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Novel method utilizing microbial treatment for cleaner production of diosgenin from *Dioscorea zingiberensis C.H. Wright* (DZW)

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HIGHLIGHTS

- A *Bacillus pumilus* HR19 with the great ability to secrete pectinase was screened.
- A novel microbial treatment for cleaner production of diosgenin was developed.
- The principle behind the microbial treatment is disclosed.
- The consumptions of water, acid and organic solvent were reduced significantly.
- The diosgenin yield was increased by 6.21%.

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ABSTRACT

A novel method utilizing microbial treatment for cleaner production of diosgenin from *Dioscorea zingiberensis C.H. Wright* (DZW) was presented. A new *Bacillus pumilus* HR19, which has the great ability to secrete pectinase, was screened and applied in the microbial treatment. Low-pressure steam expansion pretreatment (LSEP) was employed in advance to assist microbial treatment efficiently in releasing saponins, which are the precursors of diosgenin. Compared with the traditional process of acid hydrolysis, this novel process reduced the consumptions of water, acid and organic solvent by more than 92.5%, 97.0%, 97.0%, respectively, while simultaneously increasing the diosgenin yield by 6.21%. In addition, the microbial treatment was more efficient than enzymatic treatment, which arised from that microorganisms could be induced to secrete related enzymes by the compositions of DZW and relieve product inhibition by utilizing enzyme hydrolysates.

1. Introduction

In China, *Dioscorea zingiberensis C.H. Wright* (DZW) is a plant cultivated especially used for the production of diosgenin. (Liu et al., 2010). As it is known, diosgenin (25R-Spriost-5-en-3β-OH) is one of the most important steroidal sapogenins in the steroid pharmaceutical industry, and is mainly used as the starting material for the partial synthesis of oral contraceptives, sex hormones and other steroids (Zhang et al., 2009).

Diosgenin mainly exists in plant cells in the form of the ligand of saponin, with its C3 and C26 linked to sugar chains via saponin bonds. Saponins exist in the cells which are wrapped tightly by large amounts of lignocellulose and starch (Zhu et al., 2010). The most majority of saponins in DZW cells are water-soluble (Lu, 2010).

So far, some progress has been made for the cleaner production of diosgenin, most of which focused on removing most of the fibers and starch in plant tubers by physical or biological methods before acid hydrolysis (Huang et al., 2008; Liu et al., 2010; Wang et al., 2008), or on evaluating the effects of starch recycling and focusing on the optimal period for starch recycling. It should be noted that previous studies devoted to obtaining diosgenin employed the hydrolysis of saponins in the solid phase (Huang et al., 2008; Peng et al., 2011; Zhu et al., 2010), which could decrease the COD in wastewater by over 50% during the pretreatments. However, the large amount of acid wastewater generated remains a critical

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problem in the diosgenin industry because there is still a large amount of material present before the acidolysis.

Since most saponins in DZW are soluble in water, it will make sense to develop a method to release and enrich saponins in aqueous phase before acid hydrolysis, so as to significantly reduce the amounts of acid and organic solvents used, as well as the amount of waste water generated. To promote the release of saponins from DZW tissues, it is essential to loosen the plant cell walls by a variety of physical and biological methods. Among different pretreatment methods, steam explosion has been favored by researchers because it is low cost, pollution-free, and energy saving (Jacquet et al., 2011). More importantly, steam explosion can effectively disrupt the lignin structure and the crystalline structure of cellulose effectively (Qiu and Chen, 2012).

In order to meet the needs of large-scale production, a moderate low pressure steam expansion technique was applied in this study.

Since microorganisms can secrete enzymes rapidly responding to the ambient environment and reduce product inhibition effects through metabolism, microbial treatment is suggested to be more effective than direct enzymatic treatment in enzymatic hydrolysis. In addition, the high cost of enzymes can be reduced when microbial treatment is employed in place of enzymatic treatment. Therefore, it is of great interest to screen microorganisms which can efficiently secrete related enzymes that can disintegrate DZW compositions, and to apply them in microbial treatment to promote the release of saponins.

In this work, a new *Bacillus pumilus* HR19 was screened and applied to promote the release of saponins, after a low-pressure steam expansion pretreatment (LSEP) had been conducted to break the cell walls, which was expected to enhance the efficiency of the following microbial treatment. This novel microbial treatment method represents an efficient approach to the cleaner production of diosgenin. In addition, it can also provide inspirations for the extraction and separation of other drugs derived from lignocellulose-rich plants.

2. Methods

2.1. Materials and chemicals

The dried *D. zingiberensis C.H. Wright* (DZW) tubers used for diosgenin production were obtained from the Zhuxi county of Hubei province, China. The raw material was washed and then air dried. The dried DZW tubers were then crushed with a Chinese medicine grinder to pass through a 40-mesh screen for further treatment.

Diosgenin (98%) was purchased from Sigma, USA, while cellulase (80000 IU/g), pectinase (30000 IU/g), xylanase (5000 IU/g), β-glucosidase (40 IU/g), α-amylase (2000 IU/g) and glucoamylase (10000 IU/g) were obtained from Wuhan Huameihua International Technology Co., Ltd. All the other chemicals were of analytical grade.

2.2. An overview of the novel process for diosgenin production

The microbial treatment was employed to release saponins from DZW tissues for diosgenin production. The specific procedures are shown in Fig. 1, and briefly described as follows: The grinded DZW samples were first treated with the low-pressure steam expansion pretreatment (LSEP). Then, the consequent DZW slurry was treated with microorganisms to release saponins from intracellular environment. After liquefaction and saccharification, the mixtures were separated into solid residues and supernatant by centrifugation. The supernatant, which was rich of water-soluble saponins, was further purified by using microfiltration (MF) and nanofiltration (NF) membrane separation technique to enrich saponins which can be converted to diosgenin via acid hydrolysis. The detailed descriptions of LSEP, microbial treatment and enrichment of saponins are given in subsequent sections, respectively.

As comparison, the traditional process for diosgenin production was also conducted (Huang et al., 2008). Dried and ground tuber powder was first hydrolyzed by acid. After filtration and centrifugation, the solid residue was collected and dried. Soxhlet extraction using petrol as solvent was then performed to extract diosgenin from the dried and hydrolytic tuber powder.

2.3. Low-pressure steam expansion pretreatment (LSEP)

LSEP was carried out in a pressure equipment fitted with a high pressure container, storage tank, heating pan, gas vent line, high temperature and pressure ball valve, a pressure gauge, and a temperature gauge (Zhang et al., 2008). The DZW powder prepared with and without water added was placed in the storage tank and treated at 0.05–0.20 MPa with water-saturated steam.

2.4. Enzymatic treatment

DZW samples (50 g) after LSEP were loaded into a 1000 mL Erlenmeyer flask containing 300 mL of deionized water. The pH of the mixture was adjusted to 4.0–4.2 with a 0.1 M HCl solution. In the combined enzyme treatment, cellulase, pectinase and xylanase were quantified accurately and dispersed in buffer to obtain predefined enzyme loadings of 200 IU/g dry DZW powder. Sole

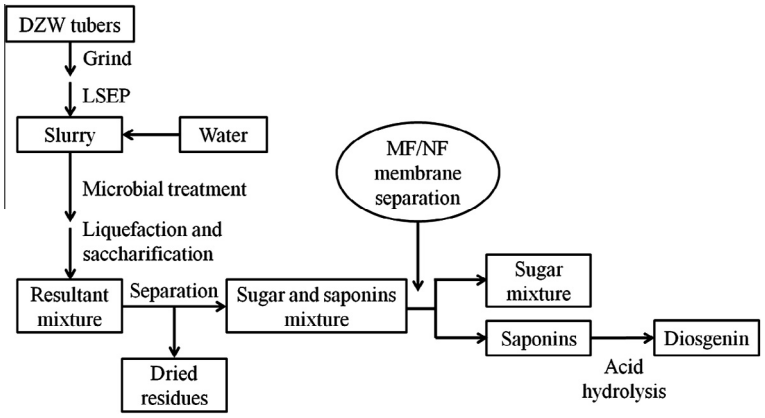

Fig. 1. Flow chart of the new process proposed for diosgenin production.

enzyme treatment was carried out with only cellulase, pectinase or xylanase quantified in the same way as for the combined enzyme treatment. All the treatments were applied for 12–24 h with agitation at 50 °C. Each treatment was repeated three times under the same conditions.

2.5. Microbial treatment

The strains were first screened preliminarily according to their ability to secrete pectinase from rot of DZW during storage (Chatterjee et al., 1995). Then based on the release degree of saponins and the processing time, the optimal microorganism(s) was (were) carefully selected from the strains obtained in the first step to be applied in the subsequent microbial treatment.

The strains were stored in LB or PDA flask at 4 °C and subcultured routinely at an interval of 4 weeks. The inoculum was either grown in LB on a shaking incubator at 150 rpm at 37 °C for 10 h, or in PDA on a shaking incubator at 150 rpm at 28 °C for 28 h.

The microbial treatment was carried out in 1000 mL Erlenmeyer flasks with 50 g of DZW samples after LSEP and 300 mL deionized water. From 10% to 20% of inoculum was aseptically inoculated in each flask, which was not sterilized. The cultures were maintained at 30–38 °C at a shaking speed of 150 rpm for 1, 2, 3, 4, 5 or 6 h, respectively, and then boiled on a hot plate for 10 min to inactivate the hydrolytic enzymes. All the cultures were grown in triplicate.

2.6. Enrichment of the water-soluble saponins

Liquefaction and saccharification of the above-mentioned mixtures (Sections 2.4 and 2.5) were achieved via a double enzyme method (Lu et al., 2009). Then, the mixture was cooled to room temperature before centrifugation at 5000 rpm for 8 min. The supernatant was rich in water-soluble saponins and reducing sugars. A ceramic microfiltration membrane (SJM-filter M-012, 0.22 μm, 0.5 m^2, China) and an organic nanofiltration membrane (GE-DK2540F1073, 150–300 Dalton, 0.5 m^2, America) were used to filter the supernatant and enrich the water-soluble saponins. Since NF can intercept high molecular weight substances like saponins ($M > 800$), the NF system was designed to filter reducing sugars for the enrichment of saponins. The residue was dried at 60 °C for subsequent chemical analysis.

2.7. Analysis method

The amounts of cellulose, hemicellulose and lignin in the residues from the cultures maintained for respectively 1, 2, 3, 4, 5 and 6 h were determined according to the procedures described in previous work (Goering and Van Soest, 1970). The degradation of each composition was characterized in terms of composition as weight percentages of the total mass of the residue for different treatment times. The starch content was calculated according to the amount of reducing sugar, determined by the DNS method (Miller, 1959).

Cellulase activity was determined according to the method of Mandels and Sternberg (1976). Pectinase activity was determined by the method of Oliveira et al. (2006). α-Amylase activity was measured by the method developed by Bernfeld (1955). The xylanase assay was performed by measuring the formation of xylose, characterized by an absorbance signal at 540 nm (Mosier et al., 2005). β-Glucosidase activity was determined according to the method proposed by Li et al. (2013).

Diosgenin was quantified by high-performance liquid chromatography (HPLC) analysis (Zhang et al., 2009). The yields of diosgenin were presented in the form of gram of product/100 g of dried DZW. The release degree of saponins under the different treatment conditions was expressed as the ratio of the content of diosgenin in the liquid phase to that in DZW powder.

2.8. Optimization of the microbial treatment conditions by orthogonal design

Orthogonal design based on three levels and three variables was applied to optimize the dosage of inoculum (10–20%), treatment time (3–5 h) and temperature (30–38 °C) during pretreatment for the maximum release of water-soluble saponins and diosgenin production. p-Values of less than 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Low-pressure steam expansion pretreatment (LSEP)

3.1.1. Optimal conditions for the steam expansion pretreatment

To explore the optimal conditions under which the steam expansion pretreatment should be conducted, the influence of the ratio of water to DZW powder and the operation pressure used on the release degree of saponins were studied (data not shown).

It was found that the ratio of water to DZW powder could have some influence on the efficiency of steam expansion. When no water was added to the DZW powder, the osmosis of water vapor was weakened to the extent that it was not powerful enough to break the bonds to lignin. On the contrary, when too much water was added to the DZW powder, less vapor acted on the lignin bonds (Zhang et al., 2008). In both cases, the release degrees of saponins were quite low because of the decreased degradation of lignocelluloses. On the other hand, the operation pressure could also affect the efficiency of steam expansion. It is important to choose an appropriate operation pressure which is powerful enough to break the cell wall structure of the DZW tissues and save energy as much as possible simultaneously. Based on the above considerations, the optimal conditions for the steam expansion pretreatment were concluded as follows: volume-to-mass ratio of water to DZW powder (mL/g) fixed at 5:1, and an operation pressure set at 0.15 MPa.

3.1.2. Influence of LSEP on the release degree of saponins

To investigate the influence of LSEP on the release degree of saponins, the DZW powders subjected to LSEP (0.15 MPa and 5:1 DZW powder to water ratio) and without the pretreatment were used in the process, respectively. It was shown that LSEP significantly increased the release degree of saponins from 19.5% to 39.8%. Previous studies have shown that steam explosion treatment of straw can help loosen the structure of lignocellulose, making it more vulnerable to enzymes and microorganisms (Alvira et al., 2010). Moreover, a wet explosion pretreatment followed by enzymatic hydrolysis was reported to be superior for releasing high concentrations of monomeric sugars from loblolly pine (Rana et al., 2012).

SEM imaging was performed to compare the morphology of the samples obtained with and without LSEP. The starch cells not subjected to LSEP were intact in shape and tightly packed. On the contrary, the cells after LSEP were well dispersed and partly broken due to cavitation effects caused by steam expansion. The adhesion between starch cells was presumably disrupted by the high temperature and pressure during the LSEP. These morphological changes caused by LSEP could increase the contact area between the enzymes and their substrates, which was likely to promote the release of saponins from DZW tissues.

Safranin fast green staining and paraffin sectioning were also carried out, to further investigate the effects of LSEP on the cell wall structure of the plant tissues and to show the changes in

Table 1
Effect of different treatments on the diosgenin yield and the release degree of saponins in the liquid phase.

Parameters	E	PX	P	X	C
[a]	2.65 ± 0.07	2.25 ± 0.06	2.65 ± 0.07	2.29 ± 0.08	1.49 ± 0.05
[b]	69.80 ± 1.63	59.30 ± 1.32	69.60 ± 1.45	60.40 ± 1.65	39.20 ± 1.52
[c]	24	24	12	12	24

E: Combination of pectinase, xylanase and cellulase; PX: combination of pectinase and xylanase; P: pectinase; X: xylanase; C: control.
[a] Diosgenin yield (g/100 g of dried DZW).
[b] The release degree of saponins (%).
[c] The time required (h).

intracellular substances before and after LSEP. It is well known that safranin is a basic dye, which can stain the nucleus and lignified cell walls red, while solid green is an acid dye, which can dye the cytoplasm and cell walls green. After LSEP, the basic peripheral lignified cell wall was nearly dissolved, and the inside fibrosis cell wall was swelled or even partly broken under the influence of high temperature and pressure. In contrast, there were thick layers of sclerenchyma surrounding the plant cells which were not treated with LSEP. This result also shows that after LSEP the lignocellulosic structure of the DWZ was damaged, which facilitated the accessibility of the steam-expanded material to the various enzymes (Jurado et al., 2009). It should be noted that there was much material released into the extracellular matrix, dyed red by safranin, after LSEP, which again suggests that LSEP could greatly favor the release of saponins.

3.2. Enzymatic treatments

3.2.1. Effect of different enzymatic treatments

The release degrees of saponins and diosgenin yields in the liquid phase from the subsequent steps under different enzymatic treatments were shown in Table 1. The total diosgenin yield (including the liquid and solid phases) was 3.80 ± 0.09 g/100 g of dried DZW powder while it was 3.06 ± 0.06 g/100 g of dried DZW powder by the traditional method. It should be noted that the different enzymatic treatments didn't affect the total amount of saponins released but affected its distribution in the solid phase vs. the liquid phase. Besides, it is interesting to note that the release degree of saponins with pectinase (P) treatment was relatively high, which was close to that under combination of pectinase, xylanase and cellulose (E) treatment but higher than those under combination of pectinase and xylanase (PX) treatment and xylanase (X) treatment. This indicates that pectinase is the key enzyme to degrade the DWZ samples.

3.2.2. Process analysis of E treatment

Since the release degree of saponins was still insufficient, the diosgenin yield in this method was lower compared with the conventional method (3.06 ± 0.06 g/100 g of DZW powder). To find out the reason why the saponins could not be released to the liquid phase completely, the dynamic changes in the activity of the various enzymes were determined every 3 h. As expected, the activity of all three enzymes decreased with time. Especially after 9 h, the activity of the enzymes decreased more quickly and became almost undetectable after 24 h (Fig. 2a). This could be attributed not only to product inhibition but also to the inhibition by intermediates and impurities produced in the enzymatic reaction (Andrić et al., 2010). Previous studies have shown that coincident with thermochemical one-step steam explosion pretreatment, the generation of by-products such as furfural, 5-hydroxymethyl furfural (HMF), and soluble ligninacetic can significantly inhibit enzymatic hydrolysis (Zhang et al., 2012).

In addition, composition analysis of DZW samples was performed. It is known that lignin is a major hindrance in the enzymatic hydrolysis of lignocellulosic materials (Kim et al., 2003).

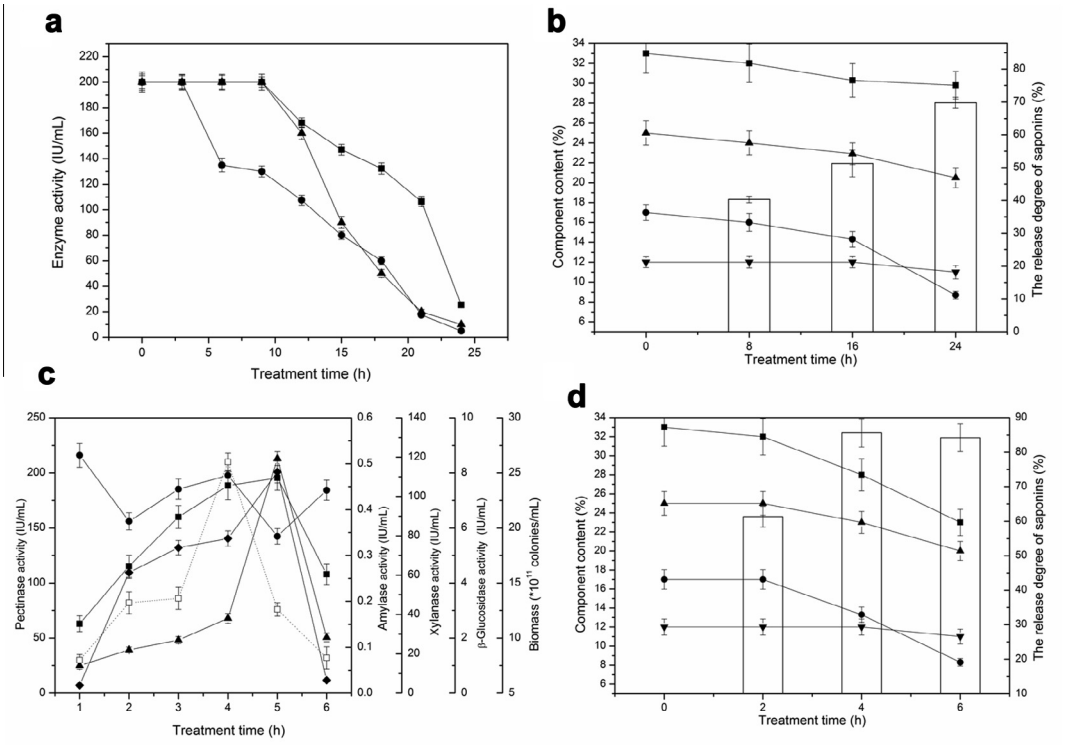

Fig. 2. Dynamic change analysis of the different enzymes, the composition and release degree of saponins in the enzymatic and microbial treatments. (a) (●) Pectinase activity, (■) cellulase activity, (▲) xylanase activity in enzyme treatment; (c) (●) pectinase activity, (■) β-glucosidase, (▲) xylanase activity (♦) amylase activity, (□) biomass in microbial treatment; (b) and (d) (■) starch content, (▲) hemicellulose content, (●) cellulose content, (▼) pectinase content in the enzymatic and microbial treatments, and the histogram denotes the release degree of saponins.

Fig. 2b shows that the degradations of cellulose, hemicellulose and starch were very limited, and especially the degradation of lignin, which could restrict the release of saponins. Thus it is crucial to develop a better way to split and degrade the saponin wrapping substances. Since it is the attenuation of enzyme activity and product inhibition that seem to affect enzymatic hydrolysis, it was suggested that microorganisms that can secrete degrading enzymes could be useful in the process instead of the enzymatic treatments.

3.3. Microbial treatment

3.3.1. Screening of microorganisms

To promote the release of saponins, it is first required to destruct the cell walls and the pectin between the cells, and then to break down the starch, proteins and other substances which thread the saponins. According to the results of the enzymatic treatments, pectinase is the key enzyme to degrade the DWZ samples after LSEP.

The microorganisms were screened in two steps. Firstly, they were screened preliminarily based on their ability to secrete pectinase from rot of DZW during storage (Chatterjee et al., 1995). Six bacteria and two fungi which can secrete pectinase greatly were isolated on that basis. Then, a new bacterium was selected from the above microorganisms by considering the release degree of saponins and the processing time comprehensively.

This new bacterium was identified by 16S rRNA analysis and was named *B. pumilus* HR19. The phylogenetic tree of *B. pumilus* HR19 was given in E-supplement (Fig. S1). It has been stored in China Center for Type Culture Collection (CCTCC), and the accession No. is M2013309.

Bacillus pumilus HR19 is a gram-positive, spore-forming bacillus. The bacteria show a rod-like morphology with the length between 1.7 and 2.0 μm and the width between 0.7 and 0.9 μm. Motile cilia are present on bacterium surfaces in large numbers. The spores emerging near the middle of bacteria are ellipsoidal, with the sporangium slightly inflated.

3.3.2. Optimal conditions for the microbial treatment

It has been observed that the fermentation time of the ZDW materials has some effects on the release degree of saponins. A series of fermentation times, i.e. 6, 12, 18 and 24 h, were applied and their effects on the the release degree of saponins were studied. It was found that the release degree of saponins already reached a relatively high level when the fermentation time was set to 6 h. In order to maximize the release degree of saponins and to reduce the production cost, orthogonal design was applied to explore the optimal conditions for the microbial treatment. *B. pumilus* HR19 is a facultative anaerobic bacterium, which does not require much dissolved oxygen, strictly speaking. In addition, the pH in the medium can be regulated through the metabolism of the bacterium during the initial period of the treatment. Thus in the orthogonal experiments, the factors oxygen and pH were not considered, but the treatment time (A), dosage of inoculums (B), and temperature (C) were selected as the variables to study their effects on the release degree of saponins.

From Table 2, it can be seen that the influencing factors of A, B, C were in descending order. It was evident that the release degree of steroidal saponins increased as the dosage of inoculum increased. On the other hand, the treatment time and temperature showed a synergistic effect on the release degree of steroidal saponins. It should be noted that further increases in the release degree could not be achieved when the two variables were increased further.

Values of "$p > F$" < 0.05 indicated which model terms were significant. In this case, A and B were significant terms which indicated that the changes in treatment time and dosage of inoculum directly influenced the release degree of steroidal saponins. Accordingly, the optimal conditions determined for the microbial treatment were: treatment time of 4 h, inoculum dosage of 15.0%, and temperature of 34.0 °C. Under these conditions, the experimental release degree of steroidal saponins achieved was as high as 85.60 ± 1.38%, which was 15.80% higher than the highest release degree achieved under enzyme treatment (69.80 ± 1.63%).

The optimal treatment time of the microbial treatment is much shorter compared with the previously published studies, in which the treatment time was 72 h or more (Virupakshi et al., 2005; Archana and Satyanarayana, 1997; Sindhu et al., 2006). It indicates this novel microbial treatment process can save much time, which is advantageous especially in industrial application. On the other hand, it has been reported that the optimal inoculum was 15% (v/w) in the production of xylanase by *Bacillus licheniformis* A99 (Archana and Satyanarayana, 1997), and the optimal temperature was 35 °C for the production of xylanase by *B. pumilus* (Virupakshi et al., 2005), which are well accordant with the optimal conditions for the microbial treatment in the present work.

3.3.3. Mechanism of microbial treatment with B. pumilus HR19

The dynamic change analysis of various enzymes was monitored according to the following procedure in the microbial treatment, to gain insight into the mechanism of the microbial treatment.

The extracellular enzymes produced during microbial treatment were first extracted and assayed to determine the activities of hydrolytic enzymes. Extracts from non-inoculated DZW samples showed no obvious enzyme activity. The enzyme activities of pectinase, xylanase, β-glucosidase and amylase produced by *B. pumilus* HR19 in the DZW broth were shown in Fig. 2c. It can be seen that the pectinase activity remained at a high level of more than 140 IU/mL throughout the entire process. The reason for this might be that

Table 2
Orthogonal design for optimization the conditions for the release degree of saponins and the diosgenin yield.

Run	A: time (h)	B: inoculum dosage (%)	C: temperature (°C)	Release degree of saponins (%)	Diosgenin yield (%)
1	3	10	30	64.92	2.467
2	3	15	34	67.99	2.584
3	3	20	38	67.94	2.582
4	4	10	34	83.56	3.175
5	4	15	38	85.27	3.240
6	4	20	30	84.75	3.221
7	5	10	38	79.57	3.024
8	5	15	30	80.24	3.049
9	5	20	34	81.21	3.086
k_1	66.95	76.02	76.64		
k_2	84.53	77.83	77.59		
k_3	80.34	77.97	77.42		
R_j	17.58	1.95	0.95		
Optimal lever	A_2	B_2	C_2		

Table 3
Comparison of parameters for the traditional and the new methods.

	Traditional method (kg/kg diosgenin)	New method (kg/kg diosgenin)
Herb use	140	120
Water use	>393	30
Acid use	17	0.51
Wastewater	>518	25
COD	80,000	25,000
Total sugar yield	0	15.0

The values were given for production of 1.0 kg of diosgenin, based on a statistical survey of diosgenin production plants in the Shiyan region of Hubei Province, central China (traditional method) and our pilot-scale experiments in a 200 L fermenter under the aforementioned optimal conditions (new method).

the pectin in DZW was exposed after LSEP and played the role as an inducer to stimulate *B. pumilus* HR19 to secrete pectinase (Klug-Santner et al., 2006).

The changes in the activities of xylanase, β-glucosidase and amylase with time showed similar trends. Xylanase is considered to be able to decompose the cell walls of the DZW raw material as well as β-glucan, which consequently reduces the viscosity of the material and promotes the release of active substances (Kumar and Wyman, 2009). It is noted that the release degree of saponins under the microbial treatment was higher than that under the PX treatment (Table 1), a possible explanation for which is that xylanase can improve the enzymatic efficiency when it is added at the appropriate time (Kumar and Wyman, 2009). β-Glucosidase, which catalyzes the hydrolysis of β-glucosidic bonds in polysaccharides to release glucose from the non-reducing end, is involved in the synthesis and degradation of cellulosic compositions in plants, animals, and bacteria (Li et al., 2013). Since the starch of DZW samples was wrapped by cellulose, hemicellulose and lignin fragments, a small amount of available starch may not be sufficient to stimulate high amylase activity (Curn et al., 1997). Therefore, amylase showed a very low activity compared with pectinase and xylanase.

It can be seen from Fig. 2c that, to support their growth, the microbes secreted large amounts of related enzymes to degrade DZW since no other nutrient substance was present in the broth. However, the glucose produced by polysaccharide hydrolysis could be hardly detected during the first 5 h. This suggests that microbes utilized the hydrolysates to maintain their growth and multiplication (Martin et al., 2002), which can relieve product inhibition effectively. Consequently, the enzyme activities showed an upward trend at this stage. It is remarkable that microbial biomass showed a sharp decline after the fourth hour. This is probably because that the hydrolysates became insufficient to support the growth and multiplication of such a large amount of microbes during that time. Besides, another reason was that the accumulated saponins could show a toxic effect on the microbes (De Geyter et al., 2012). It is interesting to note that the glucose content in the broth was monitored to be ca. 0.2 g at the end of the sixth hour, which indicated that with the toxic influence of saponins, the fewer microbes could not utilize the hydrolysates completely. As a result, enzyme activities decreased sharply during the sixth hour because of the reoccured product inhibition effects.

Since this could not further increase the release degree of saponins, additional nutrients were not added to support the microbial growth, which would also make it easier for the subsequent separation step.

Composition analysis of the DZW samples with the microbial treatment was conducted. As shown in Fig. 2d, with the exception of lignin, which was barely reduced, the cellulose, hemicellulose and starch contents were reduced from 17.0% to 8.3%, from 25.0% to 20.0% and from 33.0% to 23.0% in 6 h, respectively. This suggests that *B. pumilus* HR19 can effectively catabolize these components as carbon source but can hardly use lignin (Kim et al., 2003).

Inconceivably, in comparison with the combination enzyme treatment, the degradation of the major components in the microbial treatment was more efficient even in 20 h less of time (Fig. 2b, d). Moreover, the release degree of saponins was 15.80% higher in the microbial treatment than that in the enzymatic treatment. It is reasonable that microbes could fully utilize enzymatic hydrolysates to relieve product inhibition to maintain a high enzyme activity, thus improved the degradation efficiency of DZW as well as promoted the release of saponins.

In addition, the impact of the tendency of microorganisms on DZW was also studied. The *Gfp* gene was introduced into the *B. pumilus* HR19 cells (Miller and Lindow, 1997). The GFP fluorescence of the transformed *B. pumilus* HR19 (named HR19-gfp) was monitored under a microscope at a wave-length of 490 nm.

HR19-gfp was also used in the microbial treatment. The fluorescence microscopy observations showed that a large number of microorganisms gathered around the DZW raw material in the microbial treatment, while the enzymes and substrates were uniformly distributed in the enzymatic treatment. This suggests that the tendency of the microorganisms could accelerate enzymatic hydrolysis. The more easily for the microbes to get close to the cell, the higher degradation degree of the cell wall can be achieved (Ding et al., 2012). As a result, the microbial treatment is more effective than the enzymatic treatment even with a treatment time 20 h shorter.

3.4. Comparison of the traditional method and the new process

The traditional and the new processes with 200 L pilot-scale were comparatively studied. The results of concerned parameters were listed in Table 3. In comparison with the traditional process, the diosgenin yield in the new process increased by 6.21%, and the consumptions of water, acid and organic solvents decreased by more than 92.5%, 97.0% and 97.0%, respectively. In addition, a sugar mixture was produced, which is a good carbon source for the fermentation industry (Lu et al., 2012). A 3-ton pilot plant-scale experiment of this novel process has also been conducted, and the results were very close to those achieved in 200 L scale. This indicates the novel process is reasonable, stable and practicable in the scale-up application. Through the new process, financial benefits would be also expected. These come mainly from the significant decrease in the use of DZW, water, acid and organic solvents, the decrease in wastewater discharge, and the production of sugar. The extra cost mainly comes from the use of membrane separation technique. Overall, this new method therefore has a great value in industrial applications.

4. Conclusion

This study has demonstrated that microbial treatment is a very competitive approach for the cleaner production of diosgenin when an ideal strain and process are utilized. The novel process involving a prior LSEP has a dual aim: not only for better contact between the microorganisms and the substrate, but also for the facilitation of components degradation. The results show that the novel method enhances product yield and thus brings economic benefits when this technology is applied industrially. More meaningfully, the dosages of water, acid and organic solvents are significantly reduced, thus greatly reducing environmental pollution problems.

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