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Description

[0001] The present invention relates to a *Bacillus subtilis* composition characterized by fast germination and outgrowth in bile salts (simulated gut environment) and by producing an enzyme or an amino acid selected from the group consisting of phenylalanine, valine, threonine, tryptophan, isoleucine, methionine, leucine, lysine, cysteine, tyrosine, histidine and arginine. The *Bacillus subtilis* composition may be used as supplement in animal feed where it has a probiotic (health and growth promoting) effect and increases the digestion and availability of nutrients from animal feeds.

BACKGROUND ART

[0002] Probiotic bacteria such as *Bacillus subtilis* and *Bacillus licheniformis* are used in the animal feed industry as supplement to the diet. Their usage is related to the ability of bacillus to replace or reduce the use of antibiotics, which are used as growth promoters in the animal feed industry.

[0003] Christian Hansen A/S, Denmark commercializes an example of such a probiotic growth-promoting product under the trade name GalliPro® (deposited as DSM 17231). GalliPro® is a *Bacillus subtilis* spore cell composition.

[0004] Besides the suggested mode of actions (e.g. immune modulation, gut flora modifier) probiotic bacillus are able to produce many beneficial components, such as enzymes, which are excreted in the gastro intestinal tract (GIT) when used as animal feed supplement. Enzymes such as phytase are excreted and improve the digestion and better uptake of animal feed (higher digestibility). The diet (feed) is mostly composed of plant origin such as grains, corn, soybean, soy oil and amino acids. Overall these effects contribute to the production of cost effective animal products.

[0005] Probiotic bacilli are also able to produce other beneficial components such as essential amino acids.

[0006] Bacillus spores can pass the acidic gastric barrier and germinate and outgrow within the gastrointestinal (GIT) of the animals. This has great advantages, since when ingested they can excrete numerous types of beneficial components, e.g. bacteriocins and also excrete useful essential amino acids. Moreover, the bacillus spores are thermostabile during a feed pelletizing process and are thereby an excellent delivery system to get both bacteriocins and e.g. essential amino acids into the GIT.

[0007] In the survival and proliferation process of bacillus in GIT, the role of bile is important. Bile is produced in the liver and stored in the gallbladder. Bile contains water, lecithin, bilirubin and biliverdin and bile salts.

[0008] It is known from the literature that bile has some negative influences on the survival and germination and outgrowth of bacillus spore cells to vegetative cells in the GIT of animals. Therefore research is ongoing to find probiotic bile resistant Bacillus strains.

[0009] The article (Antonie Van Leeuwenhoek. 2006 Aug; 90(2): 139-46. Epub 2006 Jul 4) describes isolation of a number of Bacillus samples/cell directly from the intestine of chickens. The isolated bacillus cells were tested for probiotic activity. The six bacilli with highest probiotic activity were tested for bile salt resistance and it was found that a specific highly probiotic bacillus has a relatively high level of bile salt resistance.

[0010] In this article there is no special focus on any time periods for the testing of bile resistance. In the experimental part the bacillus spore cells are simply tested for resistance after 5 days of presence in bile salt (see paragraph "Simulated small intestinal fluid tolerance test" on page 141).

[0011] US2003/0124104A describes that probiotic conventional bacillus endospores are sensitive to low concentration of bile salts, i.e. spore germination and/or rehydration is inhibited by the presence of even low concentrations of bile salts. This is contrary to other bacteria such as enteric pathogens, such as *E. coli* or *S. aureus* (see section [0014] to [0015]). In view of this it is suggested to screen/select for bacillus spores that are resistant to the inhibitory activity of bile salts, and as a result, germinate into vegetative cells, which then colonize the colon (see [0019]).

[0012] The working examples are all in presence and no real experimental data of actually screened specific Bacillus cell are provided in the description.

[0013] Further the bile salt screening conditions are relatively generically described. In particular there are no indications of any time periods for the selections of bile resistance. Said in other words, based on the only broad/generic teaching of this document one may select Bacillus cells that only can outgrow (germinate) slowly, i.e. are capable of germinating from spores to vegetative cells after e.g. 20 hours in presence of relevant amount of bile salt.

[0014] In this document there is no description or suggestion to select for bacillus cells that can outgrow (germinate) rapidly, i.e. capable of germinating and outgrowing from spores to vegetative cells reaching a defined growth point within a certain time interval in presence of a relevant amount of bile salt.

[0015] In summary, the prior art references relating to selection/screening of bile resistant bacillus cells are not focusing on rapid outgrowth/germination from spore cells to vegetative bacillus cells.

[0016] International PCT application with application number PCT/EP2008/057296 was filed 11/06/2008. Applicant is Chr. Hansen A/S and it was NOT PUBLISHED at the priority date of this present application.

[0017] The priority application of PCT/EP2008/057296, EP07111939.0 was published on 07-01-2009 as EP2011858A1, and PCT/EP2008/057296 was published on 15-01-2009 as WO2009/007192, after the priority date of

this present application (19-12-2008) but prior to the filing date of this present application (16-12-2009)

[0018] PCT/EP2008/057296 and EP2011858A1 describe novel bacillus spores characterized by having an improved/rapid speed of germination and outgrowth from spore to vegetative cell in presence of a bile salt medium.

[0019] The bacillus spores as described herein have the same improved/rapid speed of germination and outgrowth from spore to vegetative cell as described in PCT/EP2008/057296 and EP2011858A1.

[0020] PCT/EP2008/057296 and EP2011858A1 only describe bacillus vegetative cells that are producing phytase in an increased amount as compared to the reference bacillus cell DSM 19467.

[0021] As can be seen below, in first aspect and claim 1 herein is disclaimed such high producing phytase bacillus cells of PCT/EP2008/057296 and EP2011858A1.

[0022] When there below is referred to prior art this shall be understood as prior art made available to the public (e.g. published articles/patents) at the filing date of this present application.

SUMMARY OF THE INVENTION

[0023] The problem to be solved by the present invention is to provide a *Bacillus subtilis* composition which can excrete high amounts of beneficial compounds in the gastro intestinal tract (GIT) of an animal.

[0024] The solution is based on that the present inventors have developed a selection method for the identification of improved *Bacillus subtilis* compositions.

An important step of the herein described selection method is to specifically screen/select for *Bacillus subtilis* spore cells with improved/rapid speed of germination and outgrowth from spores to vegetative cells in the presence of bile salts.

[0025] As described above, the prior art has described methods for selecting bacillus cells capable of growing in presence of bile salts, but the prior art screening/selection methods do NOT focus on the speed of germination and outgrowth in the presence of bile salt. Accordingly, the prior art selected bile resistant bacillus cells do not germinate and grow fast enough to comply with the speed of germination and outgrowth criteria as described herein. For instance, bacillus cells isolated directly from the intestine of e.g. chickens (as e.g. described in the Antonie Van Leeuwenhoek article discussed above) in the gut environment are not selected (under natural pressure) to germinate and outgrow rapidly in the intestine.

[0026] As shown in working examples herein this is also true for the commercially available Bacillus composition GalliPro®, which simply germinates and outgrows too slowly and does not reach the defined growth point within the first 20 hours in presence of physiological levels of bile salts to comply with the speed of germination and outgrowth criteria as described herein. GalliPro® is a *Bacillus subtilis* composition that is commercially successful.

[0027] The herein described strain deposited as DSM 19467 was selected by using GalliPro® as a starting strain and a selective pressure method and a subsequent isolation for rapid germination and outgrowth from spores to vegetative cells in presence of bile salt as described herein.

[0028] See e.g. table 1 for further details (GalliPro® may herein also be termed DSM 17231). In Figure 1 herein this is illustrated schematically.

[0029] In summary, it is believed that no prior art describes an isolated *Bacillus subtilis* composition, which comprises from 10^5 to 10^{12} CFU/g bacillus cells, wherein the cells of the bacillus composition complies with the rapid germination and outgrowth in the presence of bile salt criteria as described herein.

[0030] Without being limited to theory, the present inventors have identified that rapid germination and outgrowth is a very important aspect of the invention as bacillus spores, which are resistant to bile but do not germinate and outgrow fast enough, will be excreted before any positive characteristics, such as essential amino acid production, can be made in significant amounts by the vegetative bacillus cells.

[0031] Bacillus spores germinating too slowly will simply pass through the gastro intestinal tract (GIT) before the bacteria can produce any significant amount of e.g. essential amino acids.

[0032] After a number of detailed tests and analysis, the inventors therefore chose to work with a time range up to 20 hours and select the fastest germinating and outgrowing spores within this time period in presence of high physiological concentrations of bile salts. Without being limited to theory and based on the herein disclosed detailed experimental work, the present inventors have identified that it is important to have a rapid germination and outgrowth within the first 18 and 19 hours in the presence of 4 and 6 mM bile salt, respectively.

[0033] The present inventors then identified that once *Bacillus subtilis* cells, with rapid germination and outgrowth in bile salt medium, have been selected these cells are highly useful as starting cells for mutagenesis to obtain new cells with improved amino acid production.

[0034] As illustrated schematically in figure 1 and example 4, the rapidly outgrowing bile resistant selected strain, DSM 19467, was used as starting strain for classical mutation and the strains producing high amounts of amino acids were selected. As can be seen in example 4, some of the selected strains produce at least 5 times more of the amino acid leucine than DSM 19467 and GalliPro®.

[0035] As discussed above, in our earlier applications PCT/EP2008/057296 and EP2011858A1, DSM 19467 was

used to make new high phytase producing strains - see for instance example 4 of PCT/EP2008/057296 and EP2011858A1.

[0036] Accordingly, one may see DSM 19467 as a very useful kind of e.g. "intermediate" to make new bacillus cells with improved properties such as e.g. higher phytase or amino acid production or any other relevant compound of interest.

[0037] Further, it is evident to the skilled person that once the inventors herein have disclosed the test assay for testing rapid germination and outgrowth of example 1 plus the identified strain DSM 19467 it will be routine work for the skilled person to select other *Bacillus subtilis* cells complying with the criteria of the first aspect herein - i.e. other alternative *Bacillus subtilis* cells with similar characteristic as DSM 19467.

[0038] Accordingly, a first aspect of the invention relates to a *Bacillus subtilis* composition, which comprises from 10^5 to 10^{12} CFU/g *Bacillus subtilis* spore cells, wherein the *Bacillus subtilis* composition is characterized by:

(i): the *Bacillus subtilis* spores have a rapid germination and outgrowth from spore to vegetative cell in presence of a bile salt medium comprising 4 mM bile salts and in presence of a bile salt medium comprising 6 mM bile salts, defined by that the *Bacillus subtilis* spores reach a vegetative cell growth point of 0.4 OD_{630} within less than 18 and 19 hours, respectively, wherein the vegetative cell growth point is the point in the growth curve where the OD value starts to increase (due to growth of the vegetative cells) in a continuous way and reaches an OD_{630} of 0.4;

(I): wherein the bile salt medium is the non-selective Veal Infusion Broth (VIB) medium of example 1 herein supplemented with a bile salt mixture comprising the conjugated bile salts taurodeoxycholate and glycodeoxycholate and the deconjugated bile salt deoxycholate in the proportions 60% of the taurodeoxycholate, 30% of the glycodeoxycholate and 10% of deoxycholate; and

(II): wherein the OD assay analysis is performed by the following steps:

(a): filling a well in a microtiter plate with 0.150 ml bile salt medium having 10^8 *Bacillus subtilis* spores per ml medium (i.e. this is time zero); and
(b): incubating the plate at 37°C under atmospheric conditions and measuring the OD_{630} values, using a spectrophotometer and with agitation before each reading, to get a representative growth curve over time;

and

(ii) the *Bacillus subtilis* vegetative cells are producing an enzyme or an amino acid selected from the group consisting of phenylalanine, valine, threonine, tryptophan, isoleucine, methionine, leucine, lysine, cysteine, tyrosine, histidine and arginine in an amount that is higher than the reference *Bacillus subtilis* cell DSM 19467, wherein both the vegetative celle and reference *Bacillus* subtilis celle DSM 19467 are grown under identical conditions,

and with the proviso that the *Bacillus subtilis* vegetative cells are NOT cells as described in claim 1 of PCT/EP2008/057296 and the priority application thereof,
EP07111939.0, published as EP2011858A1, wherein the bacillus vegetative cells of item (ii) of claim 1 of PCT/EP2008/057296 and EP2011858 A1
are producing phytase in an amount of at least 1.25 times more than the reference bacillus cell DSM 19467, wherein the produced phytase amount is measured by the phytase assay of example 2 of PCT/EP2008/057296 and EP2011858A1 after 4 hours growth at 37°C in the non-selective Heart Infusion Broth (HIB) medium of example 2 of PCT/EP2008/057296 and EP2011858A1; and
wherein the phytase assay analysis is performed by the following steps:

(a): making a overnight culture of bacillus vegetative cells in a enriched culture medium; and
(b): transferring a 1% inoculum from the overnight culture to HIB medium (i.e. this is time zero) and incubation at 37°C until phytase activity measurement.

[0039] As discussed above, *Bacillus subtilis* cells with spores that comply with criteria (i) of first aspect can in principle e.g. be used as a starting strain to screen for *Bacillus subtilis* vegetative cells that may e.g. have higher/improved production of any compound of interest - such as e.g. an enzyme of interest.

[0040] As discussed above, the reference bacillus cell DSM 19467 is selected for rapid germination and outgrowth in presence of bile salt by using GalliPro® as starting strain. DSM 19467 is not selected for improved production of a compound of interest (e.g. an amino acid). Without being limited to theory, it is believed that the herein relevant production of the vast majority of commercially relevant compounds of interest (e.g. phytase or an amino acid) of DSM 19467 corresponds to GalliPro® production of the same compound.

[0041] In relation to point (i) the vegetative cell growth point for GalliPro® is at least 20 hours after incubation in 4 and 6 mM bile salt and for the novel DSM 19467 strain, as described herein, it is after 14 and 15 hours in 4 and 6 mM bile salts, respectively (see figure 2 and working example 3 herein).

[0042] It is here relevant to note that the present inventors also tested the commercially available product CALSPORIN® (Calpis Co., Ltd., Japan) to determine the vegetative cell growth point under the conditions of point (i) of first aspect. As for GalliPro® the commercial product CALSPORIN® is a *Bacillus subtilis* composition used as a probiotic feed additive. The vegetative cell growth point under the conditions of point (i) of first aspect for CALSPORIN® was more than 20 hours at 4 and 6mM bile salts, respectively. This is considerably more than the 18 and 19 hours required under point (i) and this illustrates that commercially available products have hitherto not been selected for rapid germination and outgrowth. As discussed above, "natural" bacillus cells have not been under any selective pressure to get rapid germination and outgrowth. Without being limited to theory, it is therefore believed that "natural" bacillus cells are not complying with the conditions of point (i) of first aspect.

[0043] Both the bile resistance [of point (i)] and essential amino acid assay [of point (ii)] are based on commercially available standard elements (such as e.g. standard media, bile salts; standard OD measurements and standard tests).

[0044] The reference bacillus cell is deposited as DSM 19467 and is therefore publicly available.

[0045] The *Bacillus subtilis* cell GalliPro® is deposited as DSM 17231 (named "GalliPro®") and is therefore publicly available.

[0046] Accordingly, based on the detailed assay description herein (see e.g. example 1 herein for bile resistance assay and example 2 herein for amino acid assay) the skilled person is routinely able to repeat these assays to objectively determine whether a specific *Bacillus subtilis* cell of interest complies with the bile resistance [of point (i)] and amino acid [of point (ii)] levels of the first aspect of the invention.

[0047] The novel *Bacillus subtilis* composition as described herein may be used as a probiotic supplement to animal feed. The dose and administration may be done according to the art as for instance as done for prior art GalliPro® bacillus compositions.

[0048] Accordingly, a second aspect of the invention relates to a method for feeding an animal comprising administering the *Bacillus subtilis* composition of first aspect and herein described related embodiments to an animal in conjunction with other animal feed ingredients.

[0049] A third aspect of the invention relates to a method for screening and isolating a *Bacillus subtilis* cell comprising the following steps:

(a): selecting and isolating from a pool of individual *Bacillus subtilis* spore cells a *Bacillus subtilis* spore cell that is capable of germinating and outgrowing so rapidly that it reaches a vegetative cell growth point within less than 18 and 19 hours under the conditions of point (i) of first aspect;

(b): making a vegetative *Bacillus subtilis* cell from the isolated spore cell of step (a) and mutating the selected and isolated cell to get a pool of individual *Bacillus subtilis* vegetative cells;

(c): selecting and isolating from the pool of individual *Bacillus subtilis* vegetative cells of step (b) a *Bacillus subtilis* vegetative cell that is capable of producing an enzyme or an amino acid selected from the group consisting of phenylalanine, valine, threonine, tryptophan, isoleucine, methionine, leucine, lysine, cysteine, tyrosine, histidine and arginine; and

(d): analyzing the vegetative *Bacillus subtilis* cell of step (c) to confirm that it has maintained the rapid germination and outgrowth of step (a) and isolating the selected *Bacillus subtilis* cell.

[0050] It is evident to the skilled person that once the inventors herein have disclosed the relevant test assays (in particular the assay for testing rapid germination and outgrowth of example 1) plus the reference strain DSM 19467 it will be routine work for the skilled person to select other *Bacillus subtilis* cells complying with the criteria of the first aspect herein.

[0051] As discussed herein, by using the screening/selection method as described herein the inventors have selected and isolated a number of improved *Bacillus subtilis* cells.

[0052] Embodiment of the present invention is described below, by way of examples only.

DEFINITIONS

[0053] All definitions of herein relevant terms are in accordance of what would be understood by the skilled person in relation to the herein relevant technical context.

[0054] The term "bacillus cell" or "*Bacillus subtilis* cell" relates herein to both a bacillus or *Bacillus subtilis* spore cell and a bacillus or *Bacillus subtilis* vegetative cell.

[0055] The term "bacillus spore" or "*Bacillus subtilis* spore" in relation to bacillus or *Bacillus subtilis* spore cell relates herein to a spore that according to the art may be characterized as a dormant, tough, non-reproductive structure produced

by bacillus bacteria. The primary function of spores is generally to ensure the survival of a bacterium through periods of environmental stress. They are therefore resistant to ultraviolet and gamma radiation, desiccation, lysozyme, temperature, starvation, and chemical disinfectants. Spores are commonly found in soil and water, where they may survive for long periods of time. The spore coat is impermeable to many toxic molecules and may also contain enzymes that are involved in germination. The core has normal cell structures, such as DNA and ribosomes, but is metabolically inactive. When a bacterium detects that environmental conditions are becoming unfavorable it may start the process of sporulation, which takes about eight hours.

[0056] The term "bacillus vegetative cell" or "*Bacillus subtilis* vegetative cell" relates to functional vegetative bacillus or *Bacillus subtilis* cells, which can divide to produce more vegetative cells.

[0057] The term "germination and outgrowth" relates to that bacillus spores germinate and outgrow to bacillus vegetative cells. As known to the skilled person reactivation of the spore occurs when conditions are favorable and involves germination and outgrowth. Germination involves the dormant spore starting metabolic activity and thus breaking hibernation. It is commonly characterized by rupture or absorption of the spore coat, swelling of the spore, an increase in metabolic activity, and loss of resistance to environmental stress. Outgrowth follows germination and involves the core of the spore manufacturing new chemical components and exiting the old spore coat to develop into a functional vegetative bacterial cell, which can divide to produce more cells. Growth curves (OD versus time) of bacillus cells show distinct growth phases. As the spores are transferred to a nutrient rich medium the germination is initiated followed by a temporary decrease in OD (phase I), which is due to the release of dipicolinic acid and consequently hydration of the spore coat. In the second phase (phase II = outgrowth phase) there is a period with a relatively little change in OD, until the spores are developed into a functional vegetative bacterial cells, which can divide to produce more cells and thereby give a continuous increase in OD value. The point when one starts to get the continuous increase in OD values reaching an OD of 0.4 is herein termed "vegetative cell growth point".

[0058] The term "optical density" is defined as a measure of optical absorbance using a spectrophotometer. Optical density (OD) is the absorbance of an optical element for a given wavelength λ per unit distance. If OD is e.g. measured at wavelength 630 nm it may be referred to as OD_{630}.

DRAWINGS

[0059]

Figure 1: In this figure the steps to get to the herein novel improved strains are illustrated. The working examples herein were started from DSM 17231 (GalliPro®), which was classically mutated and screened/selected for rapid germination and outgrowth in presence of bile salt to get the novel selected strain DSM 19467. DSM 19467 was used as starting strain for classical mutation and strains producing high amounts of amino acids were selected.

Figure 2a and 2b: These figures show clearly the improved rapid germination and outgrowth of DSM 19467 bacillus spores of the present invention as compared to DSM 17231 in presence of 4 and 6 mM bile salt as described herein.

DETAILED DESCRIPTION OF THE INVENTION

A relevant compound of interest:

[0060] As discussed above, the term "a relevant compound of interest" of point (ii) should be understood broadly. As discussed above, bacillus cells with spores that comply with criteria (i) of first aspect - such as e.g. DSM 19467 - can in principle e.g. be used as a starting strain to screen for bacillus vegetative cells that may e.g. have higher/improved production of any compound of interest - such as e.g. an enzyme of interest.

[0061] Suitable examples of a compound of interest may be enzymes such as e.g. mannanase. A compound of interest could also be an amino acid such as leucine.

[0062] According to the first aspect the *Bacillus subtilis* vegetative cells are producing an enzyme or an amino acid selected from the group constiting of phenylalanine, valine, threonine, tryptophan, isoleucine, methionine, leucine, lysine, cysteine, tyrosine, histidine and arginine in an amount that is higher than the reference bacillus cell DSM 19467, wherein both the vegetative cell and reference bacillus cell DSM 19467 are grown under identical conditions.

[0063] In example 4 herein one can see an example of this embodiment - in example 4 herein is described vegetative cells with at least 5 times higher production of the amino acid leucine as compared to DSM 19467.

[0064] Further, as discussed above - in example 4 of PCT/EP2008/057296 and EP2011858A1 is described similarly for phytase - i.e. vegetative cells with higher production of phytase as compared to DSM 19467.

Bacillus composition:

[0065] The term "bacillus composition" or *Bacillus subtilis* composition" shall be understood according to the art. It is herein understood as a bacillus or *Bacillus subtilis* composition comprising a number of bacillus or *Bacillus subtilis* spore cells with a characteristic of interest.

[0066] The bacillus or *Bacillus subtilis* composition comprises B. *subtilis.* In essence the composition shall simply comprise the amount of *Bacillus subtilis* spore cells given in the first aspect herein, wherein the *Bacillus subtilis* cells comply with the criteria given in the first aspect.

[0067] As known to the skilled person, herein commercially relevant bacillus spore cell compositions are generally made by fermentation. The obtained spore cells are generally concentrated, dried, mixed with a carrier and packed into a suitable container.

[0068] The relevant e.g. 10^5 to 10^{12} CFU/g *Bacillus subtilis* cells of the composition may be present in a commercially relevant form known to the skilled person.

[0069] Accordingly, in an embodiment 10^5 to 10^{12} CFU/g *Bacillus subtilis* spore cells of the composition are present as dried (e.g. spray dried) cells or as frozen spore cells.

[0070] In a preferred embodiment the *Bacillus subtilis* composition comprises from 10^6 to 10^{12} CFU/g *Bacillus subtilis* spore cells, more preferably from 10^7 to 10^{12} CFU/g *Bacillus subtilis* spore cells.
The term "CFU/g" relates to the gram weight of the composition as such, including suitable relevant additives present in the composition. It does not include the weight of a suitable container used to package the *Bacillus subtilis* composition.

[0071] An embodiment relates to that the *Bacillus subtilis* composition is packaged into a suitable container.

[0072] As known to the skilled person a commercially relevant bacterial composition generally also comprises other relevant additives such as e.g. one carrier/ingredient of the group belonging to whey, whey permeate, calcium carbonate/limestone and anti caking agents such as aluminum silicates and kieselgur (diatomaceous earth).

[0073] Beside the herein relevant bacillus cells the composition may also comprise other relevant microorganisms of interest such as e.g. lactic acid bacteria of interest.

Bacillus cell

[0074] The bacillus cell is a *B. subtilis* cell.

Assay to select for rapid germination and outgrowth in the presence of bile salt

[0075] As discussed above the bile resistance assay of point (i) of first aspect is based on commercially available standard elements (such as e.g. standard media, bile salts; standard OD measurements).

[0076] Accordingly, based on the detailed assay description herein (see e.g. example 1 herein) the skilled person is routinely able to repeat this assay to objectively determine whether a specific *Bacillus subtilis* spore cell of interest complies with the rapid germination and outgrowth from spore to vegetative cell criteria as described in point (i).

[0077] In point (i) it is explained that vegetative cell growth point is the point in a growth curve starting with 10^8 spores/ml corresponding to OD of around 0.2-0.3 until the time where the OD value has increased (due to growth of the vegetative cells) in a continuous way and has reached OD 0.4. This is in accordance with how a skilled person would understand such a vegetative cell growth point and based on a growth curve the skilled person may routinely determine this, within a limited variability of around $\pm$ 30 minutes, as explained herein.

[0078] Working example 1 herein provides a detailed description of a bile resistance assay suitable to select for rapid germination and outgrowth in the presence of bile salt. The detailed conditions of this example 1 is herein a preferred assay to determine if a *Bacillus subtilis* spore cell of interest complies with the criteria of point (i) of first aspect.

[0079] The term "bile salt" relates to the salt of bile acids. Bile acids are steroid acids found predominantly in the bile of mammals. They are produced in the liver by the oxidation of cholesterol, and are stored in gallbladder and secreted into the intestine in the form of salts. They act as surfactants, emulsifying lipids and assisting with their absorption and digestion. The bile salts used in example 1 were prepared mimicking the physiological concentrations and compositions of porcine bile salts. As known to the skilled person porcine bile salts compositions may herein be considered as relatively "harsh" conditions as compared to avian bile salt compositions.

[0080] The term "bile salt medium" relates to medium comprising relevant bacillus growth ingredients such as relevant nutrients and bile salt.

Vegetative cell growth point - in bile salt assay - point (i) of first aspect

[0081] As said above, in relation to point (i) of first aspect the *Bacillus subtilis* spore cells, as described herein, have a germination and outgrowth from spore to vegetative cell that is so rapid that they reach a vegetative cell growth point

of 0.4 OD within less than 18 and 19 hours at 4 and 6 mM bile salts, respectively.
As said above, the novel DSM 19467 strain reaches the vegetative cell growth point after 14 and 15 hours incubation in 4 and 6 mM bile salt, respectively.

[0082] Accordingly, in a preferred embodiment the *Bacillus subtilis* spores reach the vegetative cell growth point after 17 and 18 hours incubation in 4 and 6 mM bile salt under the conditions of point (i) of first aspect, more preferably the *Bacillus subtilis* spores reach the vegetative cell growth point after 15 and 16 hours incubation in 4 and 6 mM bile salt under the conditions of point (i) of first aspect.

[0083] As explained above and shown schematically in figure 1 the herein described DSM 19467 strain was selected by using the commercially available GalliPro® as a starting strain for mutagenesis and selection for rapid outgrowth in presence of bile salt as described herein.

[0084] GalliPro® is a composition comprising *Bacillus subtilis* cells and the *Bacillus subtilis* is deposited as DSM 17231. Accordingly, GalliPro® may herein be seen as a reference strain.

[0085] As said above, the vegetative cell growth starting point for GalliPro® is after 20 hours incubation in 4 and 6 mM bile salts under the conditions of point (i) of first aspect. Accordingly, in an embodiment the *Bacillus subtilis* spores reach the vegetative cell growth point at least 3 hours earlier than the reference *Bacillus subtilis* spores cells deposited as DSM 17231 ("GalliPro®") under the conditions of point (i) of first aspect, more preferably the *Bacillus subtilis* spores reach the vegetative cell growth point at least 4 hours earlier than the reference *Bacillus subtilis* spores cells deposited as DSM 17231 ("GalliPro®") under the conditions of point (i) of first aspect, and most preferably the *Bacillus subtilis* spores reach the vegetative cell growth starting point at least 5 hours earlier than the reference *Bacillus subtilis* spores cells deposited as DSM 17231 ("GalliPro®") under the conditions of point (i) of first aspect.

Essential amino acids

[0086] As known to the skilled person an amino acid may be an amino acid selected from the group consisting of: phenylalanine, valine, threonine, tryptophan, isoleucine, methionine, leucine, lysine, cysteine, tyrosine, histidine and arginine.

[0087] In a preferred embodiment the amino acid is at least one amino acid selected from the group consisting of: phenylalanine, valine, threonine, tryptophan, isoleucine, methionine, leucine, and lysine.

[0088] In more preferred embodiment the amino acid is at least one amino acid selected from the group consisting of: valine, isoleucine and leucine.

[0089] A herein very relevant amino is leucine.

[0090] As understood by the skilled person, the bacillus vegetative cells may produce higher amount of more than one amino acid, such as e.g. higher amount of two or three or more different amino acids.

Amino acid assay

[0091] As discussed above the amino acid assay of point (ii) of first aspect is based on standard commercially available elements (such as e.g. standard media, standard test).

[0092] Accordingly, based on the detailed assay description herein (see e.g. example 2 herein) the skilled person is routinely able to repeat this assay to objectively determine whether a specific *Bacillus subtilis* vegetative cell of interest complies with the produced amino acid amount as described in point (ii).

[0093] Working example 2 herein provides a detailed description of an amino acid assay. The detailed conditions of this example 2 are herein a preferred amino acid assay to determine if a bacillus vegetative cell of interest complies with the criteria of point (ii) of first aspect.

Produced amount of essential amino acid - point (ii) of first aspect

[0094] In relation to point (ii) of first aspect, the *Bacillus subtilis* vegetative cells are preferably producing at least one essential amino acid in an amount of at least 2 times more than the reference Bacillus cell DSM 19467 under the conditions of point (ii) of first aspect.

[0095] In a more preferred embodiment in relation to point (ii) of first aspect, the *Bacillus subtilis* vegetative cells are preferably producing at least one essential amino acid in an amount of at least 4 times more than the reference Bacillus cell DSM 19467 under the conditions of point (ii) of first aspect.

A method for feeding/administering bacillus spores to an animal

[0096] As said above a second aspect of the invention relates to a method for feeding an animal comprising administering the *Bacillus subtilis* composition of first aspect and herein described related embodiments to an animal in con-

junction with other animal feed ingredients.

[0097] The animal may be any animal of interest. Preferably, the animal is an animal selected from the group consisting of poultry, ruminants, calves, pigs, rabbits, horses, fish and pets.

[0098] When administering GalliPro® according to the art it is normally done in a dose from around 10^4-10^8 CFU/g feed, commonly 10^5-10^6 CFU/g feed or in doses equivalent to normal feed intake/kg live weight animal.

[0099] Alternatively the bacillus spores may be administered to the animal in one of the following ways:

(1): put it into drinking water for animals;
(2): sprayed onto animals; or
(3): application via paste, gel or bolus.

A method for screening and isolating a novel bacillus cell

[0100] As said above, the third aspect relates to a method for screening and isolating a novel *Bacillus subtilis* cell.

[0101] In the method of the third aspect is selected for a *Bacillus subtilis* cell capable of fulfilling the conditions of point (i) and (ii) of the first aspect.

[0102] As understood by the skilled person, the specific herein detailed described bile resistance and amino acid amount assay (see e.g. example 1 herein for bile resistance assay and example 2 herein for amino acid assay) parameters may be changed to make a alternative screening method that still obtains the main goals as described herein, i.e. a *Bacillus subtilis* cell that is capable of fulfilling the conditions of point (i) and (ii) of the first aspect.

[0103] In a preferred embodiment, bile resistance assay of example 1 is used in step (a) of the screening method of third aspect and the essential amino acid assay of example 2 is used in step (c) of the screening method of third aspect.

[0104] In step (d) of the screening method of third aspect a vegetative *Bacillus subtilis* cell is isolated. This vegetative *Bacillus subtilis* cell may be used to make bacillus spores from.

[0105] Accordingly, the screening method of third aspect may be followed by an extra step (e), wherein the isolated *Bacillus subtilis* vegetative cell of step (d) is fermented to make from 10^5 to 10^{12} *Bacillus subtilis* vegetative cells and these 10^5 to 10^{12} *Bacillus subtilis* vegetative cells are used to make 10^5 to 10^{12} *Bacillus subtilis* spore cells, which are isolated to give a *Bacillus subtilis* composition, which comprises from 10^5 to 10^{12} CFU/g *Bacillus subtilis* spore cells. The end result of step (e) is a novel *Bacillus subtilis* composition, which comprises from 10^5 to 10^{12} CFU/g *Bacillus subtilis* spore cells, and wherein the *Bacillus subtilis* cells are capable of fulfilling the conditions of point (i) and (ii) of the first aspect.

[0106] Accordingly, a *Bacillus subtilis* composition, which comprises from 10^5 to 10^{12} CFU/g *Bacillus subtilis* spore cells capable of fulfilling the conditions of point (i) and (ii) of the first aspect may be obtained by the screening method of third aspect.

[0107] In step (b) of the screening method of third aspect is made mutations of the earlier selected bile resistant *Bacillus subtilis* cell to select for strains producing high amounts of amino acids in step (c). As understood by the skilled person this may e.g. by classical mutation (e.g. by chemical treatments or UV) of specific exchange of genes to make a so-called Genetic Modified Organism (GMO).

Deposited strains

[0108] A sample of the *Bacillus subtilis* strain has been deposited at DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Maschroder Weg 1b, D-38124 Braunschweig) under the accession number DSM 19467 with a deposit date of June 27, 2007. The deposit has been made under the conditions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure.

EXAMPLES

EXAMPLE 1: *Bile resistance assay*

Medium:

[0109] The medium was a standard non-selective commercial available medium Veal Infusion Broth (VIB) (Difco, 234420).

At the filing date of the present application the product catalogue ("Difco™/BBL™ Manual) from the provider BD Diagnostic Systems (www.bd.com) read in relation to the Veal Infusion Broth:
"Infusion from lean veal and peptone provide the nitrogen, vitamins, carbon and amino acids in veal infusion media. Sodium chloride maintains the osmotic balance of the formulations"; and

The medium was prepared according to manufacture instructions by suspending 25 g of the Veal Infusion Broth powder in 1 L of purified water (2.5% solution) and heat with frequent agitation and boil for 1 minute to completely dissolve the powder. A 2.5% Veal Infusion Broth solution comprised per liter:

Lean Veal, Infusion: 10g
Proteose Peptone: 10 g
Sodium Chloride 5 g

[0110] The medium was distributed into sterile bottles and autoclaved for 15 min at 121°C.

Bile salt solutions/medium:

[0111] Mixtures of bile salts were prepared mimicking the physiological composition and concentration of bile salts in pig bile and the bile salts were dissolved in the Veal Infusion Broth medium as prepared above to give a final bile salt concentration of 8 mM.
The conjugated bile salts were taurodeoxycholate (Sigma T-0875, U.S.) and glycodeoxycholate (Sigma G-9910, U.S.) and the deconjugated bile salt deoxycholate (Sigma D-5670 U.S.) and the final 8 mM mixed bile salt solution contained 60% of the taurodeoxycholate, 30% of the glycodeoxycholate and 10% of deoxycholate. Before autoclaving for 15 minutes at 121°C, the solutions were adjusted to pH 7.4 using sodium hydroxide. The prepared 8 mM bile salt medium, were diluted to get bile salt concentrations of 0, 1, 2, 4, 6 and 8 mM.
[0112] The bile salts were added to the Veal Infusion Broth medium in a concentrated form.
[0113] Accordingly, the final amount of lean veal infusion, Proteose Peptone and Sodium chloride were essentially as for the 2.5% Veal Infusion Broth medium before the bile salts were added.

Spore suspensions

[0114] To distinguish between vegetative cells and spores and to ensure pure spore products for inoculation, the spore counts of the bacillus product were determined using +/- heat treatment at 80 °C for 10 min. After heat treatment and subsequent cooling to room temperature, serial 10-fold dilutions were conducted in saline peptone water. Duplicates of Tryptose Blood Agar plates (Difco 0232-01) were inoculated with 0.1 ml from the appropiate decimal dilutions. The plates were incubated at 37°C until the next day. Based on preceding spore count determinations of the products, spore suspensions were prepared in sterile distilled water to reach final calculated spore concentration of 10^8 CFU/ml. The counts of vegetative cells and spores in the final inocula were determined using the method described above. The final concentration of 10^8 CFU/ml corresponded to a start OD_{630} at 0.2-0.3.

Growth measurement: optical density measurements

[0115] Sterile flat bottom 96 well microtiter plates were used (Greiner Bio-one GmbH, Germany). Each well was filled with 0.150 ml VIB inoculated with spores (~$1x10^8$ spores per ml equivalent/corresponding to a start OD_{630} ~ 0.2-0.3) and the plates were incubated for 20 hours at 37°C with a 1 minute shaking cycle of intensity 4 (high) before each reading.
[0116] To avoid condensation on the inside of the plate cover, the lids were exposed to a dilute solution of Triton X-100.
[0117] The germination and outgrowth kinetics of Bacillus strains were measured using a spectrophotometer at wavelength 630nm (OD_{630}) (Bio-tek Instruments, Inc. VE). Readings were performed with 10 minute intervals and analyzed using the KC4™ software (Bio-tek Instruments, Inc., USA). After 20 h, data were exported to Excel® spreadsheets for further analysis, imported in SAS version 9.0 and statistically analyzed.

EXAMPLE 2: *Amino acid assay*

[0118] The method to measure and quantify the amino acids produced by the bacillus cells used in this study is a standard GC-MS method for aqueous samples, using methyl chloroformate as derivatization agent.

Growth of Bacillus cells

[0119] The Bacillus cells are inoculated and grown in a minimal salts growth medium at 37°C, 150 rpm and grown for 2 days and amount of amino acid is then measured in the supernatant as described below.
[0120] The bacillus cells are propagated in a Minimal Salts Medium according to Chapman (1972) with the following composition:

$(NH_4)_2SO_4$ (Merck 1.01217.1000)	1 g/l
K_2HPO_4 (Merck 1.05101.1000)	7 g/l
KH_2PO_4 (Merck 1.04873.1000)	3 g/l
$MgSO_4 \cdot 7H_2O$ (Merck 1.05886.1000)	0.1 g/l

Autoclaved for 15 min at 121°C and added autoclaved glucose to a final concentration of 0.5 %.

[0121] Incubation is done in tubes with 10 ml medium for 2 days at 37 °C and 150 rpm.

Amino acid assay

[0122] The amino acid assay is carried out on cell supernatants, since the amino acids are secreted to the media. Samples are sterile filtered and kept at -20 °C until analysis.

Reagents:

[0123]

Reagent 1: Internal standard solution. Norvaline 1 mM: 0.0172 g Norvaline + 100 ml MQW
Reagent 2: Methanol/Pyridine 32/8 (v/v) *(Catalysator)*
Reagent 3: Methyl Chloroformate p.a. (MCF) *(Derivatization agent)*
Reagent 4: 1 % MCF/$CHCl_3$ (v/v) *(Extraction):* 1 ml Methyl Chloroformate p.a. + Chloroform ad 1000 ml.

Sample preparation:

[0124]

- Pipette 150 µl (25 µl + 125 µ MQW) sample into 2 ml injection vial.
- Add 150 µl IS
- Add 200 µl 1-Methanol/Pyridine 32/8 % (v/v). Mix well.
- Add 25 µl MCF (Methyl Chloroformate). Mix well until gas development occurs.
- Add 500 µl 1 % MCF/$CHCl_3$ (v/v), cap and mix vigorously. Phase separation occurs within minutes. If phase separation is too slow, centrifuge the vial (500 rpm/10 min).

[0125] If Norvaline is used as antimetabolite, an external standard or another suitable internal standard should be used instead, and the 150 µl IS substituted with either MQW or sample.

[0126] Samples are run on GC-MS with a standard amino acid column and protocol.

EXAMPLE 3: *Selection of bile resistant Bacillus subtilis cell DSM 19467*

[0127] The starting bacillus cell was the *Bacillus subtilis* cell GalliPro®.

[0128] GalliPro® was mutagenized to get a pool of new individual bacillus cells. Spores were made and selected for rapid germination and outgrowth from spore to vegetative cell in presence of a bile salt medium comprising 4 and 6 mM bile salt a described in example 1 above.

[0129] *Bacillus subtilis* cell DSM 19467 was selected.

[0130] Table 1 below shows germination and outgrowth data.

[0131] Time (hours) from 10^8 CFU/ml corresponding to OD 0.2-0.3 until OD 0.4 is reached (mean of 3 replicates).

B. subtilis	4 mM bile	6mM bile
Existing product GalliPro®(DSM 17231)	>20	>20
Bile tolerant (DSM 19467)	13h 40m	15h
Commercial product: Calsporin	>20	>20

[0132] Some of the data of this example was made by testing phytase overexpressing DSM 19489. But for the technical result of this example this is herein relatively irrelevant since DSM 19467 has germination and outgrowth roughly as

DSM 19489. See PCT/EP2008/057296 and EP2011858A1 for further details.

Conclusion

[0133] DSM 19467 is a bile resistant strain and clearly germinating and outgrowing faster than GalliPro®.

EXAMPLE 4: *Selection of amino acid over-producing Bacillus cells from DSM 19467*

[0134] The starting bacillus cell was the *Bacillus subtilis* cell DSM 19467 selected in example 3.

[0135] DSM 19467, either wildtype or mutants produced by, e.g., UV-mutagenesis, was grown on Minimal Salts Medium agar, described in example 2B above and added 1,5 % agar, containing amino acid analogues in suitable inhibitory amounts. Depending on the amino acid to be over-expressed various amino acid analogues could be used, e.g., norvaline or 4-aza-DL-leucine for overproducing leucine (Bardos, 1974, Topics in Current Chemistry 52, 63-98). Colonies resistant to the amino acid analogue were picked, grown in Minimal Salts Medium and assayed for amino acid production. The vegetative cells were selected for producing high amount of amino acid by using the GC-MS method described in example 2B above.
High amino acid producing *Bacillus subtilis* cell was selected.

Results of amino acid measurements

[0136] A number of strains were selected which were producing the essential amino acid leucine in an amount that was significant higher than the reference bacillus cell DSM 19467.

[0137] A number of the selected strains produced at least 5 times more leucine than DSM 19467.

Conclusions:

[0138] This example shows that one can routinely - based on the instructions herein - screen and identify a strain, which produces at least one essential amino acid (here exemplified by leucine) in an amount that was significant higher than the reference bacillus cell DSM 19467.

[0139] DSM 19467 is originating from GalliPro® and is not selected for high essential amino acid production. Accordingly, it is believed that GalliPro® produces roughly the same amount of essential amino acid as DSM 19467.

EXAMPLE 5: *Bile resistance "check" of high essential amino acid producing bacillus cells.*

[0140] Preferred high essential amino acid producing bacillus cells selected in example 4 are re-checked for their ability of rapid germination and outgrowth from spore to vegetative cells as described in example 1.

[0141] The results are that they - as expected - have maintained roughly the same good rapid germination and outgrowth as the starting cell DSM 19467 used to obtain them.

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[0142]

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- **BARDOS.** *Topics in Current Chemistry,* 1974, vol. 52, 63-98 **[0135]**

PATENTKRAV

1. *Bacillus subtilis*-sammensætning, som omfatter fra 10^5 til 10^{12} CFU/g *Bacillus subtilis*-sporeceller, hvor *Bacillus subtilis*-sammensætningen er kendetegnet ved:

(i): at *Bacillus subtilis*-sporerne har en hurtig spiring og fremvækst fra spore til vegetativ celle i nærvær af et galdesaltmedium, som omfatter 4 mM galdesalte, og i nærvær af et galdesaltmedium, som omfatter 6 mM galdesalte, defineret ved, at *Bacillus subtilis*-sporerne når et vegetativt cellevækstpunkt på 0,4 OD_{630} inden for mindre end henholdsvis 18 og 19 timer, hvor det vegetative cellevækstpunkt er det punkt på vækstkurven, hvor OD-værdien begynder at stige (som følge af vækst af de vegetative celler) på en kontinuerlig måde og når en OD_{630} på 0,4;

(I): hvor galdesaltmediet er det ikke-selektive VIB-medium (Veal Infusion Broth medium) fra eksempel 1 heri suppleret med en galdesaltblanding, som omfatter de konjugerede galdesalte taurodeoxycholat og glycodeoxycholat og det dekonjugerede galdesalt deoxycholat i andelene 60% af taurodeoxycholatet, 30% af glycodeoxycholatet og 10% deoxycholat; og
(II): hvor OD-assay-analysen udføres ved følgende trin:

(a): fyldning af en brønd i en mikrotiterplade med 0,150 ml galdesaltmedium med 10^8 *Bacillus subtilis*-sporer pr. ml medium (dvs. dette er tiden nul); og
(b): inkubering af pladen ved 37°C under atmosfæriske betingelser og måling af OD_{630}-værdierne, ved anvendelse af et spektrofotometer og med omrøring før hver aflæsning, for at opnå en repræsentativ vækstkurve over tid;

og

(ii) at de vegetative *Bacillus subtilis*-celler fremstiller et enzym eller en aminosyre valgt fra gruppen bestående af phenylalanin, valin, threonin, tryptophan, isoleucin, methionin, leucin, lysin, cystein, tyrosin, histidin og arginin i en mængde, som er større end for reference-*Bacillus subtilis*-cellen DSM 19467, hvor både den vegetative celle og reference-*Bacillus subtilis*-cellen DSM 19467 dyrkes under identiske betingelser,

og med det forbehold, at de vegetative *Bacillus subtilis*-celler IKKE er celler som beskrevet i krav 1 i PCT/EP2008/057296 og den tilhørende prioritetsansøgning, EP07111939.0, publiceret som EP2011858A1, hvor de vegetative *Bacillus*-celler fra

punkt (ii) ifølge krav 1 i PCT/EP2008/057296 og EP2011858 A1 fremstiller phytase i en mængde, som er mindst 1,25 gange større end for reference-*Bacillus*-cellen DSM 19467, hvor den fremstillede phytasemængde måles ved hjælp af phytaseassayet fra eksempel 2 i PCT/EP2008/057296 og EP2011858A1 efter 4 timers vækst ved 37°C i det ikke-selektive HIB-medium (Heart Infusion Broth medium) fra eksempel 2 i PCT/EP2008/057296 og EP2011858A1; og
hvor phytaseassay-analysen udføres ved følgende trin:

(a): frembringelse af en overnatskultur af vegetative *Bacillus*-celler i et beriget dyrkningsmedium; og
(b): overførsel af et 1% inokulum fra overnatskulturen til HIB-medium (dvs. dette er tiden nul) og inkubering ved 37°C indtil måling af phytaseaktivitet.

2. *Bacillus subtilis*-sammensætning ifølge krav 1, hvor *Bacillus subtilis*-sporecellerne i sammensætningen er til stede som tørrede (f.eks. spraytørrede) sporeceller.

3. *Bacillus subtilis*-sammensætning ifølge krav 1 eller 2, hvor *Bacillus subtilis*-sporerne når det vegetative cellevækstpunkt mindst 3 timer tidligere end reference-*Bacillus subtilis*-sporecellerne, som er deponeret som DSM 17231 ("GalliPro®") under betingelserne fra punkt (i) ifølge krav 1.

4. *Bacillus subtilis*-sammensætning ifølge et hvilket som helst af kravene 1 til 3, hvor enzymet fra punkt (ii) ifølge krav 1 er mannanase.

5. *Bacillus subtilis*-sammensætning ifølge et hvilket som helst af kravene 1-4, hvor de vegetative *Bacillus subtilis*-celler fra punkt (ii) ifølge krav 1 fremstiller mindst én aminosyre valgt fra gruppen bestående af phenylalanin, valin, threonin, tryptophan, isoleucin, methionin, leucin, lysin, cystein, tyrosin, histidin og arginin i en mængde, som er større end for reference-*Bacillus subtilis*-cellen DSM 19467, hvor den fremstillede aminosyremængde måles ved hjælp af GC-MS-standardmetoden baseret på aminosyreassayet fra eksempel 2 heri efter to dages vækst ved 37°C i det som standard kendte minimalsaltsvækstmedium fra eksempel 2 heri.

6. Fremgangsmåde til fodring af et dyr, hvor fremgangsmåden omfatter indgivelse af *Bacillus subtilis*-sammensætningen ifølge et hvilket som helst af kravene 1 til 5 til et dyr sammen med andre dyrefoderingredienser.

7. Fremgangsmåde til fodring af et dyr ifølge krav 6, hvor dyret er et dyr valgt fra gruppen bestående af fjerkræ, drøvtyggere, kalve, grise, kaniner, heste, fisk og kæledyr.

8. Fremgangsmåde til screening for og isolering af en *Bacillus subtilis*-celle, hvor fremgangsmåden omfatter følgende trin:

(a): selektion og isolering fra en pulje af individuelle *Bacillus subtilis*-sporeceller af en *Bacillus subtilis*-sporecelle, som er i stand til at spire og vokse frem så hurtigt, at den når et vegetativt cellevækstpunkt inden for mindre end 18 og 19 timer under betingelserne fra punkt (i) ifølge krav 1;

(b): frembringelse af en vegetativ *Bacillus subtilis*-celle ud fra den isolerede sporecelle fra trin (a) og mutation af den selekterede og isolerede celle for at opnå en pulje af individuelle vegetative *Bacillus subtilis*-celler;

(c): selektion og isolering fra puljen af individuelle vegetative *Bacillus subtilis*-celler fra trin (b) af en vegetativ *Bacillus subtilis*-celle, som er i stand til at fremstille et enzym, der ikke er phytase eller en aminosyre valgt fra gruppen bestående af phenylalanin, valin, threonin, tryptophan, isoleucin, methionin, leucin, lysin, cystein, tyrosin, histidin og arginin af interesse; og

(d): analyse af den højproduktive vegetative *Bacillus subtilis*-celle fra trin (c) for at bekræfte, at den har bevaret den hurtige spiring og fremvækst fra trin (a), og isolering af den selekterede *Bacillus subtilis*-celle.

DRAWINGS

Figure 1: *B.subtilis* strains

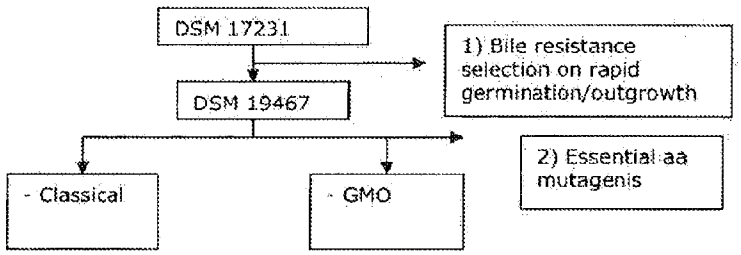

Figure 2A and 2B

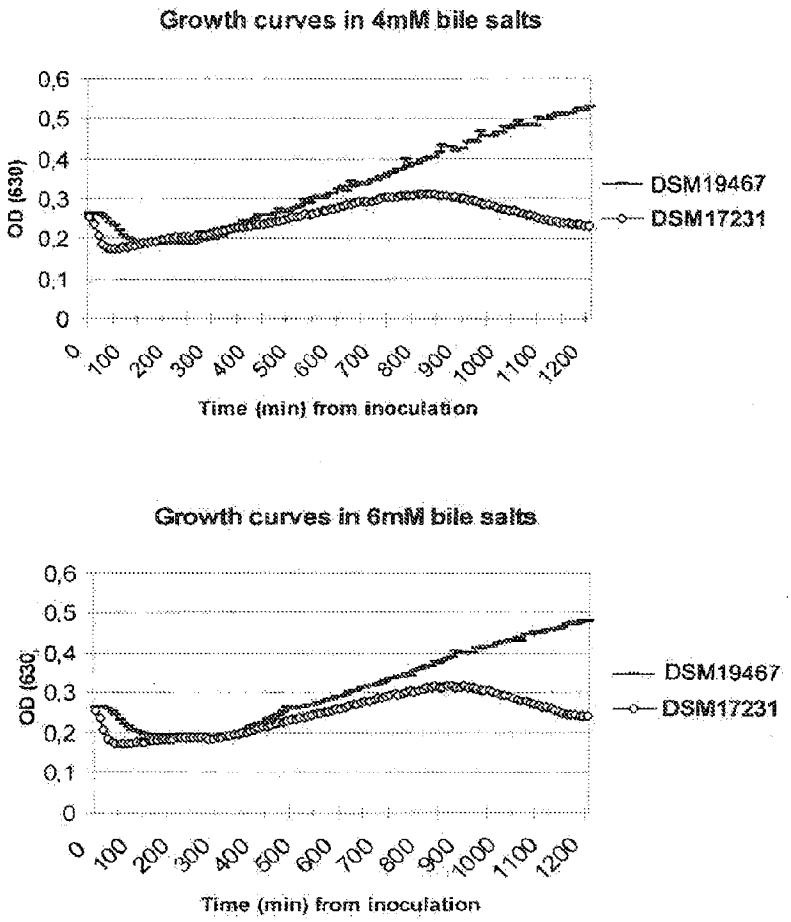

Figure 2A (4mM) and 2B (6mM). Time (min) from 10^6 spores/ml until OD 0.4 $_{630}$ is reached