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(54) Title: METHOD FOR PRODUCING INOSITOL AND INORGANIC PHOSPHATE

(57) Abstract: The present invention relates to a method for producing inositol and inorganic phosphate, comprising the steps of (a) providing a composition comprising phytate, phytic acid, and/or phytin; (b) hydrolysing the phytate, phytic acid, and/or phytin to provide a composition comprising inositol phosphate and inorganic phosphate; (c) separating inorganic phosphate from the composition provided in step (b) to provide a composition enriched in inositol phosphate and a composition comprising inorganic phosphate; (d) hydrolysing the inositol phosphate obtained in step (c) in order to provide a composition comprising inositol and inorganic phosphate.



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## Method for producing inositol and inorganic phosphate

### 5 Technical field

The present invention relates to a method for the simultaneous production of inositol and inorganic phosphate, particularly by hydrolysing phytate in co-products of bioethanol production, including wheat yeast concentrate, corn concentrate, and sorghum concentrate. These co-products are typically employed as animal feed, which makes the present method  
10 well-suited to reduce phytate levels in animal feed and thereby avoid phosphate pollution.

### Background of the invention

Ethanol can be produced by processing starch containing biomass such as wheat, corn, and sorghum. The ethanol production process results in several important co-products, including  
15 for example wheat yeast concentrate, corn concentrate and sorghum concentrate, and dried distillers grain and solubles (DDGS).

These concentrates contain valuable nutrients, and are therefore commonly used as animal feed for dairy and beef cattle, poultry, swine, and as pet food. However, they also contain  
20 high levels of phosphate, mainly in phytate form. Phytates are found in most cereal seeds and is the main storage form of plant P.

However, phosphate (P) in phytate form cannot be digested by nonruminant animals such as poultry and swine, which results in significant amounts of phytate P in their excreta.  
25 Phytates can strongly bind to divalent minerals and proteins, preventing their assimilation by the digestive system. In view thereof, phytates are also known as antinutrients. In addition, the phytate P-rich manure can cause P pollution in soil and surface water.

On top of that, in order to guarantee skeletal integrity and growth of the swine and poultry,  
30 inorganic P supplements are also added to the feed, resulting in a further increased P content in the animal excreta and worsening of potential P pollution.

In view of the above-mentioned nutritional and environmental problems, Nouredдини (2010, Biosource Technology 101; 9106-9113) has proposed a process for the degradation of  
35 phytates in the corn wet milling process, wherein phytates are hydrolysed to produce inorganic phosphate and myo-inositol.

However, the process of Nouredдини is costly, complex and requires multiple steps. Moreover, the resulting concentration in both inorganic phosphate and myo-inositol is rather low.

5 It is an objective of the present disclosure to overcome one or more of the above-mentioned problems, and to provide a method for the production of inositol and inorganic phosphate which is more efficient and effective, and which at the same time can produce animal feed with low levels of phytate P in order to reduce environmental problems.

## 10 **Summary of the invention**

The present invention provides a method for simultaneously producing inositol and inorganic phosphate, wherein the method preferably is an integrated part of an ethanol production process.

15 As starting material, the method uses a co-product of the ethanol production process, i.e. a stream containing phytate, phytic acid, and/or phytin. For example, this can be wheat yeast concentrate, corn concentrate or sorghum concentrate, depending on the raw material used in the ethanol production. The method then uses a hydrolysis step to convert the phytate, phytic acid, and/or phytin to inositol and inorganic phosphate.

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The present inventors found that the method is drastically improved by employing a separation step, preferably by means of a nanofiltration element, which separates inorganic phosphate from the reaction mixture during the hydrolysis step, and recirculates the partly hydrolysed phytate (i.e. a mixture of inositol phosphates, e.g. IP5, IP4, IP3, IP2, and/or IP1)

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back to the reaction mixture.

In this way, inhibition of the hydrolysis reaction by inorganic phosphate is prevented, particularly in the case of enzymatic hydrolysis, while at the same time a valuable product is isolated, namely inorganic phosphate.

30

The present method produces valuable products in high concentrations, namely inositol and inorganic phosphate, and at the same time greatly contributes to the prevention of P pollution by lowering phytate P content in animal feed.

35

As further important improvements, the present inventors found that an enzymatic hydrolysis step can be improved by using a synergetic combination of phytase(s) and/or acidic phosphatase(s), which can optionally be immobilized. For this, the present method does not

require to perform a partial hydrolysis and subsequently a complete hydrolysis under different reaction conditions. Instead, the present method allows to perform the complete hydrolysis in a single step. Additionally, it was found that inositol can be isolated very efficiently by means of anti-solvent crystallization also in combination with chromatography.

5

For example, the present method can be performed in a continuous stirred tank reactor (CSTR) containing immobilized phytase and immobilized acid phosphatase, or in a continuous flow reactor set-up (CFR) comprising two column reactors with (1) immobilized phytase and (2) immobilized acid phosphatase linked in series. The effluent of the CSTR or

10 CFR system, respectively, can be fed to a nanofiltration element (see Example 5) to separate the released free inorganic phosphate from the inositol and inositol phosphates. The inorganic phosphate-free solution enriched in inositol and inositol phosphates is recycled to the enzyme reactors, to complete the hydrolysis. At the end, inositol can be isolated from the enriched solution by anti-solvent crystallization.

15

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the

20 element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

### Detailed description of the invention

The present invention relates to a method for producing inositol and inorganic phosphate, comprising the steps of:

25

- (a) providing a composition comprising phytate, phytic acid, and/or phytin,
- (b) hydrolysing the phytate, phytic acid, and/or phytin to provide a composition comprising inositol phosphate (and/or inositol) and inorganic phosphate;
- (c) preferably separating inorganic phosphate from the composition provided in step (b) to
- 30 provide a composition enriched in inositol phosphate, i.e. a mixture of IP5, IP4, IP3, IP2, and/or IP1, (and/or inositol) and a composition comprising inorganic phosphate;
- (d) hydrolysing the inositol phosphate obtained in step (c) to provide a composition comprising inositol and inorganic phosphate.

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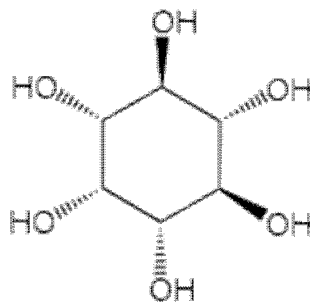
Steps (b), (c), and/or (d) are not necessarily performed in this order. For example, it is particularly preferred to perform steps (b) and (d) simultaneously and/or in the same reactor, although it is also possible to perform said steps in separate reactors. The step of separating

the inorganic phosphate can thus be performed during at least part of the process of converting the phytate, phytic acid and/or phytin to IP5, IP4, IP3, IP2, IP1 respectively, and eventually to inositol, e.g. during at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 90, or 100% of the time applied for the hydrolysis reaction.

5

Inositol (or cyclohexane-1,2,3,4,5,6-hexol) is a compound with formula  $C_6H_{12}O_6$  or  $(-CHOH-)_6$ , a derivative of cyclohexane with six hydroxyl groups:

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15

Inositol exists in nine possible stereoisomers, of which cis-1,2,3,5-trans-4,6-cyclohexanehexol, or myo-inositol (also referred to as meso-inositol or i-inositol), is the most widely occurring form in nature. Inositol is a sugar alcohol with about half the sweetness of sucrose.

20

Myo-inositol is the structural basis for a number of secondary messengers in eukaryotic cells, i.e. various inositol phosphates. In addition, inositol serves as an important component of the structural lipids phosphatidylinositol (PI) and its various phosphates, the phosphatidylinositol phosphate (PIP) lipids.

25

Inositol can be used for diverse applications, including

- as a nutritional supplement, e.g. in (baby) food or animal feed including fish feed;
- for the prevention or treatment of medical conditions, such as treatment of depression: inositol has been proposed as selective serotonin reuptake inhibitor (SSRI);
- for increasing bone strength: inositol is thought to be essential for bone formation, osteogenesis and bone mineral density;
- for increasing insulin sensitivity which may help to improve ovarian function and reduce hyperandrogenism;
- for restoring normal ovulatory activity, oocyte and egg quality, fertilization rate, and/or sperm motility;

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- for promoting cell survival and growth; and/or
- for supporting development and function of peripheral nerves.

The method according to the present disclosure can produce inositol and inorganic phosphate in a single reaction mixture. In particular, step (b) and step (d) can be performed in a single reactor or single set of reactors, preferably by recycling the composition enriched in inositol phosphate as provided in step (c) to said reactor or set of reactors.

Accordingly, step (b) and (d) can be performed as a single step, which is an embodiment of the above-described method and which comprises the steps of:

- (a') providing a composition comprising phytate, phytic acid, and/or phytin,
- (b') hydrolysing the phytate, phytic acid, and/or phytin to provide a composition comprising inositol phosphate, i.e. a mixture of IP5, IP4, IP3, IP2, and/or IP1, and inorganic phosphate, and hydrolysing the inositol phosphate thus obtained to provide a composition comprising inositol and inorganic phosphate, wherein inorganic phosphate is removed from the reaction mixture, preferably continuously and/or during at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 90, or 100% of the time applied for the hydrolysis reaction.

In step (a) (or a'), the method starts with providing a composition comprising phytate, phytic acid, and/or phytin. The composition provided in step (a) or (a') preferably comprises at most 20, 18, 16, 14, 12, 10, 8, 6, 4, 2, 1, 0.5 wt.% other constituents next to water and the phytate, phytic acid, and/or phytin, with respect to the total weight of the composition.

Phytic acid, also known as inositol hexakisphosphate (IP6), inositol polyphosphate, (1R,2S,3r,4R,5S,6s)-cyclohexane-1,2,3,4,5,6-hexa-yl hexakis[dihydrogen (phosphate)], or phytate when in salt form, can be characterized as a saturated cyclic acid, and is the principal storage form of phosphate in many plant tissues, especially bran and seeds. It is typically found in cereals and grains. Phytin ( $C_6H_6Ca_6Mg_6O_{24}P_6$ ) is the calcium magnesium salt of phytic acid as it occurs naturally in plants.

The composition comprising phytate, phytic acid, and/or phytin according to the present disclosure may be derived from wheat, corn, and/or sorghum. For example, the composition refers to wheat yeast concentrate, corn concentrate (or corn steep water), and/or sorghum concentrate, which can be processed to Distillers Dried Grains with Solubles (DDGS) for use as animal feed. Accordingly, the present method allows to produce animal feed with reduced levels of organic P, i.e. comprising at most 5000, 4000, 3000, 2500, 2000, 1500, 1000, 800, 600, 400, 200, 100, or 50 mg total phosphate per kg of the animal feed.

Total phosphate can for example be determined by a photometric dry-ashing procedure based on the standard method as described by Pulliainen and Wallin (1994 J. AOAC Int. 77, 1557-1561). Briefly, measurements can be based on a colorimetric method wherein the colour of the treated sample reflects the concentration of P, and wherein the samples are first ashed to remove organic (C, H, O) materials. Hydrochloric acid is added to the remaining inorganic ash residue to dissolve P, and the resulting solution is used for the colour reaction based on the formation of a blue complex between phosphate and sodium molybdate, in the presence of ascorbic acid as the reducing agent. The blue colour of the complex is directly proportional to the total P.

Wheat yeast concentrate is a co-product from bioethanol production. wherein starch from grain is converted into ethanol by means of fermentation. After the ethanol has been separated, a brown-coloured protein-rich substance remains, i.e. wheat yeast concentrate. The biggest part of the wheat yeast concentrate thus obtained is commonly used as animal feed. From 800 kiloton of wheat comes 250 kiloton of wheat malt concentrate. Similarly, corn concentrate or sorghum concentrate can be obtained.

In a particularly preferred embodiment of the present disclosure, step (a) of the method further involves purifying and/or concentrating the provided composition and/or removing constituents other than phytate, phytic acid and/or phytin from the provided composition, preferably by

- solid/liquid separation to remove insoluble material, for example by means of a centrifugation step, preferably at 2000-8000 rpm; and/or
- precipitating the phytate, phytic acid and/or phytin, for example by hydroxide precipitation, i.e. through adding calcium hydroxide (or magnesium hydroxide) to the provided composition, subsequently dissolving the obtained precipitate, and optionally disposing the supernatant. For example, this can be achieved by first adjusting the pH to a range of pH 2-9, or 3-8, 3-7, or pH 4-5. Then, the composition can be mixed or agitated, and subsequently centrifuged, preferably at 2000-8000 rpm for a period of e.g. 1-30 min, or 1-10 min. Then, the supernatant can be removed, and the precipitate can be dissolved by adding e.g. HCl.

Hydroxide precipitation as described above has considerable advantages, particularly when compared to chromatographic purification, since it allows to start the method of the present disclosure with a composition having a higher concentration and purity of phytate, phytic acid and/or phytin, and therefore the resulting products, i.e. inositol and inorganic phosphate will be obtained in higher concentrations and purity as well.

In step (b) and/or (d) (or b') of the method according to the present disclosure, a hydrolysis step is performed, preferably in a single (set of) containers, more preferably in a continuous stirred tank reactor, or in a continuous flow reactor which may comprise two column reactors. The container or reactor(s) is preferably set up to allow hydrolysis of phytic acid and/or inositol phosphate to inositol. Accordingly, the reaction mixture is preferably under conditions allowing such hydrolysis.

Hydrolysis of the phytate, phytic acid, and/or phytin according to the present disclosure refers to the hydrolytic cleavage of at least one, two, three, four, five, or six phosphate group(s) of the phytate, phytic acid, and/or phytin in order to obtain the different inositol phosphates being (myo-)inositol pentakis-, tetrakis-, tris-, bis- and monophosphate, i.e. IP5, IP4, IP3, IP2, and IP1 respectively, and eventually inositol. Additionally, inorganic phosphate is obtained, which refers to the anion  $\text{PO}_4^{3-}$ , and which can form a salt together with for example hydrogen or calcium, magnesium, sodium, kalium, or ammonium.

During hydrolysis step (b) and/or (d) (or b'), the concentration of inositol and inorganic phosphate in the reaction mixture will increase, while the concentration of phytic acid and/or inositol phosphates will decrease. Preferably, the method does not provide for further hydrolysis steps apart from step (b) and/or (d) (or b').

The method according to the present disclosure may comprise additional steps, for example - step (e) (or c') of separating the inorganic phosphate from the composition provided in step (d) (or b') to provide a composition comprising inositol and a composition comprising inorganic phosphate. This step thus lowers the concentration of inorganic phosphate of the starting composition, i.e. the composition provided in step (d) (or b'), such that a composition comprising inositol is provided which contains at most 1000, 800, 600, 400, 200, 100, 50, 40, 30, 20, 10, 5, or 1 mg inorganic phosphate (free phosphate) per kg of the composition. Inorganic phosphate content can be determined as described by Nouredini et al (2009, Bioresour. Technol. 100 731-736).

In step (c) (or b') and/or step (e) of the method according to the present disclosure, a separation step is performed to separate and/or remove inorganic phosphate from the reaction mixture, e.g. by (nano)filtration, ion exchange chromatography, size exclusion chromatography, electrodialysis. A particular advantage thereof is that a composition enriched in inositol is provided, with reduced inorganic phosphate content so as to significantly reduce product inhibition during the hydrolysis reaction. In this regard, enriched means that the proportion of inositol relative to inorganic phosphate is increased relative to

the starting composition. In addition, inorganic phosphate is isolated. The method according to the present disclosure preferably does not involve further separation steps such as by column chromatography.

- 5 The separation step according to the present method lowers the concentration of inorganic phosphate of the starting composition, i.e. the composition provided in step (d), such that a composition comprising inositol and/or inositol phosphate is provided which contains at most 1000, 800, 600, 400, 200, 100, 50, 40, 30, 20, 10, 5, or 1 mg inorganic phosphate (free phosphate) per kg of the composition.

10

In a preferred embodiment, the separation step in step (c) (or b') and/or step (e) is performed by membrane filtration, preferably by a nanofiltration element, more preferably a nanofiltration element with a pore dimension of at most 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 40, 60, 80, 100, or between 1-10, 1-15 nanometer. For example, a DOW Filmtec NF270-400  
15 nanofiltration element at 1-200, or 20-120, or 50-90, 65-75 psi may be used, during e.g. 0.5-600 min, preferably 1-100 min or 20-40 min, at a temperature of 0-100, preferably 1-50, or 5-35 degrees Celsius.

20

The nanofiltration element will produce a retentate comprising phytate, inositol and/or inositol phosphate which can be recycled to the reaction mixture, while the permeate will comprise inorganic phosphate.

25

It will be clear that the composition provided in step (b) and/or the enriched composition provided in step (c) according to the method of the present disclosure may further comprise inositol, phytate, phytic acid, and/or phytin (as well as inorganic phosphate).

30

The method according to the present disclosure may further comprise step (f) (or c' or d') of precipitating the inositol from the composition comprising inositol provided in step (d) or step (e) (or b'), preferably by anti-solvent crystallisation, by selectively removing solvent from the reaction mixture (i.e. water) and/or adding anti-solvent to the reaction mixture, thereby allowing the precipitation of inositol.

35

Preferably, the anti-solvent crystallisation is performed as described in WO2012128624. Briefly, the process for the crystallisation of the water-soluble inositol from the reaction mixture comprises the following steps: 1) providing, preferably in a crystallisation vessel, the water-soluble inositol in a mixture of water and a solvent in which the inositol has a lower solubility than in water; 2) preferably, passing vapour phase of the mixture (preferably at

elevated temperature such as between 30-100, or 40-80 or 50-70 degrees Celsius) through a sorption zone containing a water vapour sorbent to selectively adsorb water from the vapour phase to obtain a vapour phase depleted in water and enriched in the solvent and water-saturated water vapour sorbent; 3) enriching the mixture in the crystallisation vessel in solvent by recycling at least part of the vapour phase depleted in water and enriched in the solvent to the crystallisation vessel or withdrawing vapour phase depleted in water from the process and adding solvent from an external source to the crystallisation vessel; 4) allowing solid crystals of the water-soluble inositol to precipitate from the solution in the crystallisation vessel, preferably at a crystallisation temperature; and 5) preferably discharging precipitated solid crystals of the water-soluble inositol from the crystallisation vessel and discharging a solution of non-crystallised water-soluble inositol in water-solvent mixture from the crystallisation vessel.

It will be clear that the inositol produced according to the present disclosure typically will be myo-inositol.

In a preferred embodiment, the hydrolysis in step (b) (or b') and/or step (d) is acid hydrolysis (or chemical proton hydrolysis). Acid hydrolysis can be seen as a process in which a protic acid is used to catalyse the cleavage of a chemical bond via a nucleophilic substitution reaction, typically with the addition of the elements of water (H<sub>2</sub>O). For this, the pH of the reaction mixture is preferably adjusted to pH 1-8, or 2-6, more preferably 3-5 or 3.5-4.5 and/or the temperature of the reaction mixture is adjusted to 50-200, or 75-175 or 125-175 or 130-170 degrees Celsius, preferably for a period of 0.5-80, or 1-80, 1-50, 10-40, 20-30 hours, and preferably subsequent cooling to a temperature of 10-100, or 15-40 or 15-30 degrees Celsius, at a pH in the preferred range of pH 3-10, or 4-9, or 6-8.

In a particularly preferred embodiment of the present disclosure, the hydrolysis in step (b) (or b') and/or step (d) is enzymatic hydrolysis, which allows for very efficient conversion of the phytate, phytic acid and/or phytin into inositol. A particular advantage of enzymatic hydrolysis is that it does not require heating and cooling steps, nor adjustment of the pH, while high concentrations of inositol can be obtained.

For example, the enzymatic hydrolysis can be performed by using at least one phytase and/or at least one acidic phosphatase, preferably the combination and/or in a single container/reactor. The phytase may also be provided in a container/reactor different from the container/reactor where the acidic phosphatase is provided, preferably wherein the effluent of the phytase-containing reactor is the input for the acidic phosphatase-containing

reactor. The separation step, e.g. the nanofiltration element may be provided in between, or after these reactors.

In particular, hydrolysis of phytate (myo-inositol hexakisphosphate) to myo-inositol can be achieved by enzymatic catalysis using an enzyme preparation rich in phytase, that can catalyze the partial hydrolysis of phytate to myo-inositol 2-monophosphate (IP1), and/or an acidic phosphatase that can hydrolyse the myo-inositol 2-monophosphate further to release myo-inositol and inorganic phosphate.

Notably, phytate hydrolysis by phytases occurs in a step-wise process, with the release of myo-inositol pentakis-, tetrakis-, tris-, bis- and monophosphates, depending on the type and the substrate specificity of the enzymes used.

Plant, fungal and bacterial phytases can be used. Most suited is a phytase with broad substrate specificity, like phytase derived from *Aspergillus fumigatus*, *Emmericella nidulans* and/or *Myceliophthora thermophila*, that can readily hydrolyse phytate and catalyse the intermediate hydrolysis of myo-inositol phosphates and accumulate myo-inositol 2-monophosphate as major product, and/or a phytase with high specificity for phytate, such as a phytase from *Aspergillus niger* and *Escherichia coli*.

For the hydrolysis of myo-inositol 2-monophosphate, an acidic phosphatase from *A. niger* can be used and is particularly preferred.

Particular combinations of a phytase and acid phosphatase can act in synergy to hydrolyse phytate in order to produce myo-inositol and inorganic phosphate, preferably at conditions of pH and temperature that favour the activity of both enzymes, e.g. pH of at least 1, 2, or at least 2.5 and/or below pH 9, 8, 7 or 6 and/or temperature of 20-80 degrees Celsius, or 30-70, or 40-60, 45-55 °C.

The at least one phytase can be a fungal or bacterial phytase, particularly an phytase from *Aspergillus fumigatus*, *Emmericella nidulans*, *Myceliophthora thermophila*, *Aspergillus niger* and/or *Escherichia coli*; and/or the at least one acidic phosphatase can be a fungal or bacterial acidic phosphatase, particularly an acidic phosphatase from *Aspergillus niger*.

Preferably, a combination of enzymes is used which provide synergistic conversion reactions. In this regard, it was found that the following combinations of at least one phytase and at least one acidic phosphatase provide for a synergistic conversion efficiency:

- *Aspergillus niger* phytase and *Aspergillus niger* acid phosphatase;
- 5 - *Aspergillus fumigatus* phytase and *Aspergillus niger* acid phosphatase;
- *Myceliophthora thermophila* phytase and *Aspergillus niger* acid phosphatase; and/or
- *Escherichia coli* phytase and *Aspergillus niger* acid phosphatase.

Said synergistic conversion reactions preferably are performed in one container/reactor at conditions of pH and temperature that favour the activity of both enzymes, e.g. a pH of within 3 pH units, preferably within 2 pH units, more preferably within 1 pH unit, of the pH optimum of both enzymes, and at a temperature of within 10 °C, preferably within 5 °C, more preferably within 2 °C, of the temperature optimum of both enzymes. Most preferred conditions for the combination of:

15        *Aspergillus niger* phytase and *Aspergillus niger* acid phosphatase is between 2.5-6.5 pH and 30-55 °C, preferably about pH 4.5 and 40 °C;

*Aspergillus fumigatus* phytase and *Aspergillus niger* acid phosphatase is between 4-6 pH and 40-55 °C, preferably about pH 5 and 45°C;

*Myceliophthora thermophila* phytase and *Aspergillus niger* acid phosphatase is  
20 between 3-6.5 pH and 30-55°C, preferably about pH 4.5 and 40°C;

*Escherichia coli* phytase and *Aspergillus niger* acid phosphatase is between 2.5-6.5 pH and 30-55 °C, preferably about pH 5.5 and 50°C.

Alternatively and/or additionally, the at least one phytase and/or at least one acidic  
25 phosphatase as used according to the present disclosure can be immobilized e.g. to a solid support, or to solid particles of at least 0.1, 0.5, 1, 2, 4, 6, 8, 10, 15 mm, or at most 15, 20, 25, 30, 50, 60, 70, 80, 100 mm, preferably by adsorption, encapsulation, cross-linking as cross-linked enzyme aggregates, (covalent) binding to a solid support and/or attachment on magnetic active particles.

30 According to the present disclosure, phytate hydrolysis can thus be performed in e.g. a continuous stirred tank reactor (CSTR) containing (immobilized) phytase and (immobilized) acid phosphatase, or in a continuous flow reactor set-up preferably comprising two column reactors with (1) (immobilized) phytase and (2) (immobilized) acid phosphatase, preferably  
35 linked in series (CFR).

The effluent of the CSTR or CFR system, respectively, can be fed to a nanofiltration element (see Example 5) to separate the released free inorganic phosphate from the reaction mixture comprising inositol and inositol phosphates (and phytate). The inorganic phosphate-free solution enriched in inositol and inositol phosphates can be recycled to the enzyme reactor(s), to complete the hydrolysis. At the end, inositol can be separated from the reaction mixture.

### Brief description of the figures

Figure 1 relates to a preferred embodiment of the present disclosure. The figure shows a Continuous Stirred Tank Reactor (CSTR), wherein phytate (1) is introduced and hydrolysis is carried out with immobilized enzymes, using an immobilized phytase (A) and an immobilized acid phosphatase (B). The effluent (2) of the CSTR is fed to a nanofiltration element (C) to separate the released free phosphate (3) from the inositol and inositol phosphates. The phosphate-free solution enriched in inositol and inositol phosphates (4) is recycled to the CSTR, to complete the hydrolysis. At the end, inositol is separated from the enriched solution by anti-solvent crystallisation (not shown).

The following Examples illustrate the different elements and embodiments of the present disclosure.

### Example 1

#### Separation of phytic acid from wheat yeast concentrate

150 gram of wheat yeast concentrate at pH 4.5 was diluted with distilled water to 300 ml and mixed thoroughly and then centrifuged in a Heraeus Megafuge 16 centrifuge at room temperature for 5 minutes at 4000 rpm. The supernatant was decanted. This supernatant of 220 ml contained 3.53 g/L total phosphate of which 0.52 g/L was free phosphate.

### Example 2

#### Precipitation of phytate and phosphate by calcium hydroxide

0.5 g calcium hydroxide was suspended in 20 ml water and slowly added to the supernatant of Example 1 which was stirred during the addition of the calcium hydroxide. After 1 hour at room temperature the treated supernatant was centrifuged during 5 minutes at 4000 rpm. The clear supernatant contained 1.04 g/L total phosphate and 0.14 g/L free phosphate. By calculation it was concluded that 67% of the total phosphate and 71% of the free phosphate was precipitated. It was concluded that the precipitated phosphate was calcium phosphate and the bound phosphate was calcium phytate.

**Example 3**Dissolving the precipitate

The precipitate of Example 2 was taken and a 1 Molar solution of HCl was added until the pH was 4.2 for more than 5 minutes. A clear solution containing phosphoric acid and phytin  
5 resulted.

**Example 4**Acid hydrolysis of phytin.

20 g (7.6 mmoles) of sodium phytate was dissolved in 200 ml of water and the pH was  
10 adjusted to 4.2 with HCl, followed by hydrolysis in a closed vessel with steam pressure at temperatures up to 150 °C, for 2 to 40 h. The time required for the treatment is less for higher pressure and higher temperature. At the end of the reaction, the solution contained inositol and phosphate salts. The mixture was cooled and the pH adjusted with lime calcium hydroxide to pH 6 – 8. The solids, consisting mainly of calcium phosphate, were separated  
15 by filtration, while inositol remained in the liquid phase. Inositol was isolated from solution by crystallisation / antisolvent crystallisation.

**Example 5**Separation of phytic acid, inositol and free phosphate

20 A solution of 35 g/L of phytic acid and 35 g/L of free phosphates at pH 4.2 from Example 3 containing also calcium and chlorine ions and inositol, was fed to a DOW Filmtec NF270-400 nanofiltration element at 70 psi during 30 minutes at room temperature. Less than 1% of non-free phosphate was analysed in the filtrate, 9% of the inositol was analysed in the filtrate, while 68% of free phosphate passed the membrane into the filtrate.

**Example 6**Anti-solvent crystallisation of Myo-inositol

75 gram of myo-inositol and 25 gram of calcium phosphate were dissolved at pH 4.5 in 1 litre water, in a water jacketed glass crystallisation vessel. The solution was heated to 60 °C  
30 and 250 ml ethanol was added. The mixture was stirred at 60 °C during 24 hours in the crystallisation vessel. During this time the vapour phase thus formed was passed through a sorption column containing Zeochem Z3-03 molecular sieve. The water from the vapour was absorbed by the molecular sieve and the vapour phase that has passed the column was continuously recycled to the crystallisation vessel by bubbling it in the liquid in the  
35 crystallisation tank. After 24 hours the volume has decreased from about 1250 ml to 550 ml, and crystals of myo- inositol could be collected by sieving with a yield of 52 gram (yield

69.3%) after washing the precipitate with 96% ethanol and drying the crystals at room temperature. After analysis, the crystals were identified as myo-inositol while less than 1% (w/w) of phosphate could be detected.

## 5 Example 7

### Enzymatic hydrolysis of phytate.

Hydrolysis of phytate (myo-inositol hexakisphosphate) to myo-inositol can be achieved by enzymatic catalysis using an enzyme preparation rich in phytase, that catalyses the partial hydrolysis of phytate to myo-inositol 2-monophosphate (IP1), and an acidic phosphatase that hydrolyses further the myo-inositol 2-monophosphate to release myo-inositol and phosphate. Phytate hydrolysis by phytases occurs in a step-wise process, with the release of myo-inositol pentakis-, tetrakis-, tris-, bis- and monophosphates, depending on the type and the substrate specificity of the enzymes used. Plant, fungal and bacterial phytases can be used. Most suited are phytases with broad substrate specificity, like phytases from *Aspergillus fumigatus*, *Emmericella nidulans* and *Myceliophthora thermophila*, that readily hydrolyse phytate and the intermediate hydrolysis myo-inositol phosphates and accumulate myo-inositol 2-monophosphate as major product, and phytases with high specificity for phytate, such as phytases from *Aspergillus niger* and *Escherichia coli*. For the hydrolysis of myo-inositol 2-monophosphate, an acidic phosphatase from *A. niger* can be used.

Any combination of a phytase and acid phosphatase can act in synergy, in one reaction vessel, to hydrolyse phytate to produce myo-inositol and phosphate, at conditions of pH and temperature that favour the activity of both enzymes, (e.g. pH higher than 2.5 and below pH 6 and temperature around 40 and 50 °C). Suitable enzymes mixtures acting in synergy for the total hydrolysis of phytate to myo-inositol are: (1) *A. niger* phytase and *A. niger* acid phosphatase, (2) *A. fumigatus* phytase and *A. niger* acid phosphatase, (3) *M. thermophila* phytase and *A. niger* acid phosphatase, (4) *E. coli* phytase and *A. niger* acid phosphatase. Hydrolysis of phytate can be carried in solution, with soluble enzymes, in a membrane reactor to separate continuously the phosphate released from the reaction mixture containing the inositol phosphates and free inositol, in order to prevent enzyme inhibition by phosphate. At the end of the reaction the inositol rich solution is separated and inositol can be isolated from the solution by antisolvent crystallisation as described in example 6, while the phosphate is precipitated from the phosphate-rich fraction using known procedures.

Alternatively, phytate hydrolysis can be carried out with immobilized enzymes, using an immobilized phytase and an immobilized acid phosphatase, as described above. The enzymes can be immobilized by adsorption, by encapsulation, by cross-linking as cross-

linked enzyme aggregates, by covalent binding to an inert solid support or by attachment on magnetic active particles. Phytate hydrolysis can be performed in a continuous stirred tank reactor (CSTR) containing the immobilized phytase and acid phosphatase, or in a continuous flow reactor set-up consisting of two column reactors with (1) immobilized phytase and (2) immobilized acid phosphatase linked in series (CFR). The effluent of the CSTR and CFR systems, respectively, is fed to a nanofiltration element (see Example 5) to separate the released free phosphate from the inositol and inositol phosphates. The phosphate-free solution enriched in inositol and inositol phosphates is recycled to the enzyme reactors, to complete the hydrolysis. At the end, inositol is separated from the enriched solution by anti-solvent crystallisation.

The Table below shows the estimated end concentrations of inorganic phosphate and myo-inositol in the reaction mixture as obtained by the method according to the present disclosure.

Table 1: Estimated concentration in  $P_i$  and myo-inositol as obtained by the method according to the present disclosure upon processing wheat yeast concentrate.

|                                                                                           | <i>Without recirculation through<br/>nanofiltration element</i> | <i>With recirculation through<br/>nanofiltration element</i> |
|-------------------------------------------------------------------------------------------|-----------------------------------------------------------------|--------------------------------------------------------------|
| <i>Acid hydrolysis</i>                                                                    | 10% (w/w)                                                       | -                                                            |
| <i>Enzymatic hydrolysis with A.<br/>niger phytase and A. niger<br/>acidic phosphatase</i> | 1% mg/g                                                         | 5%mg/g                                                       |

## CLAIMS

1. Method for producing inositol and inorganic phosphate, comprising the steps of:  
(a) providing a composition comprising phytate, phytic acid, and/or phytin,  
5 (b) hydrolysing the phytate, phytic acid, and/or phytin to provide a composition comprising inositol phosphate and inorganic phosphate;  
(c) separating inorganic phosphate from the composition provided in step (b) to provide a composition enriched in inositol phosphate and a composition comprising inorganic phosphate;  
10 (d) hydrolysing the inositol phosphate obtained in step (c) to provide a composition comprising inositol and inorganic phosphate.
2. Method according to claim 1, wherein the composition comprising phytate, phytic acid, and/or phytin is derived from wheat, corn, sorghum, soy, rapeseed and/or sunflower.  
15
3. Method according to any one of the previous claims, wherein step (a) further involves purifying and/or concentrating the provided composition and/or removing constituents other than phytate, phytic acid and/or phytin from the provided composition, preferably by precipitating the phytate, phytic acid and/or phytin.  
20
4. Method according to claim 3, wherein the precipitating is performed by hydroxide precipitation, preferably through adding calcium hydroxide and/or magnesium hydroxide to the provided composition, and dissolving the obtained precipitate.
- 25 5. Method according to any one of the previous claims, wherein step (b) and step (d) are performed in the same reactor or set of reactors, preferably by recycling the composition enriched in inositol phosphate as provided in step (c) to said reactor or set of reactors.
6. Method according to any one of the previous claims, wherein the inositol phosphate  
30 in step (b), (c), and/or (d) is a mixture of IP5, IP4, IP3, IP2, and/or IP1.
7. Method according to any one of the previous claims, wherein the method further comprises:  
- step (e) of separating the inorganic phosphate from the composition provided in step (d) to  
35 provide a composition comprising inositol and a composition comprising inorganic phosphate; and/or

- step (f) of precipitating the inositol from the composition comprising inositol provided in step (d) or step (e), preferably by anti-solvent crystallisation.

8. Method according to any one of the previous claims, wherein the separation step in  
5 step (c) and/or step (e) is performed by nanofiltration, preferably by a nanofiltration element.

9. Method according to any one of the previous claims, wherein the hydrolysis in step (b) and/or step (d) is acid hydrolysis.

10. Method according to any one of claims 1-8, wherein the hydrolysis in step (b) and/or  
10 step (d) is enzymatic hydrolysis.

11. Method according to claim 10, wherein the enzymatic hydrolysis is performed by  
using at least one phytase and/or at least one acidic phosphatase.

15

12. Method according to claim 11, wherein the at least one phytase is from *Aspergillus fumigatus*, *Emericella nidulans*, *Myceliophthora thermophila*, *Aspergillus niger* and/or *Escherichia coli* ; and/or wherein the at least one acidic phosphatase is from *Aspergillus niger*.

20

13. Method according to claim 11, wherein a combination of at least one phytase and at least one acidic phosphatase is used which is chosen from:

- *Aspergillus niger* phytase and *Aspergillus niger* acid phosphatase;

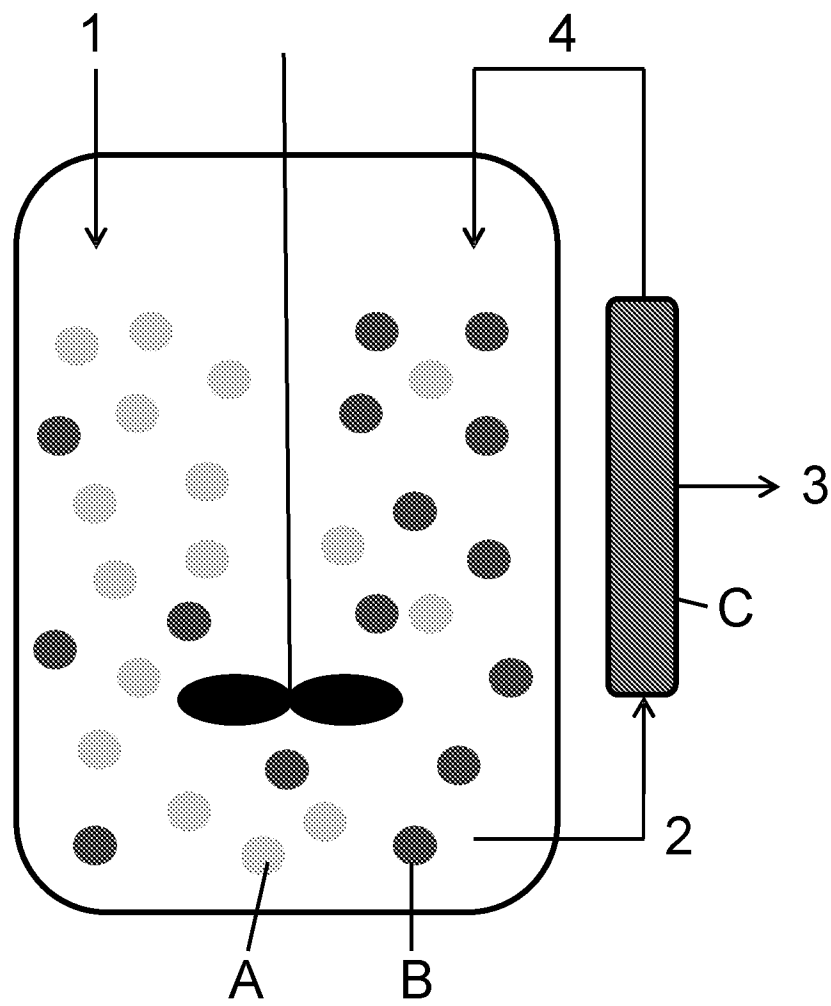
- *Aspergillus fumigatus* phytase and *Aspergillus niger* acid phosphatase;

25 - *Myceliophthora thermophila* phytase and *Aspergillus niger* acid phosphatase; and/or

- *Escherichia coli* phytase and *Aspergillus niger* acid phosphatase.

14. Method according to any one of claims 11-13, wherein the at least one phytase and/or at least one acidic phosphatase are immobilized, preferably by adsorption,  
30 encapsulation, cross-linking as cross-linked enzyme aggregates, covalent binding to a solid support or attachment on magnetic active particles.

Figure 1



## INTERNATIONAL SEARCH REPORT

International application No  
PCT/NL2019/050284

## A. CLASSIFICATION OF SUBJECT MATTER

INV. C01B25/32 C01B25/45 C12P19/46 C12P7/02  
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C01B C12P

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

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

EPO-Internal, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of Box C.



See patent family annex.

## \* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search

17 September 2019

Date of mailing of the international search report

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Name and mailing address of the ISA/

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## INTERNATIONAL SEARCH REPORT

International application No

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