein, the term “about”, in the context of concentrations of components of the formulations, typically means +/- 5% of the stated value, more typically +/- 4% of the stated value, more typically +/- 3% of the stated value, more typically, +/- 2% of the stated value, even more typically +/- 1% of the stated value, and even more typically +/- 0.5% of the stated value.

[0047] Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

[0048] Certain embodiments may also be described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the disclosure. This includes the generic description of the embodiments with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0049] The invention has been described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0050] Other embodiments are within the following claims and non-limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

EXPERIMENTAL SECTION

Enzymatic treatment of wheat bran

[0051] Wheat bran was autoclave sterilized at 121°C for 15 minutes and dried overnight in an oven. The sterilized wheat bran (19g) was placed in a 1 litre conical flask. In the “Integrated Process”, 237.5g of water, 2.85µg of recombinant *Aspergillus niger* ferulic acid esterase (AnFAE) and 95µL (0.5% (v/w)) of commercial xylanase (Megazyme) were added. This meant that the amount of ferulic acid esterase and commercial xylanase used was 0.15µg and 0.5%, respectively, per gram of wheat bran. The wheat bran to water ratio used in enzymatic hydrolysis was 1:12.5 (12.5g of water was added to 1g of wheat bran). In “Control Process”, 237.5g of water was added to wheat bran. In “Fermentation”, 19g of water was added to wheat bran. All flasks were incubated for 16 hours at 37°C. The liquid fraction was recovered, centrifuged, and kept for ferulic acid analysis. The treated wheat bran was subsequently used for solid-state fermentation. Time 0 hour was taken as before enzymatic hydrolysis and time 16 hour was after enzymatic hydrolysis.

Production of solid-state fermentation (SSF) product

[0052] Prior to starting the solid-state fermentation of the wetted wheat bran, 1 g of sterilized wheat bran in a 50ml falcon polypropylene tube was inoculated with 1ml (1.6×10^8 CFU) of DSM33837 and allowed to incubate for 3 hours at 37°C. Subsequently, the 1g of inoculated wheat bran was mixed thoroughly with the enzyme-treated wheat bran in the flask of the “Integrated Process”. For the “Control Process”, 1g of sterilized wheat bran with 1ml of water was mixed thoroughly with the water-treated wheat bran in flask. For “Fermentation”, 1g of sterilized wheat bran with 1ml of water was mixed thoroughly with pre-wetted wheat bran in flask. Fermentation was carried out for a total of 24 hours at 37°C. Following fermentation, the flasks were covered with a double-layered cheese cloth and dried in a 60°C incubator until excess moisture was completely evaporated.

Measurement of reducing sugar from solid-state fermentation (SSF)

[0053] Using the 3, 5-dinitrosalicylic acid (DNS) method as described in the art, the total reducing sugar liberated during the solid-state fermentation (SSF) was examined. 300µl of suitably diluted cell-free fermentation crude extract was added to 900µl of DNS reagent and boiled at 100°C for 5 minutes. Following which, the absorbance at 540nm (A_{540nm}) was measured and the amount of reducing sugar liberated was extrapolated from the xylose standard curve.

Colony count of viable microbes from solid-state fermentation (SSF) product post-drying

[0054] Recovery of viable microbes from the solid-state fermentation (SSF) product after drying was examined by re-hydrating 1g of dried solid-state fermentation product in 10 ml of distilled water and shaking thoroughly. The suspension was serially diluted to 10^{-4} . Diluted samples were plated onto LB (Luria Bertani) agar plates and incubated at 37°C overnight. The resultant colony-forming units (CFU) were counted and used to calculate the CFU/g of meal present in the sample.

Quantification of ferulic acid using high performance liquid chromatography (HPLC).

[0055] The concentration of ferulic acid was determined by HPLC (Shimadzu Prominence LC-20AD system equipped with a diode array detector SPD-M2A). A C18 column (5 μ m x 250mm x 4.6mm) (ODS-HYPERSIL-2, Thermo Fisher) was eluted with a gradient mobile phase consisting of 0.1% acetic acid as eluant A and 100% methanol as eluant B. The flow rate was maintained at 1ml per minute. The column oven was set at 24°C. 10 μ l of sample, appropriately diluted, were injected into the column for analysis. Peaks were monitored at 340nm. The amount of ferulic acid was quantified using a ferulic acid standard curve.

Production of recombinant ferulic acid esterase using Pichia pastoris expression system

[0056] The DNA coding for ferulic acid esterase gene from *Aspergillus niger* (AnFAE) was codon optimized and synthesized. The gene was cloned into a pPIC9K vector using EcoRI/NotI restriction sites and expressed in *Pichia pastoris* GS115. Protein expression was achieved by growing the cells in buffered minimal methanol-complex medium (BMMY; 0.1M potassium phosphate buffer pH 6.0, 1% yeast extract, 2% peptone, 1.34% YNB without amino acids, 0.4 μ g/ml biotin, 1% methanol) at 28°C with shaking (200rpm). The cells were fed with 1% methanol every 12 hours for 4 days. Subsequently, the culture was centrifuged at 4,000rpm for 30 minutes to obtain and remove the cell pellet. The supernatant containing the *Aspergillus niger* ferulic acid esterase was concentrated, and protein concentration was determined via Bradford method, using bovine serum albumin (BSA) as a standard. The cell-free supernatant was used as a crude enzyme for subsequent experiments.

[0057] The DNA sequence of the synthesized gene is as follows, with EcoRI restriction site at the 5' end and NotI restriction site at the 3' end (underlined):

GAATTCGCTTCCACTCAGGGTATTTCCGAGGACTTGTACAACAGATTGGTTGAGATGGCCAC
TATCTCCCAAGCTGCTTACGCTGACTTGTGTAACATCCCATCCACCATCATCAAGGGTGAGA
AAATCTACAACGCCCAGACCGACATTAACGGTTGGATCTTGAGAGATGACACCTCCAAAGA
GATCATCACCGTTTTAGAGGTACTGGTTCGACACCAACTTGCAGTTGGACACTAACTACA
CTCTGACCCCATTCGACACTTTGCCACAGTGTAACGACTGTGAAGTTCACGGTGGTTACTAC
ATCGGTTGGATTTCCGTTCAAGACCAGGTTGAGTCCTTGGTTAAGCAACAGGCTTCTCAGTA
CCCAGACTACGCTTTGACTGTTACTGGTCACTCTTGGGTGCTTCTATGGCTGCTTTGACAGC
TGCTCAATTGTCCGCTACTTACGACAACGTTAGACTGTACACTTTCGGTGAGCCAAGATCCG
GTAACCAGGCTTTTGCTTCTTACATGAACGACGCTTTCCAGGTGTCCTCTCCAGAGACTACTC
AGTACTTCAGAGTTACCACTCCAACGACGGTATCCCAAATTTGCCACCAGCTGAACAAGGT
TACGCTCATGGTGGTGTGAATACTGGTCTGTTGACCCATACTCCGCTCAGAACACTTTTCGTC
TGTAAGTGGTGACGAGGTTCAAGTGTGTAAGCTCAAGGTGGACAAGGTGTTAACGACGCTCA
CACTACTTACTTCGGTATGACTTCCGGTGCTTGTACTTGGCGGCCGC. (SEQ ID NO: 1)

[0058] The protein sequence of the synthesized gene is as follows (same sequence as XP_001393337.1 in protein database):

ASTQGI SEDLYNRLVEMATISQAAYADLCNIPSTIIKGEKIYNAQTDINGWILRDDTSKEIITVFRG
 TGS DTLNLQLD TNYTLTPFD TLPQCNDCEVHGGYYIGWISVQDQVESLVKQQASQYPDYALTVTG
 HSLGASMAALTA AQLSATYDNVRLYTFGEPRSGNQAFASYMND AFQVSSPETTQYFRVTHSND
 GIPNLPPAEQGYAHGGVEYWSVDPYSAQNTFVCTGDEVQCCEAQGGQGVNDAHTTYFGMTSG

5 ACTW (SEQ ID NO: 2)

Isolation and identification of microorganism strains

[0059] One gram of agricultural plant biomass and 5ml of sterile distilled water were added to a 50ml falcon polypropylene tube and incubated in a 37°C incubator. After several days, a sample of the culture was streaked on a Luria-Bertani (LB) agar plate and incubated at 37°C overnight. Sub-culture with repeated streaking was performed to obtain pure culture. Grown single colonies were picked and inoculated into LB broth overnight at 37°C, 220rpm. Next day, the culture was collected, washed with water, and inoculated at initial OD600 of 0.2 into M9 minimal medium (6.78g/L Na₂HPO₄•7H₂O, 3g/L KH₂PO₄, 1g/L NH₄Cl, 0.5g/L NaCl) containing 0.5% (w/v) beech wood xylan as sole carbon source. The tubes were incubated at 37°C, 220rpm for several days and their enzyme activity was assessed by a plate screen with Azurine-cross-linked xylan (AZCL-xylan) followed by xylanase activity assay performed using the 3,5-dinitrosalicylic acid (DNS) method. The isolates with high xylanase activity were selected and stored in 30% glycerol at -80°C for further analysis.

Identification of microorganisms

[0060] The phylogeny of isolated strains was further identified by 16S rRNA sequencing. Genomic DNA extraction was performed by the methodology as described by Hoffman et al. (1987; Gene; 57; 267-272) and used as template in PCR for 16S rRNA partial gene amplification (~500bp) using the following universal primers: WIC1P2336 (5'- CCT ACG GGA GGC AGC AG -3'; SEQ ID NO: 3) and WIC1P2337 (5'- GGA CTA CHV GGG TWT CTA AT -3'; SEQ ID NO: 4). The amplicons were purified and sequenced. Sequence similarity and homology analysis against the GenBank database were carried out using the basic local alignment search tool (BLASTN) on the National Center for Biotechnology Information (NCBI).

Description of the "Alternative Process"

[0061] In the "Alternative Process", enzyme extraction and bacterial fermentation were done simultaneously. To this end, 1g of sterilized wheat bran was inoculated with 1ml (1.6×10⁸ CFU) of DSM33837 and allowed to incubate for 3 hours at 37°C. Subsequently, the 1g of inoculated bran was mixed thoroughly with the 19g of wheat bran, 19g of water and 2.85µg of *Aspergillus niger* ferulic acid esterase in the "Alternative Process" flask. The flask was incubated for 16 hours at 37°C. Thereafter, a further 200g of water was added and incubated for another 6 hours at 37°C. The liquid fraction was removed, centrifuged, and kept for ferulic acid analysis.

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CLAIMS

1. A method of obtaining ferulic acid from bran followed by subsequent fermentation of the bran,
5 the method comprising:
 - a. combining the bran, water, and an enzyme to obtain a mixture, wherein the enzyme hydrolyses hemicellulose;
 - b. incubating the mixture of step a in an incubation step;
 - c. separating the mixture of step b into liquid and solid phases;
 - 10 d. isolating the liquid phase obtained in step c and obtaining ferulic acid from the same;
 - e. inoculating the obtained solid phase of step c with a microorganism which ferments bran;
 - f. incubating the inoculated solid phase of step e in a fermentation step; thereby obtaining fermented bran.
- 15 2. The method of any of one of the preceding claims, wherein the microorganism is selected from the group consisting of *Bacillus spp.*, *Lactobacillus spp.*, and *Aspergillus spp.*
3. The method of anyone of the preceding claims, wherein the microorganism is *Bacillus* strain DSM33837.
- 20 4. The method of any one of the preceding claims, wherein the enzyme is xylanase and/or ferulic acid esterase.
5. The method of any one of the preceding claims, wherein the ferulic acid in step d is obtained
25 using a method selected from the group consisting of distillation, vacuum distillation, batch distillation, continuous distillation, solvent extraction, and solid-phase extraction.
6. The method of any one of the preceding claims, wherein the separating of the solid and liquid
30 phases in step c is performed using a method selected from the group consisting of centrifugation, filtration, and sedimentation.
7. The method of any one of the preceding claims, wherein after the ferulic acid has been obtained from the liquid phase of step d, the remaining liquid phase is added to step a.
- 35 8. The method of any one of the preceding claims, wherein the ferulic acid from the liquid phase of step d is obtained at a yield of 0.4 mg ferulic acid per gram bran to 6 mg ferulic acid per gram bran.

9. The method of any one of the preceding claims, wherein the fermentation step is a solid-state fermentation (SSF) step.
- 5 10. The method of any one of the preceding claims, wherein the fermented bran obtained in step f is used as an animal feed ingredient.
11. The method of claim 10, wherein the bran is selected from the group consisting of wheat, maize, rice, defatted rice, sorghum, barley, rye and oat.