Notice

DESCRIPTION CN119351487A

A process for producing urolithiasis A

[0001]

Technical Field

[n0001]

This invention relates to the field of extraction and utilization technology of chemical substances in walnut green husks, and in particular to a process for producing urolithin A.

[0003]

Background Technology

[n0002]

Ellagic acid, a polyphenolic dilactone with the molecular formula C₁₄H₁₆O₁₈, is a dimer derivative of gallic acid and is widely found in various soft fruits, nuts and other plant tissues.

Ellagic acid has antioxidant and anti-aging properties, anti-inflammatory and anti-cancer effects, as well as effects such as lowering blood pressure, lowering blood sugar, and

sedation. It is a high-value-added health and beauty product and is mainly used as an additive in pharmaceuticals, health foods, and cosmetics.

Urolithiasis A, which is produced by the transformation of ellagic acid by intestinal microorganisms, is a star product for anti-aging.

Urolithin A, with the molecular formula C₁₃H₈O₄, can improve cardiac function in ischemic/reperfused mice by increasing the antioxidant activity of cardiomyocytes. It can also induce selective autophagy in mitochondria by activating the PINKI/Parkin ubiquitin-dependent pathway or BNIP3 receptor, thereby reducing the damage to mitochondrial function caused by aging. Moreover, it also plays an important role in improving human health.

[n0003]

Ellagic acid is an important source of extracts from pomegranate peel, gallnut, and other plants.

In recent years, some researchers have discovered that walnut husks also contain high levels of ellagic acid. As a result, a small number of researchers have explored its extraction from walnut husks. However, the process is relatively immature and has not yet been scaled up or industrialized. Due to the unique structure of pomegranates, the processing and extraction of pomegranate peels in existing technologies often comes at the cost of sacrificing the pomegranate seeds, and the process is relatively complex, resulting in high processing costs. In contrast, walnut husks are entirely agricultural waste, and their processing will not have any adverse effects on the more valuable walnut fruit, thus resulting in lower processing costs. Generally speaking, one pound of green-skinned walnuts contains about 4-5 ounces of green skin, and the ellagic acid content in the dry matter of the green skin is about 1%. One pound of pomegranates contains about 2 ounces of pomegranate peel, and the ellagic acid content in the dry matter of the pomegranate peel is about 1%-2%. my country's walnut production far exceeds that of pomegranates. Therefore, under the background of environmental protection and sustainable development, the processing and utilization of

walnut green husks has a broader prospect and potential. Lin'an, Hangzhou, is an important production area for hickory nuts. Its suitable climate and soil environment provide unique natural conditions for the growth of hickory nuts. The green-skinned hickory nuts produced there are not only plump kernels with high nutritional value, but also have bright green skin with moderate thickness and rich active ingredients, making them highly valuable for deep processing.

[n0004]

Currently, urolithiasis A used in food and health products is obtained through microbial transformation, which is safe and environmentally friendly, but suffers from a low conversion rate.

Therefore, existing technologies focus on screening strains with high transformation rates in order to solve the aforementioned technical problems. For example, Chinese invention patent

application No. 202211134634.7 and publication No. CN115725451A (defined as prior art 1) disclose Enterococcus faecalis FUA027 and its method and application for producing urolithin A. This strain is isolated from human intestinal tract. Its seed liquid is inoculated at a 2% inoculation amount into a fermentation medium supplemented with 1% ellagic acid for anaerobic fermentation culture. Urolithin A can be detected after 40 hours and reaches a maximum concentration of 10.8 μM after 50 hours. For example, Chinese invention patent application No. 202211409720.4 and publication No. CN 115992074A disclose a strain of Lactobacillus plantarum and its application in the production of urolithin A. This strain is derived from the feces of healthy people and is named Lactobacillus plantarum CCFM1290. When inoculated at a 2% inoculum into a culture medium containing 1.5 g/L ellagitannin, fermentation for 48 h can yield various urolithin substances, among which the content of urolithin A is 24.70±0.82 μM and the conversion rate is 8.59±0.62%. For example, Chinese invention patent application No. 202211404309.8 and publication No. CN115786190A disclose a strain of *Lactobacillus plantarum* that can produce urolithin A for anti-aging and its application. This strain is derived from the feces of healthy human beings and is listed in the catalog of edible fungi. When inoculated at a rate of 2% into a fermentation culture medium containing commercially available NFC pomegranate juice, fermentation for 48 hours can yield urolithin A with a yield of 32.01±0.97μM and a conversion rate of 10.73±0.38%.

[n0005]

However, while the aforementioned existing technologies can improve the conversion rate by relying solely on bacterial strains, the improvement is limited and cannot fundamentally solve the problem of low conversion rate.

The low conversion rate of urolithin A is mainly due to two factors: First, ellagic acid has poor hydrophilicity and is unstable in aqueous solution, resulting in a low concentration of effective substances in the initial substrate; second, the increasing concentration of urolithin A after its formation will have a product inhibition effect, which will affect the activity of microorganisms, reduce the conversion efficiency, and thus affect the formation of urolithin A. In addition, the aforementioned existing technologies all use ellagitannins, ellagic acid, or commercially available products as raw materials, which are expensive and make it difficult to industrialize even if they have a high conversion rate. Therefore, this application is submitted.

[0008]

Summary of the Invention

[n0006]

In view of the above-mentioned shortcomings of the prior art, the present invention provides a process for producing urolithiasis A.

[n0007]

A process for producing urolithiasis A includes the following steps:

[0011]

S1. Obtaining Walnut Green Husks: Select healthy, pest-free walnuts with intact green husks. After washing, pile them in a well-ventilated, shady place to allow the green husks to air dry naturally. Once the green husks soften and the moisture content is below 8%, peel them to obtain the walnut green husks. The treatment in step S1 helps to maximize the retention of ellagic acid in the walnut green husks and promote its stable existence without oxidation. The required moisture content is to concentrate the concentration of effective substances, ensuring the accuracy and reliability of subsequent results, and to prevent the walnut green husks from rehydrating and rotting, thus preserving quality. In addition, walnuts should not be harvested too late, as the green husks will have naturally cracked and partially oxidized,

leading to a decrease in the content of effective substances. Green husk walnuts should ideally be collected during the peak walnut harvest season. This ensures the freshness of the walnut green husks, guarantees the accuracy of experimental data, and allows for the utilization of the walnut green husks while reducing environmental pollution.

[0012]

S2. Preparation of fermentation substrate: The green walnut peel is crushed into a paste and added to a stabilizing solution. After stirring evenly, the resulting mixture is subjected to ultrasonic treatment. The cavitation, mechanical and thermal effects of ultrasound are used to apply pressure to the cell wall, causing the cell wall to rupture and increasing the frequency and speed of polyphenol molecules, so as to accelerate the efficient release of polyphenols from the green walnut peel. The stabilizing solution is an aqueous solution of a compound of mercapto-β-cyclodextrin and chitosan. The effective components of the stabilizing solution, mercapto-β-cyclodextrin and chitosan, have a significant adsorption effect on polyphenols.

By continuously adsorbing the released polyphenols, the remaining polyphenols in the green walnut peel are continuously released.

[0013]

S3. First Fermentation: Microbial culture A is inoculated into the fermentation substrate for anaerobic fermentation. After fermentation, solid-liquid separation is performed and the filtrate is collected. Microbial culture A is obtained by separately inoculating Bacillus belye and Bacillus coagulans into culture medium A for activation in a shaker and then mixing them. Based on ultrasonic treatment, combined with the first fermentation, polyphenols are hydrolyzed to generate ellagic acid. The extraction rate of ellagic acid is high and the extraction effect is good. Most of the ellagic acid in the green walnut skin is enriched in the filtrate. β-cyclodextrin has a hydrophilic outer edge and a hydrophobic inner cavity. The hydrophobic cavity structure can form inclusion complexes with ellagic acid, thereby reducing the contact between ellagic acid and the external environment, thus reducing its... While mitigating the risks of oxidation and hydrolysis, this process also improves the water

solubility of ellagic acid to some extent and facilitates better microbial access to ellagic acid molecules, thereby increasing its conversion rate. When β-cyclodextrin introduces thiol groups, these thiol groups can form hydrogen bonds with functional groups such as hydroxyl groups in ellagic acid, further stabilizing the structure of ellagic acid and protecting its biological activity. Under the dual effects of inclusion and hydrogen bonding, ellagic acid exists in large quantities and stably in water. Chitosan is cationic, while thiol-β-cyclodextrin is anionic. When the two are mixed, they can form a complex through electrostatic interactions. This complex helps to further improve the water solubility, stability, and bioavailability of ellagic acid.

[0014]

S4. Second fermentation: Microbial culture solution B is inoculated into the filtrate for anaerobic fermentation again. Microbial culture solution B is obtained by culturing Enterococcus faecalis in culture medium B. When Enterococcus faecalis is added, ellagic acid, which is linked to the complex through inclusion and hydrogen bonding, is converted into

urolithin A. At the same time, the released complex forms hydrogen bonds and inclusion bonds with urolithin A, making the structure of urolithin A stable and increasing its solubility in water. This invention unexpectedly found that the presence of this complex significantly increases the conversion rate of urolithin A. The reason for this is speculated to be that the combination of the complex with urolithin A can stabilize the structure of urolithin A, preventing it from being oxidized or decomposed, and can also reduce the inhibitory effect of product concentration on microbial transformation to a certain extent, i.e., the so-called product inhibition effect, thus avoiding the impact of increased urolithin A concentration on microbial activity. Under the dual effect, the conversion rate of urolithin A is significantly increased.

[0015]

S5. Extraction: After fermentation, urolithin A is extracted.

[n0008]

Compared with existing technologies, this invention, by adding thiol-β-cyclodextrin and chitosan during the preparation of fermentation substrates, increases the concentration of ellagic acid, an effective substance in the fermentation substrates, and increases the concentration of reactants, laying the foundation for the subsequent yield of urolithin A. On the other hand, it can reduce the impact of increased urolithin A concentration on microbial activity and increase the amount of product. The interaction and synergistic effect of thiol-β-cyclodextrin and chitosan significantly improve the conversion rate of urolithin A and the product yield.

This invention is safe, environmentally friendly, easy to operate, and has a simple process, making it highly promising for industrialization.

[n0009]

It should be noted that:

[0018]

(1) The crushing treatment of the green walnut skin is preferably carried out in a low-temperature, light-protected environment. If mechanical equipment is used for crushing, a slow-speed, low-temperature crusher is preferred and the exposure time should be reduced to reduce the loss of polyphenols.

Walnut husks should be crushed into a paste and used immediately. If immediate use is not possible, the husks should be kept dry and stored in a sealed, dark, and cool environment to prevent polyphenols from oxidizing.

[n0010]

(2) Thio-β-cyclodextrin is an existing technology that can be commercially available or prepared in-house.

[n0011]

(3) The stabilizing solution is an aqueous solution of a compound of mercapto-β-cyclodextrin and chitosan. There are hydrogen bonds and electrostatic attraction between mercapto-β-cyclodextrin and chitosan, but no chemical bonds. This is completely different from chitosan derivatives modified with mercapto-β-cyclodextrin.

This application unexpectedly discovered that appropriately controlling the compounding ratio of mercapto-β-cyclodextrin and chitosan, as well as the concentration of the stabilizing solution, can help adsorb the effective substances in the green walnut peel without causing flocculation.

[n0012]

(4) The preferred method for preparing the stabilizing solution is purified water, i.e. deionized water, which is obtained by distillation. Purified water that removes impurities such as

organic matter, ions and microorganisms can effectively avoid the interference of the above substances on the experimental results.

[n0013]

Preferably, in step S2, the chitosan is water-soluble chitosan, and the compound is formed by mixing mercapto-β-cyclodextrin and water-soluble chitosan at a mass ratio of (0.1-0.4):1.

Water-soluble chitosan is generally modified chitosan, such as carboxyl-modified chitosan, which has good water solubility. After dissolving in water, it can form a complex by loading mercapto-β-cyclodextrin through electrostatic interaction, and finally obtain a stable liquid with uniform composition and uniform dispersion.

[n0014]

Preferably, in step S2, the mass percentage of the stabilizing liquid is 5%-20%, for example, it can be 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20%.

[n0015]

Chitosan and mercapto-β-cyclodextrin not only have electrostatic interactions but also hydrogen bonding, and they can form an associated structure through hydrogen bonding.

The greater the amount of chitosan and thio-β-cyclodextrin added, the higher the

concentration of the stabilized solution, the more associated structures there are, and the greater the viscosity of the aqueous solution.

When the viscosity reaches a certain level, the viscous aqueous solution will hinder the transport of substances. During fermentation, the microorganisms' acquisition of nutrients and excretion of waste will be hindered, which is not conducive to the normal growth of microorganisms and the synthesis of metabolic products. Therefore, it will reduce the reaction rate and thus affect the fermentation efficiency.

Moreover, if the concentration of the stabilizing solution is too high, the excessive adsorption of effective substances by chitosan and mercapto-β-cyclodextrin will cause flocculation, making it impossible for the effective substances to exist stably in the filtrate, which will have an adverse effect on the enrichment of effective substances.

Through numerous innovative experiments, this invention has discovered that when the mass percentage of the stabilizing liquid is 5%-20%, the content of chitosan and thio-β-

cyclodextrin is moderate, which can fully exert the stabilizing effect without affecting the normal growth of microorganisms.

[n0016]

Preferably, in step S2, the material-to-liquid ratio is 1:(12-20), the ultrasonic frequency is 12-15kHz, the ultrasonic power is 150-240W, the processing time is 15-35min, during which the temperature of the mixture is maintained at 58-65°C, and after obtaining the fermentation substrate, it is naturally cooled to room temperature before proceeding to the next step.

[n0017]

In order to fully extract ellagic acid from walnut husks and dissolve it in water, the extraction temperature, extraction time, material-to-liquid ratio, and ultrasonic technical parameters all need to be optimized.

This invention has demonstrated through numerous innovative experiments that most of the ellagic acid can be extracted into the aqueous solution in 15-35 minutes at a material-to-liquid ratio of 1:(12-20), an ultrasonic frequency of 12-15kHz, an ultrasonic power of 150-240W, and an extraction temperature of 58-65°C.

[n0018]

In this embodiment, if the material-to-liquid ratio is greater than 1:12, there is too little solvent and the extraction efficiency of ellagic acid is low; if the material-to-liquid ratio is less than 1:20, there is too much solvent. Although the extraction efficiency of ellagic acid is high,

it will make subsequent processing more difficult, increasing processing costs and processing time. When the material-to-liquid ratio is 1:(12-20), such as 1:12, 1:15, 1:18, 1:20, etc., the extraction efficiency of ellagic acid is high and the subsequent processing is relatively simple. Based on this, the material-to-liquid ratio is further optimized to 1:15, at which point the extraction efficiency is the highest.

[n0019]

In this embodiment, the technical parameters of ultrasonic processing are strictly limited to: ultrasonic frequency 12-15kHz and ultrasonic power 150-240W.

If the ultrasonic frequency or ultrasonic power is too low, the cavitation effect, mechanical effect and thermal effect of the ultrasound are insufficient, which cannot effectively destroy the cell wall of the green walnut peel. The effect of accelerating the diffusion and dissolution of ellagic acid from the cell into the solvent is limited. Therefore, the ellagic acid contained in

the fermentation substrate cannot be fully extracted, resulting in a low concentration of effective substances in the fermentation substrate, which is not conducive to improving the yield of urolithin A.

If the ultrasonic frequency or power is too high, the cavitation, mechanical, and thermal effects of the ultrasound will be excessive. Although this has a positive effect on accelerating the diffusion of ellagic acid, it will adversely affect the inclusion effect of the compound on ellagic acid and the hydrogen bonding between the compound and ellagic acid. This will result in limited solubility of ellagic acid in the solvent and poor stability, which is also not conducive to obtaining fermentation substrates with high concentrations of effective substances and will not help improve the yield of urolithin A.

[n0020]

In this embodiment, the ultrasonic time can be controlled between 15 and 35 minutes,

specifically 15, 20, 25, 30, 35 minutes, etc. If the ultrasonic time is too short, the diffusion and dissolution of ellagic acid will be incomplete. If the ultrasonic time is too long, on the one hand, energy will be wasted, and on the other hand, the stability of the dissolved ellagic acid will be affected.

[n0021]

In this embodiment, the extraction temperature is 58-65°C, specifically 58, 60, 62, 63, 64, 65°C, etc. This temperature range can accelerate the release of ellagic acid from the green walnut skin and promote the dissolution of ellagic acid in water, without causing the decomposition of ellagic acid, and without adversely affecting the inclusion or hydrogen bonding between ellagic acid and the compound.

[n0022]

Preferably, in step S3, culture medium A is MRS liquid culture medium modified with corn syrup. Bacillus bellis is cultured and activated with culture medium A at (0.5-0.8) g/100 ml to obtain Bacillus bellis liquid. Bacillus coagulans is cultured and activated with culture medium A at (0.25-0.55) g/100 ml to obtain Bacillus coagulans liquid. After the culture is completed, Bacillus bellis liquid and Bacillus coagulans liquid are mixed at a volume ratio of 1:1 to obtain microbial culture solution A. The inoculum amount of microbial culture solution A in the fermentation substrate is 2v.

[n0023]

Compared to glucose, corn syrup provides microorganisms with a wider range of carbon source options.

Bacillus belyssus and Bacillus coagulans are both Bacillus species with extensive carbon source utilization capabilities. This invention has found that when corn syrup is used to replace glucose in MRS liquid culture medium in equal amounts, the two Bacillus species exhibit better growth and metabolic performance.

[n0024]

In this embodiment, both Bacillus belye and Bacillus coagulans were activated by shaking culture, with specific technical parameters as follows: temperature 36°C, rotation speed 200 rpm, and culture time 36 h.

[n0025]

Preferably, in step S3, the anaerobic fermentation temperature is 30-35°C and the time is 24-48h.

[n0026]

After the first fermentation, not only was ellagic acid enriched, but many biochemical changes produced by microbial fermentation could also modify plant components. At the same time, under the buffering environment provided by the stabilizing liquid, ellagic acid had high bioactivity.

[n0027]

Preferably, in step S4, the *Enterococcus faecalis* was purchased from the China General Microbiological Culture Collection Center (CGMCC) with accession number CGMCC NO. 24964. The specific components of culture medium B are: peptone 16.0 g/L, yeast extract 7.0 g/L, potassium chloride 5.0 g/L, starch 1.0 g/L, glucose 1.0 g/L, sodium pyruvate 1.0 g/L, arginine 1.0 g/L, sodium succinate 0.5 g/L, and cysteine. The microbial culture solution B consists of 0.5 g/L hydrochloride, 0.4 g/L sodium bicarbonate, 0.5 g/L ferric pyrophosphate, 0.005 g/L heme chloride, 0.0005 g/L vitamin K, 0.5 g/L sodium thioglycolate, and 1.0 g/L dithiothreitol. The microbial culture solution B is obtained by inoculating the *Enterococcus faecalis* into culture medium B and then culturing it at pH 6.8-7.0 and 37°C for 24 h. The inoculum amount of microbial culture solution B in the filtrate is 2 v.

[n0028]

Preferably, in step S4, the anaerobic fermentation temperature is 37°C, the time is 48-55 h, and the pH is 6.8-7.0.

[n0029]

In this embodiment, the duration of the second fermentation is best controlled between 48 and 55 hours. If the time is too short, the fermentation will be incomplete, which will not allow ellagic acid to fully participate in the reaction and will result in a low yield of urolithin A. If the time is too long, since the formation reaction of urolithin A has been basically completed, simply extending the fermentation time will not only fail to increase the content of urolithin A, but also generate a large number of byproducts in the system as the fermentation process continues, which will have an adverse effect on the stability and purity of urolithin A.

[n0030]

Preferably, in steps S3 and S4, low-speed stirring is continuously performed. The stirring speed is 80-120 rpm, such as 80, 85, 90, 95, 100, 105, 110, 115, or 120 rpm.

[n0031]

In this embodiment, continuous low-speed stirring can, on the one hand, avoid the generation of bubbles, reduce microbial damage, and protect the microbial growth environment; on the other hand, it can promote the thorough mixing of the fermentation substrate and microorganisms, ensure that the microorganisms can fully contact the fermentation substrate, and maintain the uniform distribution of microorganisms in the fermentation substrate, so as to promote interaction and synergistic fermentation among microorganisms, thereby improving fermentation efficiency and fermentation effect.

In addition, low-speed stirring can reduce the violent shearing of substances, avoid the degradation of fermentation products and the weakening of catalytic effect, thereby ensuring the quality and yield of the products.

[n0032]

Preferably, in step S5, the specific operation of the extraction is as follows: the obtained fermentation broth is extracted with an extractant, which is a mixture of CH₃CN:H₂O:HCOOH in a volume ratio of 80:19.9:0.1. After the extract is freeze-dried, it is reconstituted with ethyl acetate and then filtered through a 0.22μm filter membrane.

[n0033]

Compared with the prior art, the present invention has at least the following beneficial effects:

[0043]

(1) Using walnut green skin, a by-product of walnut processing, as a source of ellagic acid has a large output and low cost. The walnut green skin can be used directly after being crushed without the need for drying, powdering or other post-processing. The pre-treatment is simple and efficient, which not only helps sustainable development but also avoids environmental pollution. It meets the requirements of environmental protection and circular economy, and the prospects for large-scale production are bright.

[0044]

(2) Ellagic acid in walnut green skin diffuses from the inside of the walnut green skin cells to the outside under the action of ultrasound. Through the first fermentation, it is stabilized and dissolved in water by forming inclusion complex and hydrogen bonding under the action of the complex of mercapto-β-cyclodextrin and chitosan. Ellagic acid in this form is not only structurally stable, but also has high bioavailability, which provides good prerequisites for subsequent microbial transformation.

[0045]

(3) When ellagic acid begins to be converted into urolithin A, the thiol-β-cyclodextrin and

chitosan that were previously bound to ellagic acid are now bound to urolithin A and encapsulated in it. This not only stabilizes the structure of urolithin A and increases the solubility of urolithin A in water, but also reduces the inhibitory effect of product concentration on microbial transformation to a certain extent, so that the microorganisms can still maintain high activity to promote the continued generation of urolithin A.

[0046]

Attached Figure Description

[n0034]

To more clearly illustrate the technical solutions in the embodiments of this application, the accompanying drawings used in the description of the embodiments will be briefly introduced below. Obviously, the accompanying drawings described below are only some embodiments of this application. For those skilled in the art, other drawings can be obtained based on these drawings without creative effort.

[n0035]

Figure 1 shows the ultraviolet absorption spectra of the ellagic acid product and the ellagic acid standard collected after step S3 of Example 1.

[0049]

Figure 2 shows the high performance liquid chromatograms of the ellagic acid product and the ellagic acid standard collected after step S3 of Example 1.

[0050]

Figure 3 shows the ultraviolet absorption spectra of urolithin A and urolithin A standard generated in Example 1;

[0051]

Figure 4 is a liquid chromatogram of urolithin A generated in Example 1.

[0052]

Detailed Implementation

[n0036]

The technical solutions in the embodiments of this application will be clearly and completely described below with reference to the accompanying drawings. Obviously, the described embodiments are only some embodiments of this application, and not all embodiments.

Based on the embodiments in this application, all other embodiments obtained by those skilled in the art without creative effort are within the scope of protection of this application.

[n0037]

In the following examples and comparative examples, mercapto-β-cyclodextrin was prepared in-house. The specific preparation method was as follows: First, a 0.8 mol/L NaOH aqueous solution was added dropwise to a 0.15 mol/L β-cyclodextrin aqueous suspension to obtain mixed solution 1, wherein the weight ratio of NaOH to β-cyclodextrin was 1:10. Then, a 1.8 mol /L methylbenzenesulfonylchloroacetonitrile solution was added dropwise to mixed solution 1 to obtain mixed solution 2, wherein the weight ratio of methylbenzenesulfonylchloroacetonitrile to β-cyclodextrin was 1:6. Then, mixed solution 2 was stirred at room temperature until… After standing for 2 hours, the solid was collected and recrystallized and dried to obtain sulfonated β-cyclodextrin crystals. The crystals and thiourea

were then dissolved in a water-methanol mixture (specifically, methanol and water were mixed in a volume ratio of 80:20) and stirred until homogeneous to obtain mixture 3. The addition ratio of the crystals, thiourea, and water-methanol mixture was 2g:2g:80ml. Finally, 1,2-dichloroethane was added to mixture 3, wherein the weight ratio of the crystals to 1,2-dichloroethane was 1:15, to obtain mercapto-β-cyclodextrin.

[n0038]

In the following examples and comparative examples, the green-skinned walnuts were obtained from walnut growers in Lin'an, Hangzhou, and were harvested on September 7, 2024; the water-soluble chitosan was purchased from Shijiazhuang Dingmin Pharmaceutical Technology Co., Ltd.; Bacillus vesiculosus was purchased from Shanghai Xuanke Biotechnology Co., Ltd.; and Bacillus coagulans was purchased from Shanghai Jiaguan Biotechnology Co., Ltd.

All other raw materials not explicitly stated were commercially purchased, and specific manufacturers and specifications are not required.

Ellagic acid standard was purchased from Merck Chemical Technology (Shanghai) Co., Ltd., item number HY-B0183.

[n0039]

In the following examples and comparative examples, culture medium A is a corn syrup-modified MRS liquid culture medium, specifically, the glucose in the MRS liquid culture medium is replaced with an equal amount of corn syrup.

Bacillus belye was cultured with culture medium A at a ratio of 0.6 g/100 ml to obtain Bacillus belye suspension, and Bacillus coagulans was cultured with culture medium A at a ratio of 0.4 g/100 ml to obtain Bacillus coagulans suspension. After the culture was completed, Bacillus belye suspension and Bacillus coagulans suspension were mixed at a volume ratio of 1:1 to obtain microbial culture A. The inoculum amount of microbial culture A in the fermentation substrate was 2 v.

[n0040]

In the following examples and comparative examples, *Enterococcus faecalis* was purchased from the China General Microbiological Culture Collection Center (CGMCC NO. 24964). The specific components of culture medium B were: peptone 16.0 g/L, yeast extract 7.0 g/L, potassium chloride 5.0 g/L, starch 1.0 g/L, glucose 1.0 g/L, sodium pyruvate 1.0 g/L, arginine 1.0 g/L, sodium succinate 0.5 g/L, and cysteine. The microbial culture solution B consists of 0.5 g/L of sodium phosphate, 0.4 g/L of sodium bicarbonate, 0.5 g/L of ferric pyrophosphate,

0.005 g/L of heme chloride, 0.0005 g/L of vitamin K, 0.5 g/L of sodium thioglycolate, and 1.0 g /L of dithiothreitol. The microbial culture solution B is obtained by inoculating the Enterococcus faecalis into culture medium B and culturing it at pH 6.8 and 37°C for 24 h. The inoculum amount of microbial culture solution B in the filtrate is 2 v.

[n0041]

Example 1

[0059]

A process for producing urolithiasis A includes the following steps:

[0060]

S1. Obtaining the green husk of walnuts: Select healthy, pest-free walnuts with intact green husks, wash them, and pile them up in a well-ventilated and shady place to allow the green husks to dry naturally. Once the green husks soften, peel them off to obtain the green husks of walnuts.

[0061]

S2. Preparation of fermentation substrate: The green walnut peel is crushed into a paste and added to a stabilizing solution. After stirring evenly, the resulting mixture is subjected to

ultrasonic treatment to obtain the fermentation substrate. The stabilizing solution is an aqueous solution of a compound of mercapto-β-cyclodextrin and water-soluble chitosan. The mercapto-β-cyclodextrin and water-soluble chitosan are mixed at a mass ratio of 0.25:1, the mass percentage of the stabilizing solution is 12%, the material-to-liquid ratio of green walnut peel to stabilizing solution is 1:15, and the technical parameters of ultrasonic treatment are: ultrasonic frequency 13kHz, ultrasonic power 180W, time 30min, and temperature 60°C.

[0062]

S3. First fermentation: After the temperature of the fermentation substrate drops to room temperature, microbial culture A is inoculated into the fermentation substrate for anaerobic fermentation. The anaerobic fermentation temperature is 33°C and the time is 48h. The entire process is stirred at a speed of 100rpm. After the fermentation is completed, solid-liquid separation is performed and the filtrate is collected. The microbial culture A is obtained by inoculating Bacillus belye and Bacillus coagulans into culture medium A, activating them in a shaker, and then mixing them.

[0063]

S4. Second fermentation: Microbial culture solution B is inoculated into the filtrate for anaerobic fermentation again. The anaerobic fermentation temperature is 37°C, the time is 50h, the pH is 6.8, and the entire process is stirred at a speed of 100rpm. The microbial culture solution B is obtained by inoculating Enterococcus faecalis into culture medium B.

[0064]

S5. Extraction: After fermentation, the fermentation broth is extracted with an extractant. The extractant is a mixture of CH₃CN:H₂O:HCOOH in a volume ratio of

80:19.9:0.1. The extract is freeze-dried, reconstituted with ethyl acetate, and then filtered through a 0.22μm filter membrane.

[n0042]

Example 2

[0066]

Compared with Example 1, in step S2, mercapto-β-cyclodextrin and water-soluble chitosan are mixed at a mass ratio of 0.1:1.

Everything else is consistent with Example 1.

[n0043]

Example 3

[0068]

Compared with Example 1, in step S2, mercapto-β-cyclodextrin and water-soluble chitosan are mixed at a mass ratio of 0.2:1.

Everything else is consistent with Example 1.

[n0044]

Example 4

[0070]

Compared with Example 1, in step S2, mercapto-β-cyclodextrin and water-soluble chitosan are mixed at a mass ratio of 0.4:1.

Everything else is consistent with Example 1.

[n0045]

Example 5

[0072]

Compared with Example 1, in step S2, the mass percentage of the stabilizing liquid is 5%.

Everything else is consistent with Example 1.

[n0046]

Example 6

[0074]

Compared with Example 1, in step S2, the mass percentage of the stabilizing liquid is 10%.

Everything else is consistent with Example 1.

[n0047]

Example 7

[0076]

Compared with Example 1, in step S2, the mass percentage of the stabilizing liquid is 20%.

Everything else is consistent with Example 1.

[n0048]

Example 8

[0078]

Compared with Example 1, the technical parameters of the ultrasonic treatment in step S2 are: ultrasonic frequency 15kHz, ultrasonic power 180W, and time 15min.

Everything else is consistent with Example 1.

[n0049]

Example 9

[0080]

Compared with Example 1, the technical parameters of ultrasonic treatment in step S2 are: ultrasonic frequency 12kHz, ultrasonic power 240W, and time 25min.

Everything else is consistent with Example 1.

[n0050]

Example 10

[0082]

Compared with Example 1, the technical parameters of the ultrasonic treatment in step S2 are: ultrasonic frequency 14kHz, ultrasonic power 150W, and time 35min.

Everything else is consistent with Example 1.

[n0051]

Comparative Example 1

[0084]

Compared to Example 1, no stabilizing liquid was added, but all other aspects remained the same as in Example 1.

[n0052]

Comparative Example 2

[0086]

Compared to Example 1, the stabilized solution does not contain mercapto-β-cyclodextrin, but otherwise remains the same as in Example 1.

[n0053]

Comparative Example 3

[0088]

Compared to Example 1, the stabilized solution does not contain water-soluble chitosan, but all other aspects are consistent with Example 1.

[n0054]

Comparative Example 4

[0090]

Compared with Example 1, in step S2, mercapto-β-cyclodextrin and water-soluble chitosan are mixed at a mass ratio of 0.6:1.

Everything else is consistent with Example 1.

[n0055]

Comparative Example 5

[0092]

Compared with Example 1, in step S2, mercapto-β-cyclodextrin and water-soluble chitosan are mixed at a mass ratio of 0.02:1.

Everything else is consistent with Example 1.

[n0056]

Comparative Example 6

[0094]

Compared with Example 1, in step S2, the mass percentage of the stabilizing liquid is 2%.

Everything else is consistent with Example 1.

[n0057]

Comparative Example 7

[0096]

Compared with Example 1, in step S2, the mass percentage of the stabilizing liquid is 25%.

Everything else is consistent with Example 1.

[n0058]

Comparative Example 8

[0098]

Compared with Example 1, in step S2, the ultrasonic frequency is adjusted to 25 kHz.

Everything else is consistent with Example 1.

[n0059]

Comparative Example 9

[0100]

Compared with Example 1, in step S2, the ultrasonic frequency is adjusted to 8kHz.

Everything else is consistent with Example 1.

[n0060]

Comparative Example 10

[0102]

Compared with Example 1, in step S2, the ultrasonic power is adjusted to 500W.

Everything else is consistent with Example 1.

[n0061]

Comparative Example 11

[0104]

Compared with Example 1, in step S2, the ultrasonic power is adjusted to 50W.

Everything else is consistent with Example 1.

[n0062]

The filtrates collected after step S3 in each embodiment and comparative example were sampled and separated and extracted using the non-ionic macroporous resin AmberliteXAD-16, with deionized water as the eluent and ethanol as the eluent. The product enriched with ellagic acid was obtained after freeze-drying.

As shown in Figure 1, UV testing was performed on the ellagic acid standard and the ellagic acid extracted in Example 1, and both showed maximum absorption at a wavelength of 254 nm.

Ellagic acid standards and ellagic acid extracted in Example 1 were tested by HPLC. The main test conditions were as follows: a Waters 1525 liquid chromatograph was used, the column was an XBridge RC18 (250×4.6mm) with a particle size of 5μm, and the column temperature was 40°C; the mobile phase consisted of mobile phase A and mobile phase B, mobile phase A was an aqueous solution of formic acid containing 2mM ammonium acetate, and mobile phase B was methanol, with an injection volume of 10μL; the elution conditions were: flow rate 1.0mL/min, gradient elution, specifically: 0-3min, mobile phase B volume fraction 10%; 3-6min, mobile phase volume fraction increased from 10% to 30%; 6-12min, mobile phase B volume fraction maintained at 30%; 12-25min, mobile phase B volume fraction increased from 30% to 100%.

As shown in Figure 2, the elution time of Example 1 is the same as that of the ellagic acid standard.

Figures 1 and 2 clearly illustrate that the filtrate obtained after the first fermentation in

Example 1 was enriched with ellagic acid (the test results of the other examples are similar to the test results of Example 1 in Figures 1 and 2, so they are not attached separately).

Weigh the ellagic acid product and calculate the yield of ellagic acid in the walnut green husk based on the input amount and output amount of walnut green husk (yield = mass of ellagic acid / mass of walnut green husk * 100%).

The specific results are shown in Table 1.

[n0063]

UV testing was performed on the liquids after removing insoluble matter from the filter membranes of each embodiment and comparative example as test samples, as shown in

Figure 3: The UV absorption spectrum of Example 1 is very close to that of urolithin A, with both having a maximum absorption wavelength of 296 nm. The other embodiments also have test results similar to those of Example 1.

In addition, a Waters 1525 liquid chromatograph (with an XBridge RC18250 × 4.6 mm column of 5 μm particle size) was used for high performance liquid chromatography (HPLC) to determine the content of urolithin A. The test operation was the same as that in the prior art 1. The maximum absorption wavelength was determined to be 305 nm by spectral scanning. As shown in Figure 4, the peak time of Example 1 was the same as that in the prior art 1, which shows that urolithin A was indeed generated. Moreover, the peak area was larger than that in the prior art 1, indicating that the content of urolithin A was higher. The other examples also had test results similar to those of Example 1.

The content of urolithin A was quantitatively calculated using the high performance liquid chromatograms of various embodiments and comparative examples. The specific results are shown in Table 1.

[n0064]

Table 1

[0108]

[0109]

As can be seen from Table 1:

[0110]

(1) As can be seen from the test results of Examples 1-10, under the process parameters defined in this invention, the yield of ellagic acid is 0.75%-0.84%, and at least 80% of the ellagic acid in the green walnut skin is extracted, which has a very good industrialization prospect. Under this premise, after the second fermentation, the concentration of urolithin A is at least 40.2 μM and can reach up to 54.3 μM. At this time, the conversion rate of urolithin A is about 18%.

[n0065]

Based on Comparative Examples 1, 2, and 3, it can be seen that if the stabilizing solution contains only water-soluble chitosan or only mercapto-β-cyclodextrin, although it has a certain positive effect on improving the yield of ellagic acid, the degree of improvement is very limited. However, when the two substances are added in combination at a specific mass ratio, the yield of ellagic acid is significantly improved, and the final concentration of urolithin A is significantly increased.

Obviously, the increased yield of ellagic acid and the increased conversion and yield of urolithin A in this invention are the result of the mutual influence and synergistic effect of mercapto-β-cyclodextrin and chitosan, and neither can be dispensed with.

[n0066]

(2) As can be seen from the test results of Examples 1-4, Comparative Example 4 and Comparative Example 5, the mass ratio of mercapto-β-cyclodextrin to water-soluble chitosan has a significant effect on the yield of ellagic acid and the concentration of urolithin A. If the mass ratio is greater than or less than the range defined in this invention, the yield of ellagic acid and the concentration of urolithin A will decrease significantly. Obviously, if the mass ratio of the two is inappropriate, the resulting complex may have limited adsorption capacity for effective substances due to uneven composition or dispersion, which is not conducive to the extraction of ellagic acid and the generation of urolithin A.

[n0067]

(3) As can be seen from the test results of Examples 1, 5-7, Comparative Example 6 and

Comparative Example 7, the mass percentage of the stabilizer has a significant effect on the yield of ellagic acid and the concentration of urolithin A.

If its mass percentage is too high, the viscosity may be too high, leading to the flocculation of ellagic acid, which is not conducive to the formation of ellagic acid. It may also hinder the formation of urolithin A due to the unfavorable effect on the normal growth and metabolism of microorganisms.

The preferred mass percentage of the stabilizing solution is 5%-20%. Within this range, the yield of ellagic acid is high and the conversion rate of urolithin A is high.

[n0068]

(4) The test results of Example 1 and Comparative Examples 8-11 show that the frequency and power of ultrasonic treatment are strictly required when preparing fermentation substrate. Too high or too low frequency will not help to improve the yield of ellagic acid and the concentration of urolithin A.

As can be seen from Examples 8-10, the preferred ultrasonic frequency is 12-15kHz and the ultrasonic power is 150-240W.

[n0069]

In summary, this invention, by adding thiol-β-cyclodextrin and chitosan during the preparation of fermentation substrates, increases the concentration of ellagic acid, an effective substance in the fermentation substrates, and increases the concentration of reactants. On the other hand, it reduces the impact of increased urolithin A concentration on

microbial activity and increases the yield of products. The interaction and synergistic effect of thiol-β-cyclodextrin and chitosan significantly improves both the conversion rate of urolithin A and the product yield.

Compared with existing technologies, this invention has great potential for industrialization, and is safe, environmentally friendly, and has a simple process.

[n0070]

Although embodiments of the present invention have been shown and described above, it is understood that the above embodiments are exemplary and should not be construed as limiting the present invention. Those skilled in the art can make modifications, alterations, substitutions and variations to the above embodiments within the scope of the present invention.

Furthermore, those skilled in the art can combine and integrate the different embodiments or examples described herein, as well as the features of the different embodiments or examples, without contradiction.
