



Identification and a phased pH control strategy of diosgenin bio-synthesized by an endogenous *Bacillus licheniformis* Syt1 derived from *Dioscorea zingiberensis* C. H. Wright

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Received: 8 July 2021 / Revised: 7 October 2021 / Accepted: 2 November 2021 / Published online: 29 November 2021
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Abstract

Diosgenin is widely used as one precursor of steroidal drugs in pharmaceutical industry. Currently, there is no choice but to traditionally extract diosgenin from *Dioscorea zingiberensis* C. H. Wright (*DZW*) or other plants. In this work, an environmentally friendly approach, in which diosgenin can be bio-synthesized by the endophytic bacterium *Bacillus licheniformis* Syt1 isolated from *DZW*, is proposed. Diosgenin produced by the strain was identified by high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), and Fourier transform infrared spectroscopy (FTIR). The thermal gravimetric analysis (TGA) showed that the melting point of the diosgenin product was 204 °C. The optical rotation measurement exhibited that the optical rotation was $\alpha_{589}^{20} = -126.1^{\circ} \pm 1.5^{\circ}$ (chloroform, $c = 1\%$): negative sign means that the product is left-handed, which is very important to further produce steroid hormone drugs. Cholesterol may be the intermediate product in the diosgenin biosynthesis pathway. In the batch fermentation process to produce diosgenin using the strain, pH values played an important role. A phased pH control strategy from 5.5 to 7.5 was proved to be more effective to improve production yield than any single pH control, which could get the highest diosgenin yield of $85 \pm 8.6 \text{ mg L}^{-1}$. The proposed method may replace phyto-chemistry extraction to produce diosgenin in the industry in the future.

Key points

- An endophytic *Bacillus licheniformis* Syt1 derived from host can produce diosgenin.
- A dynamic pH industrial control strategy is better than any single pH control.
- Proposed diosgenin-produced method hopefully replaces phyto-chemistry extraction.

Keywords Endophytic *Bacillus licheniformis* Syt1 · Diosgenin · Isolation and identification · Batch fermentation · pH control strategy

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Introduction

Diosgenin (CAS number 512–04-9) is widely used to synthesize oral contraceptives, sex hormones, and other steroid drugs in pharmaceutical industry (Chen et al. 2015; Lepage et al. 2010). Consequently, the demands are gradually increasing in the world market; most commercial supply of diosgenin in global market is from China and North America, where diosgenin are mainly extracted from *Dioscorea* plants (Bai et al. 2015; Dawson 1991; Fernandes et al. 2003; Pan et al. 2014). *Dioscorea zingiberensis* C. H. Wright (*DZW*) is one major plant in *Dioscorea* genus, which is also traditionally called yellow ginger, and dominantly used to extract diosgenin in China (Wang et al. 2014; Zhang et al. 2012). As a result, the production of diosgenin always consumes a large number of *DZW*, which consequently results in overly depending on *DZW* resources. The status has brought about two negative social issues. Firstly, the endless excavation of *DZW* consequently causes a serious damage to the ecological environment and the degradation of *DZW* germplasm. Secondly, it often generates a lot of wastewater and acids in the phyto-chemistry extraction process of diosgenin from *DZW* (Pan et al. 2014; Wei et al. 2013; Yang et al. 2016). Some factories have been banned from extracting diosgenin from *DZW* by government for the reasons in China. Therefore, there is an urgent need to find a green, easily industrialized way to produce diosgenin.

Although several improving approaches had been reported, in which diosgenin was obtained by enzymatically hydrolyzing saponins, these methods all relied on the hydrolase system of some fungi such as *Trichoderma harzianum* or *Trichoderma reesei*, *Aspergillus fumigatus*, or *Aspergillus awamori* (Chen et al. 2018; Cheng et al. 2015; Liu et al. 2010; Zhu et al. 2010). But these methods had a premise that saponins were also extracted and purified from *Dioscorea* plants in advance, which did not solve the abovementioned problems of phyto-chemistry extraction. Moreover, most of the previous studies were limited to the Erlenmeyer flask scale rather than the batch fermentation scale.

The aims of this work were to isolate and identify an endophytic bacterium *Bacillus licheniformis* *Syt1* derived from *DZW* which can produce diosgenin without *DZW*. Moreover, an optimized pH control strategy to maximize production rate was screened in batch fermentation process. The proposed method depending on microgram fermentation to produce diosgenin would be further easy to be industrialized in factory without *DZW* cultivation in an environmentally friendly way, which is promising to replace the traditional phytochemical extraction from *DZW*.

Materials and methods

Materials and chemicals

Tubers of *DZW* were obtained from National Engineering Research Center for Phytochemistry (West). Pure water was produced by a UPHW-I-90Z model ultra-pure water instrument (Shanghai Youpu industrial Co. Ltd, Shanghai, China). Analytical grade alcohol was purchased from Nanjing Chemical Reagent Co., Ltd. Analytical grade mercury dichloride (HgCl_2), sodium chloride, sodium hydroxide and calcium chloride, and sucrose were all purchased from Tianjin Bodi Chemical Co., Ltd. Potato dextrose agar medium (PDA) was supported by Beijing Aobox Biotechnology. 120# gasoline was purchased from Shenzhen HCH Chemical Co., Ltd. Analytically pure petroleum ether, ethyl acetate, and chloroform reagents were bought from Sigma-Aldrich China. The powder of soybean cake was purchased from Tianjin Lifalong Chemical Technology Co., Ltd. HPLC-grade standard product of diosgenin (purity $\geq 98\%$) and methanol (MeOH) were purchased from Dalian melone Biotechnology Co., Ltd. DNA extraction kit and other genetic engineering reagents were from Takara China. The used primers were synthesized by Sangon Biotech of Shanghai, China.

Endophytic strains screening and target product analysis

The strong tubers of *DZW* were cleaned with sterile pure water and cut into 5 cm \times 5 cm pieces, and soaked in 75% (v/v) alcohol solution for 30 s. Subsequently, they were immersed into a 0.1% HgCl_2 solution for 5 min, and then rinsed 3 times with sterile water, and kept their surface sterile through plate culture verification. Continuously, these sterile 5 cm \times 5 cm pieces were further cut into 5 mm \times 5 mm pieces under sterile conditions. Every two pieces of them were incubated on a PDA plate at 28 °C for 3–5 days until endophytic microorganisms in tubers grew up. The obtained endophytic microorganism strains were isolated and purified on new PDA plates, then, were separately checked whether they could produce diosgenin in the following fermentation process. Every endophytic strain was individually cultured in an optimal medium consisted of 30 g/L sucrose, 6 g/L NaCl, 15 g/L CaCl_2 , and 15 g/L soybean powder, and pH was at 7.0. The whole fermentation process was maintained at 28 °C with 120r/min for 100 h under aerobic culture condition. The cultured cells were harvested by centrifugation at 5000 rpm and all cell pellets were hydrolyzed with 2 mol L^{-1} hydrochloric acid at 90 °C for 4 h. The hydrolytic liquid was

cooled to room temperature and neutralized to pH 7.0 with 1 mol L⁻¹ sodium hydroxide, and then extracted with 120# gasoline for 4 h in reflux way in a Soxhlet extractor, the gasoline phase was isolated within a separating funnel after static layering. The gasoline in gasoline phase was completely evaporated to obtain the crude extract. The crude extract was subjected to 200 mesh silica gel column chromatography (Φ30 × 300 mm) purification. In detail, the loading height of the adsorbent filler in column was 200 mm, and target diosgenin was eluted by a cosolvent (petroleum ether:ethyl acetate = 2:1, v/v). The eluate was collected, concentrated at 80 °C, and crystallized at 4 °C to obtain diosgenin-like crystal. 0.2600 g diosgenin-like crystal was accurately weighed for and dissolved in 1L methanol (0.26 mg/mL), which was analyzed by high-performance liquid chromatography analyzer (Waters 2996 diode-array detector) equipped with a Symmetry® C18 column (4.6 × 250, 5 μm). The mobile phase was MeOH/H₂O (v/v = 95/5) and the flow rate was 0.6 mL/min, and column temperature was controlled at 25 °C. Injection volume of sample was 10 μL. Detection wavelength was set at 280 nm. 0.1 mg/mL diosgenin standard solution was used as control. Each experiment was performed in triplicate under the same condition.

Physical properties identification of diosgenin-like crystal product

The melting point of the obtained diosgenin-like crystal product was determined by the Shimadzu DTG-60 (Shimadzu, Shanghai, China) using thermal gravimetric analysis (TGA) method according to the previous report (Macêdo et al. 1999). In advance, the diosgenin-like crystal product was dried for 72 h in a desiccator at an ambient temperature in advance. Then, 0.900 g dried crystal product was loaded into DTG-60 and heated with a rise rate of 10 °C min⁻¹; a nitrogen atmosphere was used, at a flow rate of 50 mL/min. The thermal gravimetric (TG) curve was analyzed with the aid of software of the instrument. The melting point was determined according to reduction in mass.

Comparisons of the Fourier transform infrared (FT-IR) spectrum of the obtained diosgenin-like crystal product with diosgenin standard product were screened by a Bruker vertex70 infrared spectrometer system (Bruker China Co., Ltd., China) according to the previous report (Zhang et al. 2009). The crystal product and standard product were both dried 2 days ago prior to FT-IR measurement. Round transparent tablet contained the crystal product sample or diosgenin standard product was subjected to 32 scans with 4 cm⁻¹ resolution in the region of 4000–500 cm⁻¹ by the Bruker vertex 70 infrared spectrometer, respectively.

NMR spectra of the obtained diosgenin-like crystal product and diosgenin standard (control group) were analyzed by

500 MHz spectrometer for ¹H NMR and 126 MHz for ¹³C NMR (Bruker, Billerica, MA, USA) according to the report (Mamay, Wahyuningrum and Hertadi 2015) with little modifications. The diosgenin-like crystal product or diosgenin standard was solved in CDCl₃ in the process, respectively.

The optical rotation of the obtained diosgenin-like crystal product was determined using a WZZ-2S digital automatic polarimeter (Shanghai, China) according to papers (Duan et al. 2018; Eibler and Vetter 2017). One percent (w/v) diosgenin-like crystal product in chloroform was prepared and determined at 589 nm under 20 °C, while pure chloroform served as blank control.

The species identification of the target strain

The strain to produce diosgenin was identified by 16S rDNA sequence with polymerase chain reaction (PCR). The genomic DNA of the strain was isolated with Takara MiniBEST Agarose Gel DNA Extraction Kit according to the instruction, which was used as the PCR amplification template; the used forward (Fw) primer was 5'-AGAGTTTGA TCCTGGCTCAGAACG-3', and the reverse (Rv) primer was 5'-TACGGCTACCTTGTTACGACTTCAC-3'. Twenty-five microliters of the PCR reactant was mixed well with 1 μL 10 μmol/L Fw primer, 1 μL 10 μmol/L Rv primer, 2.5 μL 10 × PCR buffer, 1 μL 2.5 mM dNTP mixture, 1.0 μL DNA template, 0.4 μL rTaq enzyme, and 18.1 μL nuclease-free water. PCR was carried out in the Bio-rad® ALD1244 DNA Engine (Bio-Rad, USA). The adopted PCR protocol was as follows: (1) initial denaturation temperature was 94 °C for 3 min; (2) 30 cycles, every cycle including 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; (3) final extension step was maintained at 72 °C for 5 min. The PCR product was sequenced by Shanghai Sangon Biotech of China after our purification. The obtained 16S sequence was compared with NCBI data to determine species. The phylogenetic tree was constructed by neighbor joining method using by MEGA software version 7.0 (Kumar et al. 2016; Pidiyar et al. 2003). The colony morphology and structure of the target strain was analyzed using transmission electron microscope (TEM) and atomic force microscopy (AFM) (Bruker Company, USA).

Effect of pH on diosgenin yield and pH optimization in batch fermentation

At the optimal growth temperature of this strain, pH values may play an important role to produce diosgenin in batch fermentation. Therefore, pH optimization was made to improve the diosgenin yield. Different pH tests were carried out in a fermentation medium with the same composition as the abovementioned in a 5-L automatically controlled BioFlo batch Fermenter (No.500998196, New Brunswick Scientific Co., Inc., USA). pH value of medium was in turn set at 5.5,

6.0, 6.5, 7.0, 7.5, and 8.0 in the batch fermentation; the pH during cultivation was detected by a pH electrode attached to a PID controller equipped to the fermenter, and the adjusting and stabilizing of pH values were automatically performed by adding 0.1 N hydrochloric acid or sodium hydroxide with two trickle feed pumps controlled by a computer program. The inoculation amount was 10%. The other conditions of all fermentation trials were maintained at 28 °C with 200 rpm stirring for 80 h, while 1.0 L⁻¹ min⁻¹ ventilation was given into the fermenter, 0.2% poly-siloxane was used as defoamer. Sampling was performed every 4-h interval, and the yields of diosgenin were analyzed using multi-well microplate spectrophotometry according to the previous paper (Li et al. 2012). Production rates of diosgenin were calculated by the following formula.

$$Y = \Delta C / \Delta t$$

where Y is the production rates of diosgenin, mg L⁻¹ h⁻¹; Δt is the fermentation time increment, h; ΔC is the concentration variation of diosgenin in Δt period, mg L⁻¹. Each experiment was repeated for three times. In order to maximize production rate of diosgenin, a phased pH control strategy was practiced in the fermenter according to the above effects of pH on production rates.

Statistical analysis

The results of all were analyzed by statistical software SPSS 18.0.

Results

Identification of diosgenin-like crystal product

A total of three endogenous strains were obtained. Only one strain was found to produce the diosgenin-like crystal product. The diosgenin-like crystal product was identified by HPLC. Its retention time was at 4.100 min in HPLC chromatogram as Fig. 1A showed, which coincided with that of diosgenin standard. It was preliminarily inferred that the diosgenin-like crystal product is most likely diosgenin. The purity of the diosgenin product was calculated by peak area ratio method to be ~96.20%. The melting point curve of the diosgenin-like crystal product was further determined by the thermal gravimetric analysis method (TGA). It can be seen that it began melting at 204 °C from the obtained melting point curve as Fig. 1B showed, which is consistent with previous reports (Zhang et al. 2009, 2007).

The FT-IR spectrum of the diosgenin-like crystal product was displayed in Fig. 1C. It can be seen that the spectrum shape of the crystalline product is almost the same

with that of diosgenin standard except difference in transmitted light intensity, especially same in the range between 1800 and 600 cm⁻¹, where it is the major fingerprint area of diosgenin. In other areas with typical characteristics, a large OH-stretching vibration is at 3525 cm⁻¹, which is consistent with -OH in diosgenin molecular structure as Fig. 1D showed. Four strong absorption peaks between 2950 and 2834 cm⁻¹ wavenumber values are presumed to be asymmetric stretching vibrations of four methyl groups (-CH₃) as showed in Fig. 1D. A stretch absorption peak of a -C=C- group appears at 1680 cm⁻¹, indicating one -C=C- group in diosgenin molecular structure as in Fig. 1D. About six strong absorption bands between 1458 and 1130 cm⁻¹ are inferred to be deformation vibrations of four six-membered rings and two five-membered rings in diosgenin molecular structure. Strong absorptions between 1050 and 980 cm⁻¹ should be due to -C-O-C- stretches in diosgenin molecule according to previous reports (Cavagna et al. 2010; Gong et al. 2019; Kos et al. 2003). A number of characteristic absorption bands in the range 980–500 cm⁻¹ should be out-of-plane bending vibrations of C-H bonds in the spiral skeleton of diosgenin molecule. In addition, the optical rotation of the crystal-like product was further determined to be $\alpha_{589}^{20} = -126.1^\circ \pm 1.5^\circ$ (chloroform, $c = 1\%$), which was similar with that of standard. A negative sign means the product is left-handed, which is very important for producing steroid hormone drugs using diosgenin, because left-handed stereoselectivity is closely related to drug activity (Davies 1990). NMR spectrum of the diosgenin-like crystal product and standard was recorded using 500-MHz spectrometer for ¹H NMR and 126 MHz for ¹³C NMR. The ¹H NMR spectrum data (500 MHz, CDCl₃) of the diosgenin-like crystal product are as follows: δ 5.37–5.32 (m, 1H), 4.40 (q, $J = 7.5$ Hz, 1H), 3.49 (ddq, $J = 25.7, 10.9, 3.4, 2.3$ Hz, 2H), 3.37 (t, $J = 10.9$ Hz, 1H), 2.33–2.27 (m, 1H), 2.23 (ddd, $J = 13.2, 10.8, 2.8$ Hz, 1H), 2.05–1.93 (m, 2H), 1.85 (ddt, $J = 14.2, 10.6, 5.6$ Hz, 3H), 1.81–1.70 (m, 2H), 1.70–1.51 (m, 9H), 1.51–1.39 (m, 3H), 1.28 (dq, $J = 19.8, 6.7$ Hz, 1H), 1.23–1.04 (m, 3H), 1.02 (s, 3H), 0.97 (d, $J = 7.0$ Hz, 4H), 0.78 (t, $J = 3.2$ Hz, 6H). The ¹³C NMR spectrum data (126 MHz, CDCl₃) of the diosgenin-like crystal product are as follows: δ 140.8(C-1), 121.5(C-2), 109.3(C-3), 80.9(C-4), 71.8(C-5), 66.9(C-6), 62.1(C-7), 56.5(C-8), 50.1(C-9), 42.3(C-10), 41.6(C-11), 40.3(C-12), 39.8(C-13), 37.2(C-14), 36.7(C-15), 32.1(C-16), 31.9(C-17), 31.6(C-18), 31.5(C-19), 31.4(C-20), 30.3(C-21), 28.9(C-22), 20.9(C-23), 19.4(C-24), 17.2(C-25), 16.3(C-26), 14.5(C-27). Its ¹H spectrum and ¹³C spectrum are displayed in Fig. 1E and Fig. 1F, respectively. According to the ¹H spectra, δ 7.265 ppm is the solvent peak of CDCl₃, and δ 5.37–5.32 (m, 1H) indicates that there is a double-bond H in the compound. Considering the two

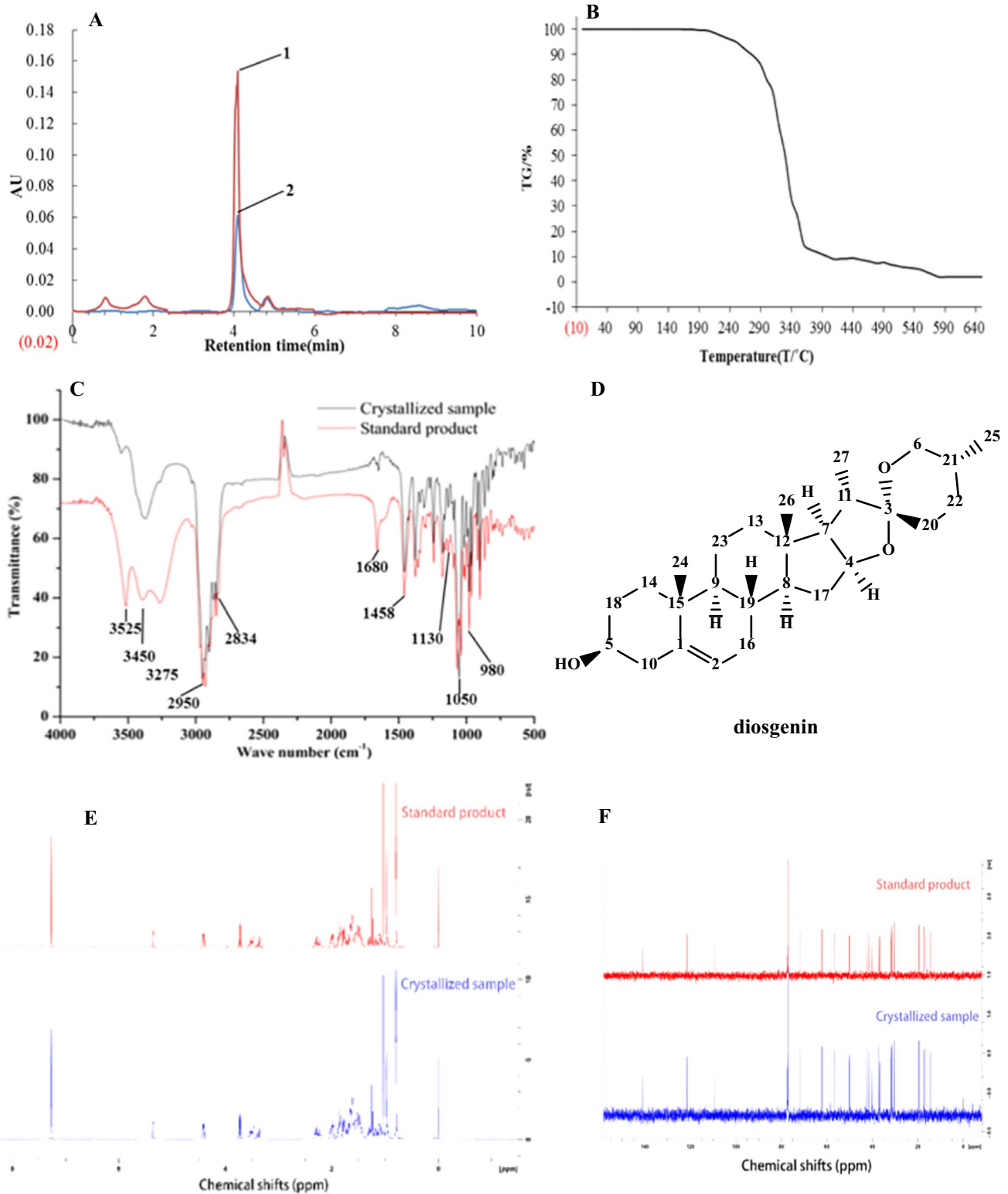


Fig. 1 The identification of the diosgenin-like crystal product. **A** Chromatograms of the diosgenin-like crystal product and diosgenin standard. Therein, peak 1: the chromatogram of the diosgenin-like crystal product; peak 2: the chromatogram of diosgenin standard product. **B** The thermal gravimetric analysis curve of the diosgenin-like

crystal product. **C** FT-IR spectra of the diosgenin-like crystal product (in black) and diosgenin standard (in red). **D** The molecular structure formula of diosgenin. **E** ¹H NMR of the diosgenin-like crystal product (in blue) and diosgenin standard (in red). **F** ¹³C NMR of the diosgenin-like crystal product (in blue) and diosgenin standard (in red)

double C bond signals of δ 140.8(C-1) and 121.5(C-2) in the ^{13}C spectrum, it can be concluded that there is a C=C double bond in this compound. The ^1H spectra δ 4.40 (q, $J=7.5$ Hz, 1H), 3.49 (ddq, $J=25.7, 10.9, 3.4, 2.3$ Hz, 2H), and 3.37 (t, $J=10.9$ Hz, 1H) indicate that there are four CH or CH_2 signals connected with oxygen in the compound. According to the four C signals connected with oxygen in the C spectrum δ 109.3(C-3), 80.9(C-4), 71.8(C-5), and 66.9(C-6), it can be deduced that there are four O atomic signals in the compound. Four characteristic methyl peaks of diosgenin are found in the high field of ^{13}C spectrum, which are δ 19.4(C-24), 17.2(C-25), 16.3(C-26), and 14.5(C-27), respectively. Compared with the ^1H and ^{13}C spectra of the compound with diosgenin standard in Fig. 1E and Fig. 1F, it can be concluded that the diosgenin-like crystal product was diosgenin ($\text{C}_{27}\text{H}_{42}\text{O}_3$).

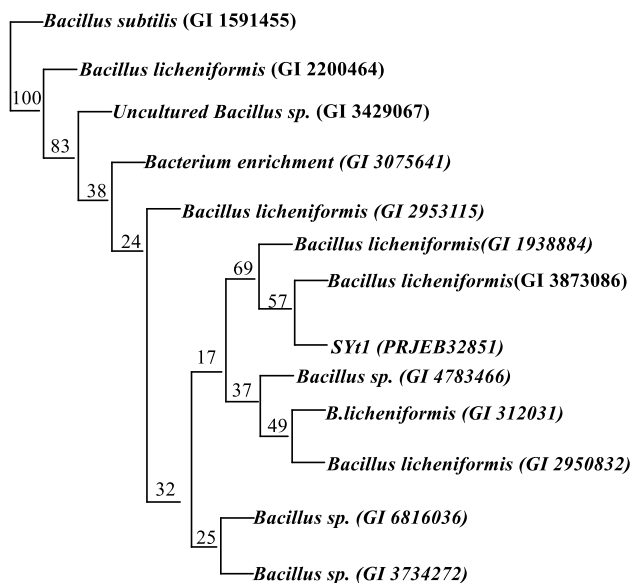
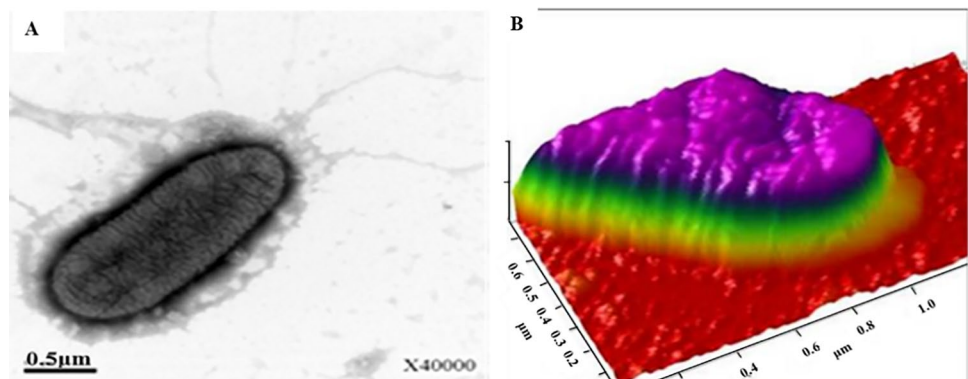


Fig. 2 Phylogenetic neighbor joining tree of strain *B. licheniformis* Syt1. The number at branch point indicates the reliability of the branch. Numbers in bracket indicate GenBank accession number

Fig. 3 The cell morphology identification of *B. licheniformis* Syt1. **A** The TEM photograph of Syt1; **B** the atomic force microscopy photograph of Syt1



The species identification of the target strain

By comparing the 16S DNA sequence (Supplementary Material 1) with the NCBI database data using phylogenetic analysis method (Accession number: PRJEB32851), the result indicated that the target strain belonged to a sub-branch of the *Bacillus licheniformis* and it shared a high sequence similarity with *Bacillus licheniformis* (GI 2,200,464) as Fig. 2 showed. The strain was therefore named *B. licheniformis* Syt1. It had been preserved in China General Microbiological Culture Collection Center (CGMCC), and the deposit number was 2334.

The TEM photograph of Syt1 is shown in Fig. 3A. It looks like a straight and round-ended rod, with peritrichous flagella indicating motility. There is no apparent capsule observed by TEM. A wrinkled and undulating surface can be clearly observed by the atomic force microscope (AFM) from Fig. 3B. It also can be observed that the shape of the strain actually is a flat rod with ciliated edges, indicating that Syt1 presented powerful adhesion. Its size is about 1.5 ~ 3.0 μm length \times 0.5 ~ 0.8 μm width according to the scale of AFM.

Effect of pH on diosgenin yield and pH optimization in batch fermentation

Effect of pH values on the production rate of diosgenin and optimization strategy are illustrated in Fig. 4. From Fig. 4A, it can be seen that the production rate of diosgenin increases with increasing fermentation time for any single pH control at first. Next, there is a maximum production rate appearing at an optimal fermentation time point, and then decreases with the extension of the fermentation time whatever for anyone fixed pH value. When pH increases by 0.5 unit in the range from 5.5 to 8.5, the corresponding maximal production rate of diosgenin is 1.69 ± 0.081 $\text{mg L}^{-1} \text{h}^{-1}$, 1.88 ± 0.074 $\text{mg L}^{-1} \text{h}^{-1}$, 1.80 ± 0.055 $\text{mg L}^{-1} \text{h}^{-1}$, 1.51 ± 0.065 $\text{mg L}^{-1} \text{h}^{-1}$, 1.17 ± 0.088 $\text{mg L}^{-1} \text{h}^{-1}$, 0.73 ± 0.045 $\text{mg L}^{-1} \text{h}^{-1}$, and 0.39 ± 0.033 $\text{mg L}^{-1} \text{h}^{-1}$, respectively; the corresponding time points are at 16 h, 24 h,

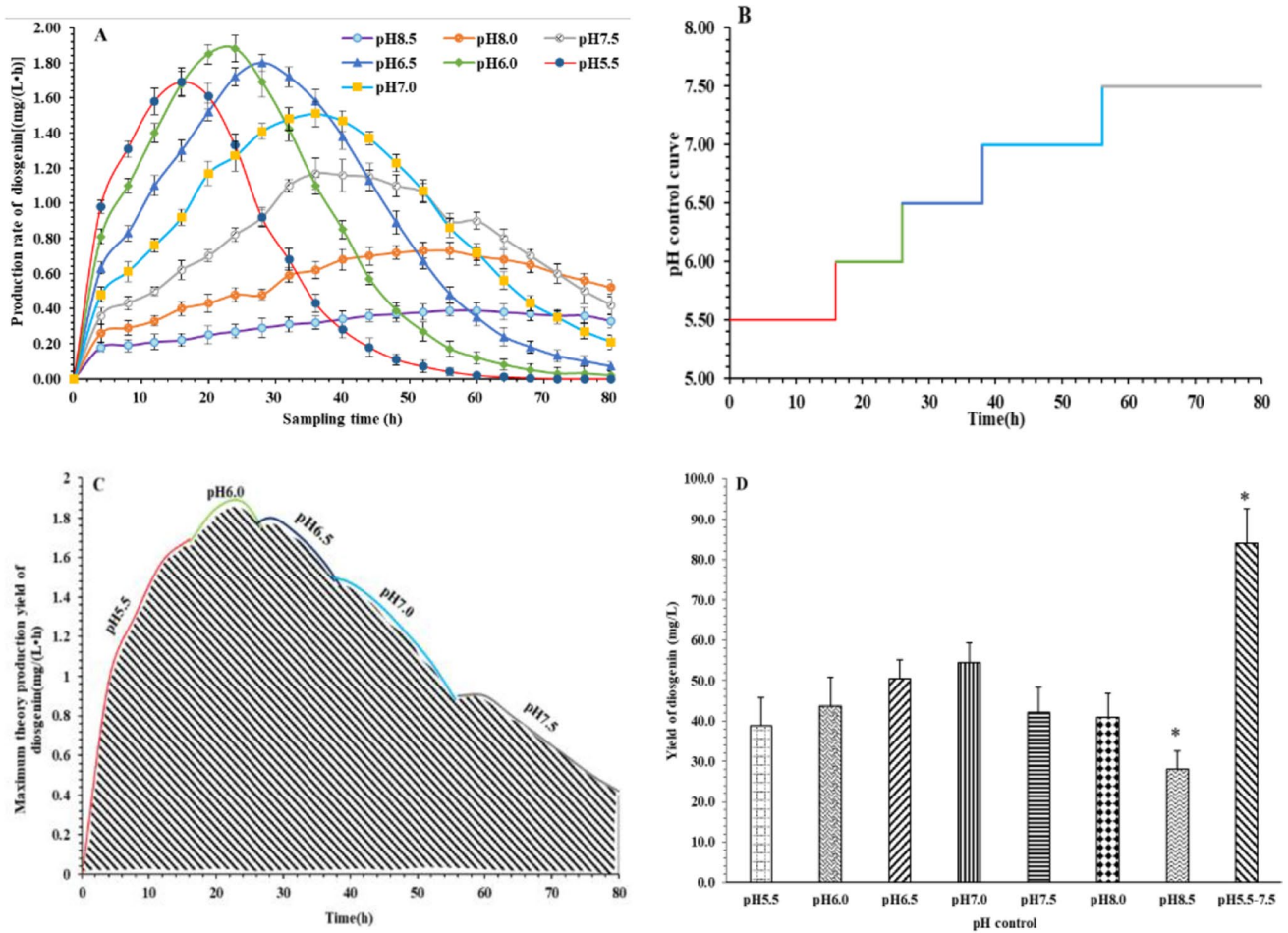


Fig. 4 Effect of pH values on the production rate of diosgenin and optimization strategy. **A** Effect of pH on production rate of diosgenin; **B** phased pH optimization control strategy; **C** schematic diagram of

32 h, 36 h, 37 h, 52 h, and 56 h, respectively. Moreover, when the fermentation time varies from 60 to 80 h, the production rate of diosgenin rapidly decreases at pH 5.5, 6.0, and 6.5, and closes to 0 at 80 h, but at that moment the production rate of diosgenin still maintains at $0.21 \pm 0.043 \text{ mg L}^{-1} \text{ h}^{-1}$, $0.42 \pm 0.050 \text{ mg L}^{-1} \text{ h}^{-1}$, and $0.33 \pm 0.036 \text{ mg L}^{-1} \text{ h}^{-1}$ at pH 7.0, 7.5, and 8.0, respectively. Therefore, a phased pH control is very beneficial to improve production rate of diosgenin.

When the phased pH control strategy as showed in Fig. 4B is used, the maximum theoretical yield of diosgenin should be the shaded area shown in Fig. 4C. The practice results of the phased pH control strategy indicated that the diosgenin yield was $85 \pm 8.6 \text{ mg L}^{-1}$, significantly higher than that of any single-pH control (yield in order from low pH to high pH: $38.9 \pm 6.8 \text{ mg L}^{-1}$; $43.7 \pm 7.1 \text{ mg L}^{-1}$; $50.4 \pm 4.8 \text{ mg L}^{-1}$; $54.4 \pm 4.9 \text{ mg L}^{-1}$; $42.1 \pm 6.3 \text{ mg L}^{-1}$; $40.9 \pm 5.9 \text{ mg L}^{-1}$; $28.1 \pm 4.5 \text{ mg L}^{-1}$) as shown in Fig. 4D. Thence, the phased pH control strategy is actually beneficial

maximum production rate of diosgenin using phased pH control strategy; **D** verification of maximum yield using phased pH control strategy

to improve the batch fermentation yield of diosgenin of SYt1.

Discussion

In this work, an endogenous *Bacillus licheniformis* SYt1 was firstly isolated from DZW, which was proved to produce diosgenin. As one of the sterol family, diosgenin was often synthesized by eukaryotes for critical cellular functions including maintaining membrane fluidity, phagocytosis, stress tolerance, and cell signaling (Riobo 2012; Wei et al. 2016), but not typically produced by bacteria, because bacteria still lacked homologs of the eukaryotic proteins required for the modifications such as demethylation and saturation in metabolic pathway. However, a few bacterial species such as γ -proteobacteria (Banta et al. 2015) and δ -proteobacteria (Bode et al. 2003) had been reported to produce cholesterol, a kind of sterol with a

similar structure to diosgenin. Bacterial sterol synthesis pathways are still unknown (Wei et al. 2016). Given the sporadic and sparse distribution of sterol synthesis in the bacterial domain, it has been suggested that bacteria most likely acquired this biosynthetic pathway through horizontal gene transfer from eukaryotes (Bode et al. 2003; Summons et al. 2006; Wei et al. 2016). This reasoning has not been confirmed. *B. licheniformis* SYt1 is one endogenous bacterium of *DZW*, and horizontal gene transfer might highly probably happen between them. However, very few information is available for discussion. If the horizontal gene transfer was true, the strain should produce saponins in its metabolic process. Unfortunately, saponins have not been determined by us in the process, which suggested that another compound similar to diosgenin in structure might be formed in the strains. A possible biosynthesis pathway of diosgenin in plant is illustrated in Fig. 5 according to the previous reports (Ciura et al. 2017; Zolfaghari et al. 2020). In this way, acetyl-CoA is converted to isopentenyl pyrophosphate (IPP) through MVA pathway, and the latter is transformed to cholesterol through subsequent 13 possible steps. Cholesterol is subsequently metabolized to be diosgenin undergoing 7-step biocatalytic reactions. All organisms must be able to produce both IPP (Thibodeaux and Liu 2017). Therefore, cholesterol probably is one critical precursor of diosgenin.

A verification experiment was performed by us through adding cholesterol as the sole carbon source in medium. The obtained results are displayed in Fig. 6. Lane 1 indicates that no conversion from added cholesterol (0.01 g/L) as sole carbon source to diosgenin happened when no living strain SYt1 was inoculated in medium. Lane 2 shows that some added cholesterol had been converted to diosgenin by inoculated strain SYt1 in medium when 0.2 g/L cholesterol was added as sole carbon source. Lane 5 shows that all cholesterol in Lane 2 had been almost converted

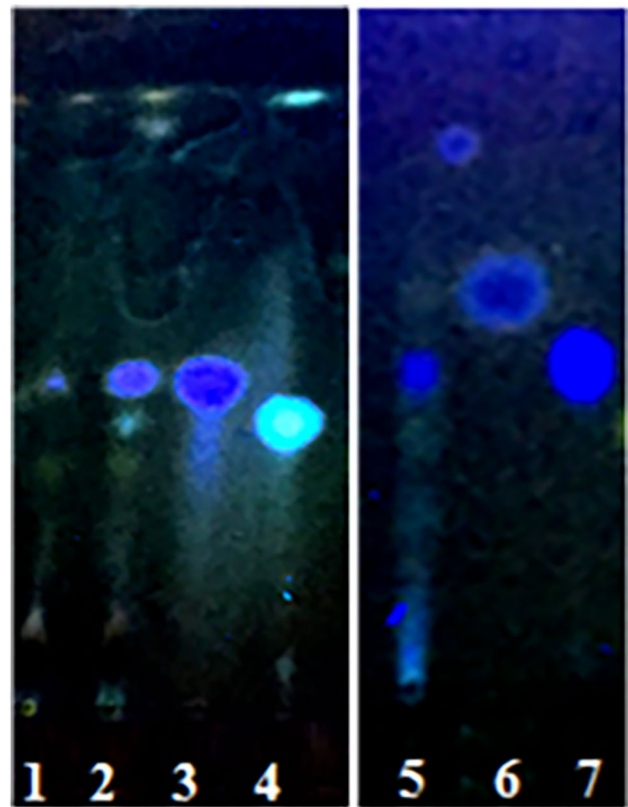
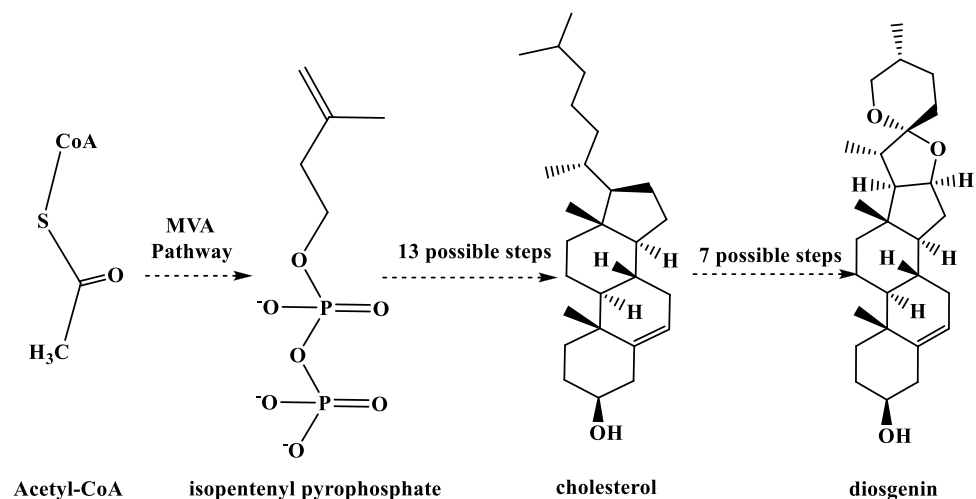


Fig. 6 Conversion evidence from cholesterol to diosgenin using thin-layer chromatography. Lane 1: control (no strain SYt1 inoculation, 0.01 g/L cholesterol addition); lane 2: part bioconversion of cholesterol to diosgenin by strain SYt1 (0.2 g/L cholesterol addition); lane 3: cholesterol standard (0.2 g/L); lane 4: diosgenin standard (0.2 g/L); lane 5: almost complete bioconversion of cholesterol to diosgenin by strain SYt1 (0.2 g/L cholesterol addition); lane 6: cholesterol standard (0.2 g/L); lane 7: diosgenin standard (0.2 g/L)

Fig. 5 Possible biosynthesis pathway of diosgenin in *fenugreek* (Ciura et al. 2017; Zolfaghari et al. 2020)



to diosgenin by the presented strain *SYt1*. Therefore, the results suggested that cholesterol may be one precursor of diosgenin. Because it was found that diosgenin production rate was directly proportional to cell biomass of *SYt1*, the potential precursors were presumed to exist in the cell wall of *SYt1*.

The biosynthesis of diosgenin was a multi-step biocatalytic reaction process, in which it required the step-by-step participation of multiple enzymes (Ciura et al. 2017; Zolfaghari et al. 2020), and the optimal pH might vary from step to step for diosgenin biosynthesis. Therefore, an optimized pH adjustment seems to be necessary to improve the yield of diosgenin. The practical results in Fig. 4 indicated that the phased pH optimization control strategy was suitable for the multi-step biocatalytic reaction process of precursors or diosgenin, while some parallel reactions may be suppressed. The dynamic pH control strategy has achieved excellent results in the batch fermentation tests of other microorganisms (Feng et al. 2014; Wu et al. 2017; Yue et al. 2021).

Admittedly, this is a complicated multi-step process. To expose this mechanism, the corresponding enzymes are needed to be further purified and simulated these biocatalytic reactions, which is a very burdensome and difficult work. However, it is a very interesting and worth exploring topic in metabolomics and genomics in the future.

Most papers about the microbial production methods of diosgenin relied on microorganisms to convert the saponins in *DZW* to diosgenin in vivo instead of acid hydrolysis process of plant in vitro (Chen et al. 2018; Cheng et al. 2015; Liu et al. 2010; Zhu et al. 2010). The proposed method is different from previous reports because it does not require *DZW* to produce diosgenin. In short, this study demonstrates that endogenous *B. licheniformis SYt1* isolated from *DZW* can produce diosgenin in fermentation processing. pH plays an important role to improve the yield of diosgenin in the process under aerobic culture conditions. A phased pH optimization control strategy from 5.5 to 7.5 can get maximal yield 85 ± 8.6 mg L⁻¹ diosgenin, which is higher than that of any single pH control. This is a new way to produce diosgenin through microorganism fermentation without *DZW* as substrate, which may replace large amounts of plant extracts to produce diosgenin in the future.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00253-021-11679-z>.

Acknowledgements We thank the colleagues in research platform of College of Life Sciences provided experimental convenience in the work.

Author contribution C. L. W. is equal to the first author. Z. Q. H. and B. F. Q. conceived the project, and designed the experiments. Z. Q. H., C. L. W., and L. F. Z. analyzed the results and wrote the manuscript. L. T. P., S. Y. H., M. J., Y. S. X., and R. C. constructed and executed all

the experiments. Z. H. L. polished the whole paper. All authors read and approved the manuscript.

Funding The work was supported by the Shaanxi Province Strategic Emerging Industry Major Product (Group) Project (No. 2015KTCQ03-05) and the Shaanxi Province Key R&D Program (2021SF-387).

Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

Conflict of interest The authors declare no competing interests.

References

- Bai Y, Zhang L, Jin W, Wei M, Zhou P, Zheng G, Niu L, Nie L, Zhang Y, Wang H, Yu L (2015) In situ high-valued utilization and transformation of sugars from *Dioscorea zingiberensis* C.H. Wright for clean production of diosgenin. *Bioresour Technol* 196:642–647. <https://doi.org/10.1016/j.biortech.2015.08.010>
- Banta AB, Wei JH, Welander PV (2015) A distinct pathway for tetrahymanol synthesis in bacteria. *Proc Natl Acad Sci U S A* 112(44):13478–13483. <https://doi.org/10.1073/pnas.1511482112>
- Bode HB, Zeggel B, Silakowski B, Wenzel SC, Reichenbach H, Müller R (2003) Steroid biosynthesis in prokaryotes: identification of myxobacterial steroids and cloning of the first bacterial 2,3(S)-oxidosqualene cyclase from the myxobacterium *Stigmatella aurantiaca*. *Mol Microbiol* 47(2):471–481. <https://doi.org/10.1046/j.1365-2958.2003.03309.x>
- Cavagna M, Dell'Anna R, Monti F, Rossi F, Torriani S (2010) Use of ATR-FTIR microspectroscopy to monitor autolysis of *Saccharomyces cerevisiae* cells in a base wine. *J Agric Food Chem* 58(1):39–45. <https://doi.org/10.1021/jf902369s>
- Chen Y, Dong Y, Chi Y, He Q, Wu H, Ren Y (2018) Eco-friendly microbial production of diosgenin from saponins in *Dioscorea zingiberensis* tubers in the presence of *Aspergillus awamori*. *Steroids* 136:40–46. <https://doi.org/10.1016/j.steroids.2018.05.005>
- Chen Y, Tang Y-M, Yu S-L, Han Y-W, Kou J-P, Liu B-L, Yu B-Y (2015) Advances in the pharmacological activities and mechanisms of diosgenin. *Chin J Nat Medicines* 13(8):578–587. [https://doi.org/10.1016/S1875-5364\(15\)30053-4](https://doi.org/10.1016/S1875-5364(15)30053-4)
- Cheng Y, Dong C, Huang C, Zhu Y (2015) Enhanced production of diosgenin from *Dioscorea zingiberensis* in mixed culture solid state fermentation with *Trichoderma reesei* and *Aspergillus fumigatus*. *Biotechnol Biotechnol Equip* 29(4):773–778. <https://doi.org/10.1080/13102818.2015.1032352>
- Ciura J, Szeliga M, Grzesik M, Tyrka M (2017) Next-generation sequencing of representational difference analysis products for identification of genes involved in diosgenin biosynthesis in *fenugreek* (*Trigonella foenum-graecum*). *Planta* 245(5):977–991. <https://doi.org/10.1007/s00425-017-2657-0>
- Davies D (1990) Chirality, drug metabolism and action. Academic Press, Salt Lake City, pp 45–51
- Dawson RF (1991) Diosgenin production in North America—a brief history. *HortTechnology* 1(1):22–27. <https://doi.org/10.21273/horttech.1.1.22>

- Duan J, Dong X, Shen Y, Gao B, Zhang Z, Gao T, Wang M (2018) Simultaneous determination of enantiomers of carfentrazone-ethyl and its metabolite in eight matrices using high-performance liquid chromatography with tandem mass spectrometry. *J Sep Sci* 41(19):3697–3705. <https://doi.org/10.1002/jssc.201701349>
- Eibler D, Vetter W (2017) Enantioseparation and optical rotation of flavor-relevant 4-alkyl-branched fatty acids. *J Chromatogr A* 1505:87–95. <https://doi.org/10.1016/j.chroma.2017.05.011>
- Feng S, Yang H, Zhan X, Wang W (2014) Novel integration strategy for enhancing chalcocopyrite bioleaching by *Acidithiobacillus sp.* in a 7-L fermenter. *Bioresour Technol* 161:371–378. <https://doi.org/10.1016/j.biortech.2014.03.027>
- Fernandes P, Cruz A, Angelova B, Pinheiro HM, Cabral JMS (2003) Microbial conversion of steroid compounds: recent developments. *Enzyme Microb Technol* 32(6):688–705. [https://doi.org/10.1016/S0141-0229\(03\)00029-2](https://doi.org/10.1016/S0141-0229(03)00029-2)
- Gong N, Wang Y, Zhang B, Yang D, Du G, Lu Y (2019) Screening, preparation and characterization of diosgenin versatile solvates. *Steroids* 143:18–24. <https://doi.org/10.1016/j.steroids.2018.11.016>
- Kos G, Lohninger H, Krška R (2003) Development of a method for the determination of fusarium fungi on corn using mid-infrared spectroscopy with attenuated total reflection and chemometrics. *Anal Chem* 75(5):1211–1217. <https://doi.org/10.1021/ac0260903>
- Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis Version 7.0 for Bigger Datasets. *Mol Biol Evol* 33(7):1870–1874. <https://doi.org/10.1093/molbev/msw054>
- Lepage C, Liagre B, Cookmoreau J, Pinon A, Beneytout JL (2010) Cyclooxygenase-2 and 5-lipoxygenase pathways in diosgenin-induced apoptosis in HT-29 and HCT-116 colon cancer cells. *Int J Oncol* 36(5):1183–1191. https://doi.org/10.3892/ijo_00000601
- Li P, Mou Y, Lu S, Sun W, Lou J, Yin C, Zhou L (2012) Quantitative determination of diosgenin in *Dioscorea zingiberensis* cell cultures by microplate- spectrophotometry and high-performance liquid chromatography. *Afr J Pharm Pharmacol* 6(15):1186–1193. <https://doi.org/10.5897/AJPP11.440>
- Liu L, Dong Y-S, Qi S-S, Wang H, Xiu Z-L (2010) Biotransformation of steroidal saponins in *Dioscorea zingiberensis* C. H. Wright to diosgenin by *Trichoderma harzianum*. *Appl Microbiol Biotechnol* 85(4):933–940. <https://doi.org/10.1007/s00253-009-2098-1>
- Macêdo RO, Barbosa-Filho JM, da Costa EM, de Souza AG (1999) Thermal behaviour of some terpenoids. *J Therm Anal Calorim* 56(3):1353–1357. <https://doi.org/10.1023/a:1010110624198>
- Mamay, Wahyuningrum D., Hertadi R (2015) Isolation and characterization of levan from moderate halophilic bacteria *Bacillus licheniformis* BK AG21. *Procedia Chemistry* 16:292–298. <https://doi.org/10.1016/j.proche.2015.12.055>
- Pan C, Zhao Y, Liu G, Dou G, Ru Z, Zhu K (2014) Development and demonstration of a cleaner process to produce diosgenin from *Dioscorea zingiberensis* based on physical separation. *J Clean Prod* 76:161–166. <https://doi.org/10.1016/j.jclepro.2013.12.074>
- Pidiyar VJ, Jangid K, Dayananda KM, Kaznowski A, Gonzalez JM, Patole MS, Shouche YS (2003) Phylogenetic affiliation of *Aeromonas culicicola* MTCC 3249T based on gyrB gene sequence and PCR-amplicon sequence analysis of cytolytic enterotoxin gene. *Syst Appl Microbiol* 26(2):197–202. <https://doi.org/10.1078/072320203322346047>
- Riobo NA (2012) Cholesterol and its derivatives in Sonic Hedgehog signaling and cancer. *Curr Opin Pharmacol* 12(6):736–741. <https://doi.org/10.1016/j.coph.2012.07.002>
- Summons RE, Bradley AS, Jahnke LL, Waldbauer JR (2006) Steroids, triterpenoids and molecular oxygen. *Philos Trans R Soc Lond B Biol Sci* 361(1470):951–968. <https://doi.org/10.1098/rstb.2006.1837>
- Thibodeaux CJ, Liu H-w (2017) The type II isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IDI-2): a model for acid/base chemistry in flavoenzyme catalysis. *Arch Biochem Biophys* 632:47–58. <https://doi.org/10.1016/j.abb.2017.05.017>
- Wang P, Ma C, Chen S, Zhu S, Lou Z, Wang H (2014) Conversion of steroid saponins into diosgenin by catalytic hydrolysis using acid-functionalized ionic liquid under microwave irradiation. *J Clean Prod* 79:265–270. <https://doi.org/10.1016/j.jclepro.2014.05.041>
- Wei JH, Yin X, Welander PV (2016) Sterol synthesis in diverse bacteria. *Front Microbiol* 7(990):1–19. <https://doi.org/10.3389/fmicb.2016.00990>
- Wei M, Bai Y, Ao M, Jin W, Yu P, Zhu M, Yu L (2013) Novel method utilizing microbial treatment for cleaner production of diosgenin from *Dioscorea zingiberensis* C.H. Wright (DZW). *Bioresour Technol* 146:549–555. <https://doi.org/10.1016/j.biortech.2013.07.090>
- Wu Y, Wang C, Zheng M, Zuo J, Wu J, Wang K, Yang B (2017) Effect of pH on ethanol-type acidogenic fermentation of fruit and vegetable waste. *Waste Manage (Oxford)* 60:158–163. <https://doi.org/10.1016/j.wasman.2016.09.033>
- Yang H, Yin H, Shen Y, Xia G, Zhang B, Wu X, Cai B, Tam JP (2016) A more ecological and efficient approach for producing diosgenin from *Dioscorea zingiberensis* tubers via pressurized biphasic acid hydrolysis. *J Clean Prod* 131:10–19. <https://doi.org/10.1016/j.jclepro.2016.05.030>
- Yue L, Chuan S, Yuanyuan W, Han D, Li K, Jinyuan M, Kaijun W (2021) Effect of pH dynamic control on ethanol-lactic type fermentation (ELTF) performance of glucose. *Environ Technol* 1–13. <https://doi.org/10.1080/09593330.2021.1942560>
- Zhang L, Zuo B, Wu P, Wang Y, Gao W (2012) Ultrasound effects on the acetylation of dioscorea starch isolated from *Dioscorea zingiberensis* C.H. Wright. *Chem Eng Process* 54(4):29–36. <https://doi.org/10.1016/j.cep.2012.01.005>
- Zhang Y, Tang L, An X, Fu E, Ma C (2009) Modification of cellulase and its application to extraction of diosgenin from *Dioscorea zingiberensis* C.H. Wright. *Biochem Eng J* 47(1):80–86. <https://doi.org/10.1016/j.bej.2009.07.006>
- Zhang YQ, Liang JH, Fu EH, Li BX (2007) Effect of modified enzymatic catalysis on the extraction of diosgenin from *Dioscorea zingiberensis* C H Wright. *Chem Eng Technol* 30(11):1488–1494. <https://doi.org/10.1002/ceat.200700234>
- Zhu Y-L, Huang W, Ni J-R, Liu W, Li H (2010) Production of diosgenin from *Dioscorea zingiberensis* tubers through enzymatic saccharification and microbial transformation. *Appl Microbiol Biotechnol* 85(5):1409–1416. <https://doi.org/10.1007/s00253-009-2200-8>
- Zolfaghari F, Rashidi-Monfared S, Moieni A, Abedini D, Ebrahimi A (2020) Improving diosgenin production and its biosynthesis in *Trigonella foenum-graecum* L. hairy root cultures. *Industrial Crops and Products* 145:112075. <https://doi.org/10.1016/j.indcrop.2019.112075>

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