

An Isolated *Bacillus Subtilis* Strain, Uses Thereof and Processes Using Said Isolated Strain

Technical Field

- 5 The present invention generally relates to an isolated strain of *Bacillus subtilis*. The present invention also relates to uses of the isolated strain and a process for hydrolysing a plant raw material, producing a non-alcoholic malted beverage or producing an alcoholic beverage using the isolated strain.

Background Art

- 10 Brewer's spent grain is the major by-product generated from the brewing process for production of ethanol-containing drinks such as beers. The wort and solid residue (spent grain) are separated by filtration. The spent grain is normally considered as a low-value waste and subsequently used to feed animals. As spent grain is produced from agricultural products, it contains components of plant cell walls, proteins and other substances such as
- 15 lipids. The components of beer spent grains have been found to be different when obtained from different sources but are generally rich in cellulose and proteins regardless of where they were obtained from. Therefore, converting spent grain to value-added chemicals has received much attention. It has been suggested that spent grain can be used as a source for phytochemicals as well.
- 20 Accumulated studies have shown that spent grain can be used as substrates or further processed into value-added chemicals. Spent grain can be used to produce oligosaccharides through auto-hydrolysis. Brewer's spent grain was converted into biogas by anaerobic digestion. Carbohydrate-degrading enzymes such as cellulases were used to treat spent grain to produce sugars which can be used as carbon sources for microbial fermentation. As
- 25 spent grain contains proteins, proteases were demonstrated to solubilize spent grain to produce amino acids that can be used in food production or converted to other chemicals. The annual production of spent grain is very high, which makes it an attractive substrate for food industries. Although studies have been conducted for treatment of spent grain from beer production, few reports were found for treatment of spent grains from other food
- 30 processing industries. To our knowledge, there is no report on treatment of cereal spent grains.

- Barley spent grain (BSG) is the by-product generated from the malting process used when producing malt extract for the manufacture of malted beverages, such as MILO® or NESTOMALT®. An objective is to utilize the barley spent grain to get the benefit of waste
- 35 reduction, improvement of nutritional benefits and flavor enhancement. For instance, this may be achieved by solubilizing the spent grains using either microorganisms or enzymes or both, and then to add the hydrolysate back into the main product stream (cereal malt extract) and to identify the nutritional value of the newly solubilized side stream.

There is a need to provide a solubilized spent grain that overcomes, or at least ameliorates, one or more of the disadvantages described above. The solubilized spent grain may have a higher level of solubilization as compared to those in the art.

There is a need to provide a bacterial species that can be used to solubilize the spent grain.

5 Summary

According to one aspect, there is provided an isolated strain of *Bacillus subtilis* selected from the group consisting of *Bacillus subtilis* B1-4 under deposit number CNCM I-5356, *Bacillus subtilis* WCB-2 under deposit number CNCM I-5357 and a mutant strain thereof.

Advantageously, the above bacterial strain may secrete or produce a biological material.
10 The biological material may be an enzyme such as a protease or a carbohydrase.

According to another aspect, there is provided a cell culture comprising an isolated strain as defined herein in a growth medium.

According to another aspect, there is provided a cell-free supernatant comprising at least one protease and at least one carbohydrase produced by an isolated strain as defined herein.

15 The cell-free supernatant may comprise a mixture of protease(s) and carbohydrase(s).

According to another aspect, there is provided use of an isolated strain as defined herein for obtaining a cell culture.

The isolated strain of *Bacillus subtilis* may be selected from the group consisting of *Bacillus subtilis* B1-4 under deposit number CNCM I-5356, *Bacillus subtilis* WCB-2
20 under deposit number CNCM I-5357 and a mutant strain thereof

According to another aspect, there is provided a use of an isolated strain as defined herein for hydrolyzing a plant raw material.

According to another aspect, there is provided use of a cell-free supernatant as defined herein for hydrolyzing a plant raw material.

25 According to another aspect, there is provided a process for hydrolysing a plant raw material comprising the step of contacting a plant raw material with an isolated strain as defined herein, a cell culture as defined herein or a cell-free supernatant as defined herein.

The isolated strain of *Bacillus subtilis* may be selected from the group consisting of *Bacillus subtilis* B1-4 under deposit number CNCM I-5356, *Bacillus subtilis* WCB-2
30 under deposit number CNCM I-5357 and a mutant strain thereof

According to another aspect, there is provided a process for producing a non-alcoholic malted beverage comprising the step of mixing a hydrolysed plant raw material with a beverage ingredient.

35 According to another aspect, there is provided a process for producing an alcoholic beverage comprising the step of alcoholic fermenting a hydrolysed plant raw material.

Definitions

The following words and terms used herein shall have the meaning indicated:

- 5 The term “isolated” when referring to the bacterial strain is to be interpreted broadly to mean that the bacterial strain was obtained and separated from its native environment in order to obtain a substantially pure culture of the bacterial strain. When the bacterial strain is in the substantially pure culture, this may mean that the bacterial strain is substantially free of other species of organisms. The bacterial strain may then be stored in the form of a culture of a single species of organism. When the bacterial strain is used, the bacterial strain may then be in a mixture with other species.
- 10 The term “mutant” is to be interpreted broadly to include derived bacterial strains where less than about 1%, less than about 0.1%, less than about 0.01%, less than about 0.001% or further less than about 0.0001% of the nucleotides in the original bacterial genome have been replaced with another nucleotide, or deleted, compared to the original bacterial strain. This may occur via genetic engineering, chemical mutagenesis or radiation (such as UV
- 15 radiation). Where a mutant is used, the mutant should be functionally equivalent to the original bacterial strain, such as being able to produce or secrete the same types of biological materials (such as proteins) as the original bacterial strain or at least produce or secrete the essential types of biological materials (such as proteins) that are used for the hydrolysis or solubilization of a plant raw material as in the original bacterial strain.
- 20 The term “cell-free supernatant” is to be interpreted broadly to refer to a liquid phase that does not substantially contain any cell components, cell fractions or bacterial cells. The cell-free supernatant may be obtained by purifying a cell-containing medium to remove any whole cells, cell fractions or cell debris that may have occurred as a result of lysing the bacterial cells. Depending on the method to purify and aggregate the cells or cell fractions,
- 25 a small amount of the cells or cell fractions may still exist in the cell-free supernatant. The cell-free supernatant may contain biological materials (such as proteins or enzymes) that are extracted from or secreted by the bacterial strains. The cell-free supernatant may be used to solubilise or hydrolyse a plant raw material.
- 30 The term “solubilization” is to be interpreted broadly to refer to an enzymatic treatment of a substrate, such as a plant raw material, where biological macromolecules (such as carbohydrates, proteins or lipids) present in the substrate are broken down into their respective shorter chain oligomers or monomers (such as monosaccharides, amino acids or fatty acids). The macromolecules may also be made soluble after becoming shorter chain oligomers or monomers. The enzymatic treatment may involve the use of enzymes secreted
- 35 by or extracted from one or more of the isolated bacterial strain as disclosed herein (and in a comparative sense the use of a commercial enzyme cocktail for solubilization). The term “solubilization” and grammatical variants thereof such as solubilizing may be used in a similar manner or interchangeably with the term “hydrolysis” and grammatical variant thereof such as hydrolyzing.
- 40 The word “substantially” does not exclude “completely” e.g. a composition which is “substantially free” from Y may be completely free from Y. Where necessary, the word “substantially” may be omitted from the definition of the invention.

Unless specified otherwise, the terms "comprising" and "comprise", and grammatical variants thereof, are intended to represent "open" or "inclusive" language such that they include recited elements but also permit inclusion of additional, unrecited elements.

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.

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.

Certain embodiments may also be described broadly and generically herein. Each of the narrower species and subgeneric 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.

Detailed Disclosure of Embodiments

Exemplary, non-limiting embodiments of the present disclosure will now be disclosed.

The isolated strain of *Bacillus subtilis* may be *Bacillus subtilis* B1-4 deposited with the depository agency Collection Nationale de Cultures de Microorganismes (CNCM) under deposit number CNCM I-5356, which was deposited on 27 September 2018. This corresponds to the identification reference of the depositor of NCC-2956. The isolated strain of *Bacillus subtilis* may be *Bacillus subtilis* WCB-2 deposited with the depository agency CNCM under deposit number CNCM I-5357, which was deposited on 27 September 2018. This corresponds to the identification reference of the depositor of NCC-2957. The isolated strain may be a mutant of the *Bacillus subtilis* B1-4 as deposited above. The isolated strain may be a mutant of the *Bacillus subtilis* WCB-2 as deposited above.

The bacterial strain may be provided in the form of a live bacterial culture or as a lyophilized bacterial population. Where the bacterial strain is provided as a lyophilized bacterial population, this may aid in storage and transportation of the bacterial strain. The lyophilized bacterial population (typically in the form of a cell pellet) can be revived using known revival techniques, such as exposing the lyophilized cell pellet to an appropriate growth or culture medium to rehydrate the pellet and thus allow the cells in the pellet to be revived.

The isolated strain in a growth medium or culture broth may form a cell culture. Thus, the cell culture may comprise an isolated bacterial strain (or mutant thereof) as defined above

in a growth medium or culture broth. The cell culture may contain a mixture of the above bacterial strains or mutants thereof or may be a mixture of the mutants of the above bacterial strains.

The bacterial strain may secrete a biological material into the cell culture medium or it may produce a biological material that is endogenous within the cell. Where the biological material is endogenous to the cell, the cell may need to be lysed in order to release the biological material into the medium. The cells, cell fractions, cell components or cell debris may then be aggregated into a pellet (for example, using centrifugation) in order to form a cell-free supernatant. As necessary, the cell-free supernatant may be subjected to further purification steps in order to remove any cell portions that may not have been removed in the previous purification step.

The cell-free supernatant may contain biological materials that are produced by the bacterial strain(s) or mutant(s) thereof. The cell-free supernatant may contain a protein such as an enzyme. The enzyme may be a protease, a carbohydrase or a lipase. Hence, the cell-free supernatant may comprise at least one protease and at least one carbohydrase produced by the above isolated strain(s).

The protease may be able to solubilize a protein in the plant raw material into the constituent amino acid monomers. The protease may include a peptidase. The protease may be a bacillopeptidase, a cell wall-associated protease, a minor extracellular protease vpr, an aminopeptidase, a subtilisin or a metalloprotease. The protease may be bacillopeptidase F, cell wall-associated protease, minor extracellular protease vpr, aminopeptidase YwaD, subtilisin E, subtilisin amylosacchariticus, putative aminopeptidase YtoP, putative aminopeptidase YsdC or extracellular metalloprotease.

The protease may be selected from the group consisting of subtilisin E, extracellular protease vpr, *bacillolysin*, and *transpeptidase*. The subtilisin E may have the accession number P04189, while the extracellular protease vpr may have the accession number P29141.

The carbohydrase may be able to solubilize a carbohydrate in the plant raw material into the monosaccharide monomer. The carbohydrase may be an amylase, an endoglucanase, an arabinanase, a xylanase, a glucuronoxylanase, galactanase, a lyase or a glucanase. The carbohydrase may be alpha-amylase, endoglucanase, extracellular endo-alpha-(1->5)-L-arabinanase 2, glucuronoxylanase XynC, arabinogalactan endo-beta-1,4-galactanase, pectin lyase, beta-glucanase or endo-1,4-beta-xylanase A.

The carbohydrase may be a beta-glucanase or an alpha-amylase. The beta-glucanase may have the accession number P04957, the alpha-amylase may have the accession number P00691.

The mixture of at least one protease and at least one carbohydrase, when used to solubilize a plant raw material, may increase the solubilization of the plant raw material, as compared to the same solubilization under the same conditions but using a different strain of *Bacillus subtilis* such as *Bacillus subtilis* ATCC21556, or by a bacterial strain that secretes proteases only. Due to the additional presence of the carbohydrase secreted by or extracted from the isolated bacterial strain as provided above, this may aid in increasing the

solubilization of the plant raw material. The cell-free supernatant as extracted from the above isolated strain(s) may be used as a whole to solubilize the plant raw material.

There is also provided use of an isolated strain as defined above for obtaining a cell culture.

5 There is also provided use of an isolated strain as defined above for hydrolyzing a plant raw material. The plant raw material may be a cereal. The cereal may be selected from barley, corn, rice, rye, oat, sorghum or wheat. The plant raw material may be spent grain from a malting process, such as spent barley grain, spent corn grain, spent rice grain, spent rye grain, spent oat grain, spent sorghum grain or spent wheat grain.

10 There is also provided use of a cell-free supernatant as defined above for hydrolyzing a plant raw material. The plant raw material may be a cereal. The cereal may be selected from barley, corn, rice, rye, oat, sorghum or wheat. The plant raw material may be spent grain from a malting process, such as spent barley grain, spent corn grain, spent rice grain, spent rye grain, spent oat grain, spent sorghum grain or spent wheat grain.

15 Exemplary, non-limiting embodiments of a process for hydrolysing a plant raw material will now be disclosed.

The process of hydrolysing a plant raw material may comprise the step of contacting a plant raw material with an isolated strain as defined above, a cell culture as defined above or a cell-free supernatant as defined above.

20 The contacting step may be undertaken at a temperature in the range of about 45°C to about 60°C, about 45°C to about 50°C, about 45°C to about 55°C, about 50°C to about 60°C, about 55°C to about 60°C, about 45°C, about 46°C, about 47°C, about 48°C, about 49°C, about 50°C, about 51°C, about 52°C, about 53°C, about 54°C, about 55°C, about 56°C, about 57°C, about 58°C, about 59°C or about 60°C.

25 The contacting step may be undertaken for a time period of about 1 to about 24 hours, about 4 to about 24 hours, about 8 to about 24 hours, about 12 to about 24 hours, about 16 to about 24 hours, about 20 to about 24 hours, about 1 to about 4 hours, about 1 to about 8 hours, about 1 to about 12 hours, about 1 to about 16 hours, about 1 to about 20 hours, about 1 to about 5 hours, about 5 to about 24 hours, about 1 to about 3 hours, about 1.5 to about 3 hours, about 2 to about 3 hours, about 2.5 to about 3 hours, about 1 to about 1.5 hours, about 1 to about 2 hours, about 1 to about 2.5 hours, about 1 hour, about 1.5 hours, about 2 hours or about 2.5 hours.

The contacting step may be undertaken at a pH in the range of about 4 to about 9, about 4 to about 5, about 4 to about 6, about 4 to about 7, about 5 to about 9, about 6 to about 9, about 7 to about 9, about 8 to about 9, about 5 to about 8, about 6 to about 8, about 6, about 7, about 7.5 or about 8.

35 The contacting step may also be known as a solubilizing step in order to break down the biological macromolecules present in the plant raw material into their respective monomers. Where the plant raw material contain proteins or carbohydrates, the proteins or enzymes secreted by or extracted from the above isolated bacterial strain(s) may solubilize or break down the protein or carbohydrates into their respective amino acid monomers or monosaccharide monomers.

40

- The solubilization efficiency may be defined as $(W_0 - W)/W_0 \times 100\%$, where W_0 refers to the dry weight of the plant raw material before solubilization and W refers to the dry weight of the solubilized plant raw material. The solubilization efficiency may be more than about 5%, more than about 6%, more than about 7%, more than about 8%, more than about 9%, more than about 10%, more than about 11%, more than about 12%, more than about 13%, more than about 14%, more than about 15%, more than about 16%, more than about 17%, more than about 18%, more than about 19%, more than about 20%, more than about 21%, more than about 22%, more than about 23%, more than about 24%, more than about 25%, more than about 26%, more than about 27%, more than about 28%, more than about 29%, or more than about 30%. The solubilisation efficiency may be in the range of about 5% to 30% or about 5% to 15%. The solubilization efficiency may be more than the amount of protein present in the plant raw material before solubilization due to the presence of the carbohydrases which aid in solubilizing the carbohydrates present in the plant raw material in addition to the proteins present in the plant raw material (which are solubilized by the proteases).
- Where the plant raw material is solubilized with the cell-free supernatant (which is essentially the enzymes extracted from or secreted by the above bacterial strain(s)), the ratio of the plant raw material (w/v) to the cell-free supernatant (mL) may be in the range of 1:1 to 1:4, such as about 1.2.
- The plant raw material may be a cereal. The cereal may be selected from barley, corn, rice, rye, oat, sorghum or wheat. The plant raw material may be spent grain from a malting process, such as spent barley grain, spent corn grain, spent rice grain, spent rye grain, spent oat grain, spent sorghum grain or spent wheat grain.
- The plant raw material may be provided in the wet form or in a dry form. Where the plant raw material is provided in the dry form and a liquid medium is required for solubilization, the dry plant raw material may be reconstituted using a suitable medium. Alternatively, if the plant raw material is provided in the wet form and the volume need to be increased for solubilization, the wet plant raw material may be added to a suitable medium. This suitable medium may not be toxic to the above bacterial strain(s) (if a viable strain(s) is used during solubilization) or may not degrade the enzymes secreted by or extracted from the above bacterial strain(s). The medium may be a food grade medium that may have been prepared from 100% food grade ingredients. Where there is a need to adjust the pH of a food grade medium, food-grade pH adjusters as known to a person skilled in the art may be used. Exemplary food-grade pH adjusters may be sodium bicarbonate (from baking soda) or vinegar.
- The solubilized liquid containing the solubilized plant raw material may then be subjected to a centrifugation step in order to concentrate the solubilized product or to filter pressing. The centrifugation step is not particularly limited but would need to be of appropriate conditions (such as temperature, centrifugation speed and duration) to form a pellet of the plant raw material. The supernatant is then decanted and the plant raw material pellet dried and cooled. The centrifugation temperature may be at room temperature or at a temperature less than 10°C such as 4°C; the centrifugation speed may be 3,000 rpm to 5,000 rpm, such as 4,000 rpm; and the centrifugation duration may be about 30 minutes to about 2 hours, such as 45 minutes.

Exemplary, non-limiting embodiments of a process for producing a non-alcoholic malted beverage will now be disclosed.

5 The process for producing a non-alcoholic malted beverage may comprise the step of mixing a hydrolysed plant raw material with a beverage ingredient. The hydrolysed plant raw material may be one which has a higher total solids content of more than 5% as compared to another plant raw material (such as spent grain) that has not been hydrolysed.

10 The process may comprise, before the mixing step, the step of contacting a plant raw material with an isolated strain as provided above, a cell culture as provided above or a cell-free supernatant as provided above in order to produce the hydrolysed plant raw material.

The process for producing a non-alcoholic malted beverage may comprise the steps of:

- a) contacting a plant raw material with an isolated strain as provided above, a cell culture as provided above or a cell-free supernatant as provided above to thereby produce a hydrolysed plant raw material; and
- 15 b) mixing the hydrolysed plant raw material with a beverage ingredient.

The process may further comprise, after the contacting step (a), a step of (c) concentrating the hydrolysed plant raw material. The concentrating step may involve evaporating the hydrolysed plant raw material in order to increase the total solids %. The total solids % may be about 60 to 90% after the concentrating (or evaporating) step.

20 The process may further comprise a step of (d) drying the hydrolysed plant raw material or the concentrated hydrolysed plant raw material. The drying step may be undertaken before the mixing step (b).

The concentrated hydrolysed plant raw material may be mixed with the dried hydrolysed plant raw material.

25 The beverage ingredient may be cocoa, coffee, milk, tea, yoghurt, a nutritional beverage, or a mixture thereof. The beverage ingredient may contain a food ingredient. The beverage ingredient may be fortified with a suitable mineral. The beverage ingredient may contain additional nutrients to increase the nutritional content of the beverage ingredient. The beverage ingredient may contain a flavour in order to appeal to consumers (such as chocolate flavour, or fruit flavour – strawberry, banana, papaya, honeydew, apple etc). The beverage ingredient may be a health tea, a cocoa drink, calcium fortified coffee beverage, calcium fortified milk, a soy drink, calcium fortified soy milk, plain milk, flavoured milk, nutritional beverage, chocolate drink etc.

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35 Exemplary, non-limiting embodiments of a process for producing an alcoholic beverage will now be disclosed.

The process for producing an alcoholic beverage may comprise the step of alcoholic fermenting a hydrolysed plant raw material.

The process may comprise, before said alcoholic fermenting step, the step of contacting a plant raw material with an isolated strain as provided above, a cell culture as provided

above or a cell-free supernatant as provided above in order to produce the hydrolysed plant raw material.

The process for producing an alcoholic beverage may comprise the steps of:

- 5 a) contacting a plant raw material with an isolated strain as provided above, a cell culture as provided above or a cell-free supernatant as provided above to thereby produce a hydrolysed plant raw material; and
- b) alcoholic fermenting the hydrolysed plant raw material.

Brief Description of Drawings

10 The accompanying drawings illustrate a disclosed embodiment and serves to explain the principles of the disclosed embodiment. It is to be understood, however, that the drawings are designed for purposes of illustration only, and not as a definition of the limits of the invention.

Fig.1

15 [Fig. 1] is a series of graphs showing the protease activities of *Bacillus subtilis* B1-4 and *Bacillus subtilis* ATCC21556 in different conditions. [Fig. 1(a)] is a graph showing the protease activities of the above *Bacillus subtilis* strains as a function of pH (pH profile). [Fig. 1(b)] is a graph showing the protease activities of the above *Bacillus subtilis* strains as a function of solubilization time at a temperature of 50°C (temperature profile). [Fig. 1(c)] is a graph showing the protease activities of the above *Bacillus subtilis* strains as a function of
20 solubilization time at a temperature of 55°C (temperature profile).

Fig.2

[Fig. 2] is a number of bar graphs showing the solubilization of spent barley grain B by the enzyme solutions of *Bacillus subtilis* B1-4 and *Bacillus subtilis* ATCC21556 strains in different media. [Fig. 2(a)] shows the solubilization of spent barley grain B by the enzyme
25 solutions of both strains at 55°C using Nutrient-NaCl medium. [Fig. 2(b)] shows the solubilization of spent barley grain B by the enzyme solutions of both strains at 55°C using Mesh B medium. [Fig. 2(c)] shows the solubilization of spent barley grain B by the enzyme solutions of both strains at 55°C using Milo medium. The bars from left to right for each sample were based on 2 hours, 4 hours, 6 hours and 24 hours.

30 Fig.3

[Fig. 3] is a bar graph showing the enzymatic hydrolysis of spent barley grain at a temperature of 50°C using a food-grade Nutrient-NaCl medium at a pH of 7.0 and at varying periods of 1 hour, 1.5 hours and 2 hours.

Fig.4

35 [Fig. 4] is a bar graph showing the solubilization percentages of the *Bacillus subtilis* B1-4 and *Bacillus subtilis* ATCC21556 strains. For each sample, the bar on the left stands for

Negative Control, the bar in the center stands for Total Solubilization while the bar on the right stands for Net Solubilization.

Fig.5

5 [Fig. 5] is a pie diagram showing the percentages of the various enzyme components in the enzyme supernatant of *Bacillus subtilis* B1-4.

Fig.6

[Fig. 6] is a bar graph showing the amino acid profiles of the liquid hydrolysate of spent barley grain B before and after solubilization using the enzyme solution from *Bacillus subtilis* B1-4 after 2 hours.

10 **Fig.7**

[Fig. 7] is a graph showing the size-exclusion chromatography of supernatant B1-4 by Yarra 1.8µm SEC-X150.

Fig.8

15 [Fig. 8] is a bar graph showing the solubilization of a number of samples as discussed in Example 4 below. For each sample, the bar on the left represents “Ave loss%” while the bar on the right represents “Net loss%”.

Fig.9

20 [Fig. 9] is a bar graph showing the solubilization of barley spent grain by commercial carbohydrase cocktails. For each sample, the bar on the left represents “Dry Matter Loss%” while the bar on the right represents “Net loss%”.

Examples

Non-limiting examples of the invention and a comparative example will be further described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of the invention.

25 **Example 1 - Characterisation of spent barley grains**

Characterization of two types of spent barley grains A and B (that were barley by-products from malt extraction processes) from Nestlé was carried out. The compositions of spent barley grains A and B were analysed using the standard protocols of National Renewable Energy Laboratory (NREL, <https://www.nrel.gov/bioenergy/biomass-compositional-analysis.html>) for Total organic carbon (TOC), water content, ash content, total lipids, starch content and monomer sugars.

35 The total protein content was determined by Bradford Assay using bovine serum albumin (obtained from Sigma-Aldrich of St. Louis, Missouri of the United States of America) as the protein standard. This was done by adding 0.05 ml of enzyme solution to 1.5 ml of Bradford Reagent (obtained from Sigma-Aldrich of St. Louis, Missouri of the United States

of America) in a cuvette. The reaction mixture was mixed and incubated at room temperature for 10 minutes before measuring the UV absorbance at 595 nm.

The compositions of spent barley grains A and B are shown in Table 1 below (each value was the average of three batches of experiments).

5 Table 1 Compositions of Spent Grains A and B

Components	Spent Barley Grain A (%)	Spent Barley Grain B (%)
Carbohydrates	42.68	42.21
- Glucan	54.8 (Starch: 3.3%)	59.51 (Starch: 5.89%)
- Xylan	28.4	25.23
- Arabinan	16.8	15.26
Protein	21.03	18.58
Lipid	9.04	9.63
Klason Lignin	24.6	20.26
Acetate	0.83	0.58
Others	1.82	8.75
Total organic content (TC-IC)	44.58	44.48
Moisture	64.87	66.51
Ash	1.19	1.12

From Table 1, it can be seen that the spent barley grains A and B contain mainly carbohydrates (about 42%), proteins (about 18 to 21%), lipids (about 9%) and lignin (about 20 to 24%). The most abundant proteins were storage proteins such as hordeins and embryo globulin.

Spent barley grain B was selected for isolation of the microorganism and for further testing.

Example 2 – Isolation of the bacterial strains

a) Strain *Bacillus subtilis* B1-4

15 Spent barley grain B (1 to 3 g) having the components shown in Table 1 above (such as 42.2% of carbohydrates, 18.6% of proteins, 9.6% of lipids and 20.3% of Klason lignin) was first inoculated into 30 mL of medium 1 that contained 5 g/L of peptone (Sigma-Aldrich of St. Louis, Missouri of the United States of America), 3 g/L of beef extract powder (Sigma-Aldrich of St. Louis, Missouri of the United States of America) and 0.5% of sodium chloride (Sigma-Aldrich of St. Louis, Missouri of the United States of America) at pH 7.4.

The mixture was incubated at 30°C with shaking at 180 rpm overnight. The overnight culture was spread on agar plates made from medium 1 with 1.5% agar (Sigma-Aldrich). Single colonies were then inoculated on skim milk agars which contained casein (1% w/v) (Sigma-Aldrich of St. Louis, Missouri of the United States of America), skim milk (1% w/v), yeast extract (2.5 g/L), dextrose (1 g/L) (Sigma-Aldrich of St. Louis, Missouri of the United States of America) and agar (15 g/L) (Sigma-Aldrich of St. Louis, Missouri of the United States of America) at pH 7.0. The presence of a clear zone around the colony was used as an indication of a protease-producing colony.

b) Strain *Bacillus subtilis* WCB-2

- Japanese white cream waffle sticks (1 to 3 g, obtained from Bourbon Corporation of Japan) was first inoculated in 30 mL of medium 1 as above at pH 7.4. The mixture was incubated at 30°C with shaking at 180 rpm overnight. The overnight culture was spread on agar plates made from medium 1 with 1.5% agar. Single colonies were then inoculated on skim milk agars. Presence of a clear zone around the colony was used as an indication of protease-producing colony.

c) Strains identification

- The strains were identified by 16S rRNA sequencing using forward (5-AGAGTTTGATCCTGGCTCAG-3) (SEQ ID No: 1) and reverse (5-TACCTTGTACGACTT-3) (SEQ ID No: 2) primers. The sequence similarity search was done for the 16S rRNA sequence using the online search tool BLAST (<http://www.ncbi.nlm.nih.gov/blast>). The unknown organisms were identified using the maximum aligned sequence through BLAST search. The strains were identified as belonging to the *Bacillus* genus and of the *Bacillus subtilis* species.

- The isolated strain obtained from spent barley grain B was named *Bacillus subtilis* B1-4 and deposited with CNCM under deposit number CNCM I-5356.

The isolated strain obtained from the Japanese white cream waffle sticks was named *Bacillus subtilis* WCB-2 and deposited with CNCM under deposit number CNCM I-5357.

d) Strain characterization

- Both the *Bacillus subtilis* B1-4 and *Bacillus subtilis* WCB-2 strains were subjected to a protease activity using Milo as a medium containing 20 g Milo powder (commercially obtained) in 1 liter of dd H₂O. *Bacillus subtilis* having the deposit number ATCC21556 was also tested and used as a reference strain. In order to extract the protease, the isolated bacteria were added to the Milo medium and cultivated at 30°C for 26 hours and the enzymes were extracted by centrifugation. To measure protease activity, 0.5 mL of enzyme solution was mixed with 2.5 mL of 1% casein (Sigma-Aldrich of St. Louis, Missouri of the United States of America) solution (Tris-HCl buffer, pH 7.0) and incubated at a temperature 55°C for 10 minutes. The reaction was terminated by adding 2.5 mL of 1% trichloroacetic acid (TCA) (Sigma-Aldrich of St. Louis, Missouri of the United States of America) and incubated at 30°C for 20 minutes. The reaction mixture was then centrifuged at 6000 rpm for 10 minutes and the supernatant was collected. 2.5 mL of alkaline solution (500 mmol/L of sodium carbonate (Sigma-Aldrich of St. Louis, Missouri of the United States of America)) and 0.5 mL of 0.5 M Folin phenol reagent (1:4 dilution of 2 M Folin

and Ciocalteu's Phenol Reagent with purified water) (Sigma-Aldrich of St. Louis, Missouri of the United States of America) was added to 1 mL of the supernatant and the mixture was incubated at 30°C. After 30 minutes, the protease activity was read at 660 nm using a spectrophotometer. Blanks were prepared by adding 2.5 mL TCA before incubating with casein. One unit of protease activity is defined as the amount of enzyme that liberated 1 µg of tyrosine per minute at pH 7.0 and at a temperature of 55°C. Table 2 shows the comparison between the protease activities of the various strains in Milo medium.

Table 2 Protease activities measured in Milo medium

Strain	Culture Age (hours)	Protease Activity (U/mL)
B1-4	26	552.8
WCB-2	26	352.7
ATCC21556	26	457.4

From Table 2 above, it is shown that *Bacillus subtilis* B1-4 had the highest protease activity among the three strains investigated. *Bacillus subtilis* B1-4 was thus used for further characterization. The effect of pH and temperature on the protease activity of *Bacillus subtilis* B1-4 were measured and compared with that of *Bacillus subtilis* ATCC21556. Here, the culture medium used was food-grade Nutrient-NaCl medium (made using 5 g/L peptone (Sigma-Aldrich of St. Louis, Missouri of the United States of America)), 3 g/L yeast extract (Sigma-Aldrich of St. Louis, Missouri of the United States of America) and 5 g/L sodium chloride (Sigma-Aldrich of St. Louis, Missouri of the United States of America)).

For the pH profiling, the enzyme was collected at optimal culture age (which refers to the culture time at which the protease activity reaches a maximum value) and mixed with 1% casein in potassium phosphate at various pH values of 5.15, 6, 7, 8 and 9 at a temperature of 50°C. This follows the protocol established by Sigma-Aldrich titled "Enzymatic Assay of Protease using Casein as a Substrate". Briefly, 0.65% (w/v) casein solution was first added into a number of vials, which were then equilibrated in a suitably thermostated water bath at 37°C for about 5 minutes. The enzyme solution (at various volumes) were then added to the casein vials, mixed and incubated at 37°C for exactly 10 minutes. 110 mM trichloroacetic acid reagent (TCA) and enzyme solution were then added, mixed by swirling and incubated at 37°C for about 30 minutes. The solution from each vial was filtered using a 0.45 µm syringe filter and added into a number of vials and mixed with 500mM sodium carbonate solution and 0.5 M Folin and Ciocalteu's Phenol Reagent. A standard curve was then prepared by pipetting 1.1 mM L-tyrosine standard, purified water, 500mM sodium carbonate solution and 0.5 M Folin and Ciocalteu's Phenol Reagent into suitable vials, which were then mixed and incubated for 30 minutes. The vials were removed and cooled to room temperature. Each vial was then filtered using 0.45 µm syringe filter into suitable cuvettes and the A660 nm of each vial was recorded. The standard curve was then obtained and used to determine the enzymatic activity.

The pH profiles of *Bacillus subtilis* B1-4 and *Bacillus subtilis* ATCC21556 is shown in Fig. 1(a). For the temperature profiling, the same assay as for the pH profiling was used but at a fixed pH of 7 with two varying temperature values of 50°C and 55°C. Fig. 1(b) thus show the temperature profile at 50°C while Fig. 1(c) shows the temperature profile at 55°C.

As can be seen from Fig. 1(a) to Fig. 1(c), the optimal pH and temperature for the protease activities of *Bacillus subtilis* B1-4 were 7.0 and 50°C respectively. The pH of the wet spent grain B was 6.5 to 6.9, Therefore, the optimal pH of 7.0 of the proteases produced by *Bacillus subtilis* B1-4 is similar with the natural pH of the wet spent grain, and will thus be favourable for the enzymatic hydrolysis of the spent grains without the need to adjust the pH of the system.

Example 3 - Solubilization of Spent Barley Grains

Spent barley grain B was used here. The spent barley grain was kept at -80°C before use. The wet spent grain was first oven-dried at 105°C for 4 hours until constant weight and then cooled down to room temperature in a desiccator. The hydrolysis was carried out without pH adjustment to avoid the use of additional chemicals. Both dry and wet spent grains were used for the solubilization experiments.

The enzymes were produced by cultivating *Bacillus subtilis* B1-4 strain at 30°C in 3 different media – Nutrient NaCl (as provided above), Mesh B (20 g/L) and Milo (20g/L) (as provided above) for 26 hours and the enzymes were extracted by centrifugation. Different amounts of spent barley grains (2 to 7%, w/v) were mixed with 20 mL of the enzyme solution obtained to test the solubilization for 2 to 24 hours at 50°C or 55°C. The spent barley grains treated with the same media as above with *Bacillus subtilis* ATCC21556 were used as reference. The enzymatic hydrolysis was stopped by centrifuging at 4°C and 4,000 rpm for 45 minutes. The supernatant was decanted and the pellet was dried at 105°C and cooled down in a desiccator. The solubilization efficiency was defined as $(W_0 - W)/W_0 \times 100\%$, where W_0 and W are the weights of the spent grain before and after the solubilization treatment, respectively.

Fig. 2(a) shows the solubilization of spent barley grain B by the enzyme solutions of both strains at 55°C using Nutrient-NaCl medium. Fig. 2(b) shows the solubilization of spent barley grain B by the enzyme solutions of both strains at 55°C using Mesh B medium. Fig. 2(c) shows the solubilization of spent barley grain B by the enzyme solutions of both strains at 55°C using Milo medium.

It can be seen from Fig. 2 that the solubilization of spent barley grain B was significantly affected by both the media used for cultivating the strains and the time used for the solubilization. The best solubilization for spent barley grain B was achieved when nutrient-NaCl medium was used to cultivate *Bacillus subtilis* B1-4, giving a solubilization of 14.1% within 2 hours.

The solubilization was then carried out at a temperature of 50°C using the same food-grade Nutrient-NaCl medium (as provided above) at a pH of 7.0 and at varying periods of 1 hour, 1.5 hours and 2 hours. The result of this is shown in Fig. 3. From Fig. 3, it can be seen that the enzyme solution of *Bacillus subtilis* B1-4 could well solubilize the wet cereal spent barley grain B. The differences in the solubilization of the spent barley grain B at 1 hour,

1.5 hours and 2 hours were not significant, and the highest solubilization of 28.5% was achieved at 2 hours. This value was higher than the protein content of 18.6% in the spent barley grain B (see protein content of spent barley grain B in Table 1 above), indicating that some other components such as lipids and carbohydrates might have been solubilized by other enzymes in addition to proteases. The net solubilisation in Fig. 3 was calculated based on the formula:

$$\text{Net solubilisation} = \text{gross solubilisation} - \text{negative control solubilisation}$$

where “negative control solubilisation” refers to the solubilisation led by enzymes that are inherent in spent grains.

Fig. 4 shows the solubilisation percentages of 100 g wet spent grain hydrolysis using *Bacillus subtilis* B1-4 and *Bacillus subtilis* ATCC21556 strains. The loading ratio of wet spent grain versus the crude enzymes was 1:2, which means that 100 g of wet spent grain solubilized in 200 mL of crude enzyme solution. The negative control used in Fig. 4 was the blank food-grade Nutrient-NaCl medium (rather than the crude enzymes) used for solubilisation.

Example 4 - Characterization of enzymes secreted by *Bacillus subtilis* B1-4

The enzymes that are secreted by *Bacillus subtilis* B1-4 in the food-grade Nutrient-NaCl medium were analysed using LC-MS and compared with those secreted by *Bacillus subtilis* ATCC21556, and shown in Table 3 (where proteins that are common to both strains are shown side-by-side). As can be seen from Table 3, *Bacillus subtilis* B1-4 secreted not only proteases but also other enzymes.

Table 3 Comparison of enzymes secreted by *Bacillus subtilis* B1-4 and *Bacillus subtilis* ATCC21556

<i>Bacillus subtilis</i> B1-4			<i>Bacillus subtilis</i> ATCC21556		
Accession	Description	M.W.	Accession	Description	M.W.
P16397	Bacillopeptidase F	154.4			
P54423	Cell wall-associated protease	96.4	P54423	Cell wall-associated protease	96.4
P29141	Minor extracellular protease vpr	85.6	P29141	Minor extracellular protease vpr	85.6
P00691	Alpha-amylase	72.3	P00691	Alpha-amylase	72.3
P24141	Oligopeptide-binding protein	61.5	P24141	Oligopeptide-binding protein OppA	61.5
P07983	Endoglucanase	55.2	P07983	Endoglucanase	55.2

P42293	Extracellular endo-alpha-(1->5)-L-arabinanase 2	52.6	P42293	Extracellular endo-alpha-(1->5)-L-arabinanase 2	52.6
P54533	Dihydrolipoyl dehydrogenase	50.4	P54533	Dihydrolipoyl dehydrogenase	50.4
P54331	Phage-like element PBSX protein XkdK	50.2	P54331	Phage-like element PBSX protein XkdK	50.2
P71004	Uncharacterized protein YwhL	49.5			
			P21880	Dihydrolipoyl dehydrogenase	49.7
P25152	Aminopeptidase YwaD	49.4	P25152	Aminopeptidase YwaD	49.4
Q45070	Glucuronoxylanase XynC	47.3	Q45070	Glucuronoxylanase XynC	47.3
O07013	Arabinogalactan endo-beta-1,4-galactanase	47.2			
P04189	Subtilisin E	39.5			
P00783	Subtilisin amylosacchariticus	39.4	P00783	Subtilisin amylosacchariticus	39.4
O34924	Putative aminopeptidase YtoP	39.2	O34924	Putative aminopeptidase YtoP	39.2
P94521	Putative aminopeptidase YsdC	39.2	P94521	Putative aminopeptidase YsdC	39.2
			O07603	Putative aminopeptidase YhfE	38.7
O34819	Pectin lyase	38.1	O34819	Pectin lyase	38.1
P39790	Extracellular metalloprotease	33.8			
P42418	Protein IolH	33.5			
			O34789	Uncharacterized protein YdjI	36.1
			P39138	Arginase	32.1
			O31436	Uncharacterized protein YbdN	31.5

			P31847	Uncharacterized protein YpuA	31.3
			P54507	Spore coat-associated protein N	28.3
P04957	Beta-glucanase	27.3	P04957	Beta-glucanase	27.3
O31803	Uncharacterized protein YncM	26.6	O31803	Uncharacterized protein YncM	26.6
O34918	Expansin-YoaJ	25.6	O34918	Expansin-YoaJ	25.6
			O05411	Uncharacterized protein YrpD	24.9
			Q795U4	UPF0173 metal-dependent hydrolase YtkL	24.8
P70961	Uncharacterized protein YwmD	24			
P18429	Endo-1,4-beta-xylanase A	23.3	P18429	Endo-1,4-beta-xylanase A	23.3
Q45538	Protein CotjC	21.7			
O32211	Stress response protein YvgO	17.6			
O34554	Uncharacterized protein YjfA	16.9			
O31649	Uncharacterized protein YjdH	15.2			
P42091	Protein CgeC	11.4			
			O34384	Uncharacterized protein YceE	20.9
			P39632	Uncharacterized protein YweA	16.7
Total	30			29	

Among the 30 enzymes/proteins produced by *Bacillus subtilis* B1-4 and 29 enzymes/proteins produced by *Bacillus subtilis* ATCC21556, only 17 of them (56.7%) are produced by both strains. Some unique proteases produced by *Bacillus subtilis* B1-4 such

as subtilisin E and extracellular metalloprotease might be responsible for the high solubilization of spent barley grain B by the enzymes produced by this strain.

Proteomic profiling was also used to analyse the enzyme components in the enzyme supernatant, followed by fractionation by size-exclusion chromatography which aimed to purify the main components. As shown additionally in Fig. 5 (which was based on a different mass spectrometry method used to quantify the amount of the enzyme components in *Bacillus subtilis* B1-4), there were three main components in the enzyme supernatant of *Bacillus subtilis* B1-4 – subtilisin E, beta-glucanase and alpha-amylase, comprising around 70% of the total protein amount, while other proteases such as minor extracellular protease vpr, bacillolysin, transpeptidase made up around 20% of the total protein amount. Therefore, the enzyme supernatant was a mixture of protease and carbohydrase, instead of being solely protease. With reference to Fig. 5, 26.44% was subtilisin E, 24.69% was beta-glucanase, 19.16% was alpha-amylase, 11.08% was others, 10.27% was minor extracellular protease vpr, 3.78% was bacillolysin, 2.23% was gamma-glutamyltranspeptidase, 1.27% was aminopeptidase YwaD and 1.09% was putative aminopeptidase YhfE.

Fig. 6 shows the preliminary analysis of the amino acid profile of the liquid hydrolysate of spent barley grain B that was solubilized using the enzyme solution from *Bacillus subtilis* B1-4 after 2 hours. From Fig. 6, it can be seen that proline and glutamic acid are the two major amino acids. The “blank” sample referred to in Fig. 6 was the blank food-grant NutrientNa-Cl medium that was used for the solubilisation of the spent grain.

Fractionation of supernatant by size-exclusion chromatography

First, a column TSKgel 3000SW was tried on Dionex - UltiMate 3000 Rapid Separation LC Systems. The buffer system was 50mM pH 6.5 MES with 200 mM Arginine, 5mM EDTA and 0.05% Sodium Azide. Injected protein amount was around 50 µg. Fractions were collected every minutes. Then, another column phenomenex Yarra 1.8 SEC-X150 was also tried. The separation was done on the same machine, while with different mobile phase: pH 6.8, 50mM potassium phosphate with 100mM NaCl at 0.3ml/min flow rate. In order to collect enough amount enzymes for solubilization, 60 cycles were run with 2.5 µg protein injected in each run.

The fractions which show same kind of activity were combined and concentrated by 10kD cutoff spin column.

Solubilization efficiency of fractions

Concentrated fractions were added to 100 mg of milled and freeze-dried spent grain powder, followed by topped up to 1ml with milliQ water and solubilized at 60 °C with agitation at 800 rpm for 2 hours. A reaction without enzyme was used as a negative control, while original B1-4 supernatant and Depol/Alcalase combined as two positive controls. Dosage of Depol 740L® and fraction 1 (peak 1) are added according to the glucanase activity in original SGB1-4, while dosage of Alcalase® and fraction 2 (peak 2) are the same with the protease activity in original B1-4.

Table 4 Enzyme dosages of fractions and commercial cocktails

	B1-4						
Enzymes	Glucanase	Protease	Amylase	Peak 1	Peak 2	Depol	Alc
Specific activity U/ml	13.61	0.398	104.05	105.7	2.8	1134	1198
Vol. of enzyme solution added in 50mg SG ml	0.9	0.9	0.9	0.12	0.13	0.0106	0.0003
Dose U /50 mg dry SG	12.249	0.3582	93.645	12.25	0.36	12.0	0.36
Dosage U/100 mg dry SG	24.50	0.72	187.29	24.5	0.72	24	0.72

After enzyme treatment, the hydrolysates were centrifuged at 14,800 rpm for 10 minutes at 4°C, and then the pellets were washed once with deionized water (Milli Q, Millipore) and dried at 105 °C until weight becomes constant. Samples were cooled down in a desiccator and weighed. The amount of remaining solids was measured to calculate the dry weight loss of each reaction.

Based on Fig. 7, peak 1 was detected as glucanase, while peak 2 still has both protease activity and glucanase activity. No activity was detected at peaks 3 and 4.

10 Solubilization efficiency of fractions

Although only two main activities were detected, the solubilization was still performed in case the degraded fragments of amylase may have some activity. There may be some enzyme activity in peak 3 and 4 other than the main activities.

Results as shown in Fig. 8 showed that when using the same dosage of glucanase and protease, the original B1-4 showed higher solubilization yield than combined Depol 740L and Alcalase. This indicated that B1-4 is very efficient.

Therefore, even if the fractions used were separate or combined, the solubilization yields were all far less efficient than whole B1-4. Possible reasons may be that amylase and other minor components were degraded by protease which also existed in B1-4. Another reason may be that some enzymes lost activity during this long 60 cycles of fractionation. This showed the advantage of using whole supernatant as compared to individual fractions.

25 Comparative Example 1 - Fermentation using commercial enzymes

100 mg of milled and freeze-dried spent grain powder was topped up to 1ml with deionized water and solubilized by 24U/g dry spent grain at 60 °C with agitation at 800 rpm for 2 hours. A reaction without enzyme was used as a negative control. Each run had two replicates.

After enzyme treatment, the hydrolysates were centrifuged at 14,800 rpm for 10 minutes at 4°C, and then the pellets were washed once with deionized water (Milli Q, Millipore) and dried at 105 °C until weight becomes constant. Samples were cooled down in a desiccator

and weighed. The amount of remaining solids was measured to calculate the dry weight loss of each reaction.

Fig. 9 shows the solubilization result of barley spent grain using commercial carbohydrase cocktails. As can be seen, the solubilization of such commercial enzyme cocktails are not as good as using the bacterial strains provided herein.

Industrial Applicability

The isolated bacterial strains *Bacillus subtilis* B1-4 and/or *Bacillus subtilis* WCB-2 (or their mutants thereof) may be used to solubilize a plant raw material. The level of solubilization in the hydrolysed plant raw material may be more than 20%. The isolated bacterial strain may be used to form a cell culture or a cell-free supernatant. In the cell-free supernatant, the supernatant may contain enzymes that were extracted from the bacterial strains, whether as secreted by the bacteria into the supernatant or by lysing the bacterial cells and extracting the endogenous protein from the cell lysis.

The isolated bacterial strains or mutant(s) thereof may be used to hydrolyse a plant raw material. The isolated bacterial strains or mutant(s) thereof may be used to produce a non-alcoholic malted beverage. The isolated bacterial strains or mutant(s) thereof may be used to produce an alcoholic beverage.

It will be apparent that various other modifications and adaptations of the invention will be apparent to the person skilled in the art after reading the foregoing disclosure without departing from the spirit and scope of the invention and it is intended that all such modifications and adaptations come within the scope of the appended claims.

Reference to Deposited Biological Material

Bacillus subtilis B1-4 was deposited with CNCM under deposit number CNCM I-5356. *Bacillus subtilis* WCB-2 was deposited with CNCM under deposit number CNCM I-5357.

Sequence Listing Free Text

SEQ ID No: 1 – forward primer 5-AGAGTTTGATCCTGGCTCAG-3

SEQ ID No: 2 – reverse primer 5-TACCTTGTTACGACTT-3

TRAITÉ DE BUDAPEST SUR LA RECONNAISSANCE
INTERNATIONALE DU DÉPÔT DES MICRO-ORGANISMES
AUX FINS DE LA PROCÉDURE EN MATIÈRE DE BREVETS

FORMULE INTERNATIONALE

DESTINATAIRE

**NESTEC S.A.
AVENUE NESTLÉ 55
CH-1800 VEVEY**

RÉCÉPISSÉ EN CAS DE DÉPÔT INITIAL
délivré en vertu de la règle 7.1 par
l'AUTORITÉ DE DÉPÔT INTERNATIONALE
identifiée au bas de cette page

NOM ET ADRESSE
DU DÉPOSANT

I. IDENTIFICATION DU MICRO-ORGANISME	
Référence d'identification donnée par le DÉPOSANT : NCC 2956	Numéro d'ordre attribué par l'AUTORITÉ DE DÉPÔT INTERNATIONALE : CNCM I-5356
II. DESCRIPTION SCIENTIFIQUE ET/OU DÉSIGNATION TAXONOMIQUE PROPOSÉE	
Le micro-organisme identifié sous chiffre I était accompagné : ☒ d'une description scientifique ☒ d'une désignation taxonomique proposée (Cocher ce qui convient).	
III. RÉCEPTION ET ACCEPTATION	
La présente autorité de dépôt internationale accepte le micro-organisme identifié sous chiffre I, qu'elle a reçu le 27 SEPTEMBRE 2018 (date du dépôt initial) ¹ .	
IV. RÉCEPTION D'UNE REQUÊTE EN CONVERSION	
La présente autorité de dépôt internationale a reçu le micro-organisme identifié sous chiffre I le (date du dépôt initial) et a reçu une requête en conversion du dépôt initial en dépôt conforme au Traité de Budapest le (date de réception de la requête en conversion).	
V. AUTORITÉ DE DÉPÔT INTERNATIONALE	
Nom : COLLECTION NATIONALE DE CULTURES DE MICROORGANISMES (CNCM) Adresse : INSTITUT PASTEUR 25 RUE DU DOCTEUR ROUX 75724 PARIS CEDEX 15	Signature(s) de la (des) personne(s) compétente(s) pour représenter l'autorité de dépôt internationale ou de l'(des) employé(s) autorisé(s) : RAQUEL HURTADO-ORTIZ Date : PARIS, LE 24 JANVIER 2019

¹ En cas d'application de la règle 6.4.d), cette date est la date à laquelle le statut d'autorité de dépôt internationale a été acquis.



BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF
MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO: NESTEC S.A. AVENUE NESTLE 55 CH-1800 VEVEY NAME AND ADDRESS OF DEPOSITOR	RECEIPT IN THE CASE OF AN INITIAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITORY AUTHORITY identified at the bottom of this page
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I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: NCC 2956	Accession number issued by the INTERNATIONAL DEPOSITORY AUTHORITY: CNCM I-5356
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified in section I was accompanied by: ☒ a scientific description ☒ a proposed taxonomic designation (indicate as applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depository Authority accepts the microorganism identified in section I, which was received by it on 27 September 2018 (date of the initial deposit) ¹	
IV. RECEIPT OF A REQUEST FOR CONVERSION	
This International Depository Authority received the microorganism identified in section I on (date of the initial deposit) ¹ and received a request for conversion of the initial deposit into one conforming to the Budapest Treaty on _____ (date of receipt of the request for conversion)	
V. INTERNATIONAL DEPOSITORY AUTHORITY	
Name: COLLECTION NATIONALE DE CULTURES DE MICROORGANISMES [FRENCH NATIONAL COLLECTION OF MICROORGANISM CULTURES] (CNCM) Address: INSTITUT PASTEUR 25 RUE DE DOCTEUR ROUX 75724 PARIS CEDEX 15	Signature(s) of the person(s) having the power to represent the International Depository Authority or of authorised official(s): RAQUEL HURTADO-ORTIZ [Signature] [Stamp of the CNCM] Date: PARIS, 24 JANUARY 2019

¹ Where Rule 6.4.d) applies, this date shall be the date on which the status of the International Depository Authority was acquired.

TRAITÉ DE BUDAPEST SUR LA RECONNAISSANCE
INTERNATIONALE DU DÉPÔT DES MICRO-ORGANISMES
AUX FINS DE LA PROCÉDURE EN MATIÈRE DE BREVETS

FORMULE INTERNATIONALE

DESTINATAIRE

M CORNELIS SCHÜLLER

IP GROUP / PATENTS

NESTEC S.A.

AVENUE NESTLÉ, 55

CH-1800 VEVEY - SUISSE

NOM ET ADRESSE DE LA PARTIE À
LAQUELLE LA DÉCLARATION SUR
LA VIABILITÉ EST DÉLIVRÉE

DÉCLARATION SUR LA VIABILITÉ

délivrée en vertu de la règle 10.2 par

l'AUTORITÉ DE DÉPÔT INTERNATIONALE

identifiée à la page suivante

I. DÉPOSANT	II. IDENTIFICATION DU MICRO-ORGANISME
Nom : NESTEC S.A. Adresse : AVENUE NESTLÉ 55 CH-1800 VEVEY	Numéro d'ordre attribué par l'AUTORITÉ DE DÉPÔT INTERNATIONALE : CNCM I-5356 Date du dépôt ou du transfert ¹ : 27 SEPTEMBRE 2018
III. DÉCLARATION SUR LA VIABILITÉ	
La viabilité du micro-organisme identifié sous chiffre II a été contrôlée le 21 JANVIER 2019 ². À cette date, le micro-organisme ☒ ³ était viable ☐ ³ n'était plus viable	

¹ Indiquer la date du dépôt initial ou, si un nouveau dépôt ou un transfert ont été effectués, la plus récente des dates pertinentes (date du nouveau dépôt ou date du transfert).

² Dans les cas visés à la règle 10.2.a)ii) et iii), mentionner le contrôle de viabilité le plus récent.

³ Cocher la case qui convient.

IV. CONDITIONS DANS LESQUELLES LE CONTRÔLE DE VIABILITÉ A ÉTÉ EFFECTUÉ ⁴	
V. AUTORITÉ DE DÉPÔT INTERNATIONALE	
Nom : COLLECTION NATIONALE DE CULTURES DE MICROORGANISMES (CNCM) Adresse : INSTITUT PASTEUR 25 RUE DU DOCTEUR ROUX 75724 PARIS CEDEX 15	Signature(s) de la(des) personne(s) compétente(s) pour représenter l'autorité de dépôt internationale ou de l'(des) employé(s) autorisé(s) : RAQUEL HURTADO-ORTIZ  Date : PARIS, LE 24 JANVIER 2019



⁴ À remplir si cette information a été demandée et si les résultats du contrôle étaient négatifs.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF
MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO:

M. CORNELIS SCHÜLLER
IP GROUP / PATENTS
NESTEC S.A.
AVENUE NESTLÉ 55
CH-1800 VEVEY, Switzerland

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY
STATEMENT IS ISSUED

VIABILITY STATEMENT

issued pursuant to Rule 10.2 by
the INTERNATIONAL DEPOSITORY AUTHORITY
identified on the next page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: NESTEC S.A. Address: Avenue Nestlé 55 CH-1800 VEVEY	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY: CNCM I-5356 Date of the deposit or of the transfer¹: 27 September 2018
III. VIABILITY STATEMENT The viability of the microorganism identified under section II was tested on 21 January 2019². On that date, the microorganism was ³ ☒ viable ³ ☐ no longer viable	

¹ Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

³ Mark the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED ⁴	
V. INTERNATIONAL DEPOSITORY AUTHORITY	
Name: COLLECTION NATIONALE DE CULTURES DE MICROORGANISMES [FRENCH NATIONAL COLLECTION OF MICROORGANISM CULTURES] (CNCM) Address: INSTITUT PASTEUR 25 RUE DE DOCTEUR ROUX 75724 PARIS CEDEX 15	Signature(s) of the person(s) having the power to represent the International Depository Authority or of authorised official(s): [signature] RAQUEL HURTADO-ORTIZ [Signature] [Stamp of the CNCM] Date: PARIS, 24 JANUARY 2019

⁴ To be filled in if this information was requested and the results of the test were negative.

TRAITÉ DE BUDAPEST SUR LA RECONNAISSANCE
INTERNATIONALE DU DÉPÔT DES MICRO-ORGANISMES
AUX FINS DE LA PROCÉDURE EN MATIÈRE DE BREVETS

FORMULE INTERNATIONALE

DESTINATAIRE

**NESTEC S.A.
AVENUE NESTLÉ 55
CH-1800 VEVEY**

RÉCÉPISSÉ EN CAS DE DÉPÔT INITIAL
délivré en vertu de la règle 7.1 par
l'AUTORITÉ DE DÉPÔT INTERNATIONALE
identifiée au bas de cette page

NOM ET ADRESSE
DU DÉPOSANT

I. IDENTIFICATION DU MICRO-ORGANISME	
Référence d'identification donnée par le DÉPOSANT : NCC 2957	Numéro d'ordre attribué par l'AUTORITÉ DE DÉPÔT INTERNATIONALE : CNCM I-5357
II. DESCRIPTION SCIENTIFIQUE ET/OU DÉSIGNATION TAXONOMIQUE PROPOSÉE	
Le micro-organisme identifié sous chiffre I était accompagné : ☒ d'une description scientifique ☒ d'une désignation taxonomique proposée (Cocher ce qui convient).	
III. RÉCEPTION ET ACCEPTATION	
La présente autorité de dépôt internationale accepte le micro-organisme identifié sous chiffre I, qu'elle a reçu le 27 SEPTEMBRE 2018 (date du dépôt initial) ¹ .	
IV. RÉCEPTION D'UNE REQUÊTE EN CONVERSION	
La présente autorité de dépôt internationale a reçu le micro-organisme identifié sous chiffre I le (date du dépôt initial) et a reçu une requête en conversion du dépôt initial en dépôt conforme au Traité de Budapest le (date de réception de la requête en conversion).	
V. AUTORITÉ DE DÉPÔT INTERNATIONALE	
Nom : COLLECTION NATIONALE DE CULTURES DE MICROORGANISMES (CNCM) Adresse : INSTITUT PASTEUR 25 RUE DU DOCTEUR ROUX 75724 PARIS CEDEX 15	Signature(s) de la (des) personne(s) compétente(s) pour représenter l'autorité de dépôt internationale ou de l'(des) employé(s) autorisé(s) : RAQUEL HURTADO-ORTIZ Date : PARIS, LE 24 JANVIER 2019

¹ En cas d'application de la règle 6.4.d), cette date est la date à laquelle le statut d'autorité de dépôt internationale a été acquis.



BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF
MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO: NESTEC S.A. AVENUE NESTLE 55 CH-1800 VEVEY NAME AND ADDRESS OF DEPOSITOR	RECEIPT IN THE CASE OF AN INITIAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITORY AUTHORITY identified at the bottom of this page
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I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: NCC 2957	Accession number issued by the INTERNATIONAL DEPOSITORY AUTHORITY: CNCM I-5357
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified in section I was accompanied by: ☒ a scientific description ☒ a proposed taxonomic designation (indicate as applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depository Authority accepts the microorganism identified in section I, which was received by it on 27 September 2018 (date of the initial deposit) ¹	
IV. RECEIPT OF A REQUEST FOR CONVERSION	
This International Depository Authority received the microorganism identified in section I on (date of the initial deposit) ¹ and received a request for conversion of the initial deposit into one conforming to the Budapest Treaty on _____ (date of receipt of the request for conversion)	
V. INTERNATIONAL DEPOSITORY AUTHORITY	
Name: COLLECTION NATIONALE DE CULTURES DE MICROORGANISMES [FRENCH NATIONAL COLLECTION OF MICROORGANISM CULTURES] (CNCM) Address: INSTITUT PASTEUR 25 RUE DE DOCTEUR ROUX 75724 PARIS CEDEX 15	Signature(s) of the person(s) having the power to represent the International Depository Authority or of authorised official(s): RAQUEL HURTADO-ORTIZ [Signature] [Stamp of the CNCM] Date: PARIS, 24 JANUARY 2019

¹ Where Rule 6.4.d) applies, this date shall be the date on which the status of the International Depository Authority was acquired.

TRAITÉ DE BUDAPEST SUR LA RECONNAISSANCE
INTERNATIONALE DU DÉPÔT DES MICRO-ORGANISMES
AUX FINS DE LA PROCÉDURE EN MATIÈRE DE BREVETS

FORMULE INTERNATIONALE

DESTINATAIRE

M CORNELIS SCHÜLLER

IP GROUP / PATENTS

NESTEC S.A.

AVENUE NESTLÉ, 55

CH-1800 VEVEY - SUISSE

NOM ET ADRESSE DE LA PARTIE À
LAQUELLE LA DÉCLARATION SUR
LA VIABILITÉ EST DÉLIVRÉE

DÉCLARATION SUR LA VIABILITÉ

délivrée en vertu de la règle 10.2 par

l'AUTORITÉ DE DÉPÔT INTERNATIONALE
identifiée à la page suivante

I. DÉPOSANT	II. IDENTIFICATION DU MICRO-ORGANISME
Nom : NESTEC S.A. Adresse : AVENUE NESTLÉ 55 CH-1800 VEVEY	Numéro d'ordre attribué par l'AUTORITÉ DE DÉPÔT INTERNATIONALE : CNCM I-5357 Date du dépôt ou du transfert ¹ : 27 SEPTEMBRE 2018
III. DÉCLARATION SUR LA VIABILITÉ	
La viabilité du micro-organisme identifié sous chiffre II a été contrôlée le 21 JANVIER 2019 ². À cette date, le micro-organisme ☒ ³ était viable ☐ ³ n'était plus viable	

¹ Indiquer la date du dépôt initial ou, si un nouveau dépôt ou un transfert ont été effectués, la plus récente des dates pertinentes (date du nouveau dépôt ou date du transfert).

² Dans les cas visés à la règle 10.2.a)ii) et iii), mentionner le contrôle de viabilité le plus récent.

³ Cocher la case qui convient.

IV. CONDITIONS DANS LESQUELLES LE CONTRÔLE DE VIABILITÉ A ÉTÉ EFFECTUÉ ⁴	
V. AUTORITÉ DE DÉPÔT INTERNATIONALE	
Nom : COLLECTION NATIONALE DE CULTURES DE MICROORGANISMES (CNCM) Adresse : INSTITUT PASTEUR 25 RUE DU DOCTEUR ROUX 75724 PARIS CEDEX 15	Signature(s) de la(des) personne(s) compétente(s) pour représenter l'autorité de dépôt internationale ou de l'(des) employé(s) autorisé(s) : RAQUEL HURTADO-ORTIZ  Date : PARIS, LE 24 JANVIER 2019



⁴ À remplir si cette information a été demandée et si les résultats du contrôle étaient négatifs.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF
MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO:

M. CORNELIS SCHÜLLER
IP GROUP / PATENTS
NESTEC S.A.
AVENUE NESTLÉ 55
CH-1800 VEVEY, Switzerland

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY
STATEMENT IS ISSUED

VIABILITY STATEMENT

issued pursuant to Rule 10.2 by
the INTERNATIONAL DEPOSITORY AUTHORITY
identified on the next page

I. DEPOSITOR Name: NESTEC S.A. Address: Avenue Nestlé 55 CH-1800 VEVEY	II. IDENTIFICATION OF THE MICROORGANISM Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY: CNCM I-5357 Date of the deposit or of the transfer¹: 27 September 2018
III. VIABILITY STATEMENT The viability of the microorganism identified under section II was tested on 21 January 2019². On that date, the microorganism was ³ ☒ viable ³ ☐ no longer viable	

¹ Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

³ Mark the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED ⁴	
V. INTERNATIONAL DEPOSITORY AUTHORITY	
Name: COLLECTION NATIONALE DE CULTURES DE MICROORGANISMES [FRENCH NATIONAL COLLECTION OF MICROORGANISM CULTURES] (CNCM) Address: INSTITUT PASTEUR 25 RUE DE DOCTEUR ROUX 75724 PARIS CEDEX 15	Signature(s) of the person(s) having the power to represent the International Depository Authority or of authorised official(s): [signature] RAQUEL HURTADO-ORTIZ [Signature] [Stamp of the CNCM] Date: PARIS, 24 JANUARY 2019

⁴ To be filled in if this information was requested and the results of the test were negative.

Claims

1. An isolated strain of *Bacillus subtilis* selected from the group consisting of *Bacillus subtilis* B1-4 under deposit number CNCM I-5356, *Bacillus subtilis* WCB-2 under deposit number CNCM I-5357 and a mutant strain thereof.
- 5 2. A cell culture comprising an isolated strain as defined in claim 1 in a growth medium.
3. A cell-free supernatant comprising at least one protease and at least one carbohydrase produced by an isolated strain as defined in claim 1.
4. The cell-free supernatant according to claim 3, wherein said protease is selected from the group consisting of subtilisin E, extracellular protease vpr, *bacillolysin*, and *transpeptidase*.
- 10 5. The cell-free supernatant according to claim 3 or 4, wherein said carbohydrase is a beta-glucanase or an alpha-amylase.
6. Use of an isolated strain as defined in according to claim 1 for obtaining a cell culture.
7. Use of an isolated strain as defined in claim 1 for hydrolyzing a plant raw material.
8. Use according to claim 7, wherein the plant raw material is a cereal.
- 15 9. Use according to claim 8, wherein said cereal is selected from barley, corn, rice, rye, oat, sorghum or wheat.
10. Use according to any one of claims 7 to 9, wherein the plant raw material is spent grain from a malting process.
11. Use of a cell-free supernatant as defined in any one of claims 3 to 5 for hydrolyzing a
- 20 plant raw material.
12. Use according to claim 11, wherein the plant raw material is a cereal.
13. Use according to claim 12, wherein said cereal is selected from barley, corn, rice, rye, oat, sorghum or wheat.
14. Use according to any one of claims 11 to 13, wherein the plant raw material is spent
- 25 grain from a malting process.
15. A process for hydrolysing a plant raw material comprising the step of contacting a plant raw material with an isolated strain as defined in claim 1, a cell culture as defined in claim 2 or a cell-free supernatant as defined in any one of claims 3 to 5.
16. The process according to claim 15, wherein said contacting step is undertaken at a
- 30 temperature in the range of 45°C to 60°C.
17. The process according to claim 15 or 16, wherein said contacting step is undertaken for a time period of 1 to 3 hours.
18. The process according to any one of claims 15 to 17, wherein said contacting step is undertaken at a pH in the range of 6 to 8.

19. The process according to any one of claims 15 to 18, wherein the plant raw material is a cereal.
20. A process for producing a non-alcoholic malted beverage comprising the step of mixing a hydrolysed plant raw material with a beverage ingredient.
- 5 21. The process according to claim 20 comprising, before said mixing step, the step of contacting a plant raw material with an isolated strain as defined in claim 1, a cell culture as defined in claim 2 or a cell-free supernatant as defined in any one of claims 3 to 5 in order to produce said hydrolysed plant raw material.
- 10 22. The process according to claim 21, comprising, after said contacting step, the step of concentrating the hydrolysed plant raw material.
23. The process according to claim 21 or 22, comprising the step of drying the hydrolysed plant raw material.
24. The process according to any one of claims 20 to 23, wherein said beverage ingredient is cocoa, coffee, milk, tea or a mixture thereof.
- 15 25. A process for producing an alcoholic beverage comprising the step of alcoholic fermenting a hydrolysed plant raw material.
- 20 26. The process according to claim 25, comprising, before said alcoholic fermenting step, the step of contacting a plant raw material with an isolated strain as defined in claim 1, a cell culture as defined in claim 2 or a cell-free supernatant as defined in any one of claims 3 to 5 in order to produce said hydrolysed plant raw material.