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Screening strains for directed biosynthesis of β-D-mono-glucuronide-glycyrrhizin and kinetics of enzyme production

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Abstract

One fungus, tentatively named *Penicillium* sp. Li-3, was screened to biosynthesize β-D-mono-glucuronide-glycyrrhizin (GAMG), directly. Using glycyrrhizin as elicitor and the sole carbon source, this strain was capable of expressing β-D-glucuronidase, one intracellular enzyme with high substrate specificity. And glycyrrhizin was hydrolyzed directly into GAMG by enzyme from *Penicillium* sp. Li-3 with high production. It was found that the mol conversion ratio of this reaction was up to 88.45%. Research about kinetics of β-D-glucuronidase production showed that the cell growth and enzyme production of this strain was partial coupled. During the expressing of target enzyme, carbon catabolite repression existed, so only glycyrrhizin was the best carbon source as well as the elicitor. It was found that the surfactant (Tween 80 0.12%) could improve the ability of enzyme production markedly. Under the condition of initial pH 4.8 of the medium and 32 °C of the culture temperature, the maximum enzyme activity of 181.53 U ml^{-1} was obtained.

Keywords: β-D-mono-glucuronide-glycyrrhizin; Screening; Biosynthesis; Kinetics; Enzyme production

1. Introduction

Glycyrrhizin(18β-glycyrrhetinic-acid-3-*O*-[β-D-glucuronopyranosyl-(1 → 2)-β-D-glucuronopyranoside], GL), the main component of licorice extract (*Glycyrrhiza glabra*), is a kind of natural edulcorant as well as one component in Oriental medicine [1–9]. Hydrolyzed one distal glucuronide, GL is modified into β-D-mono-glucuronide-glycyrrhizin (GAMG), which has higher merit than GL. As a sweetener with high sweetness and low caloric, the sweetness of GAMG is 1000 times as compared with GL. In addition, LD_{50} of GAMG is 5000 mg/kg while that of GL is 805 mg/kg [10], which means that GAMG is much safer than GL. Adding in drugs, GAMG have not only activities of antivirus, anti-inflammatory, anti-tumor, etc. [11] but also higher biological utilization ratio than GL. As for these facts, GAMG is expected to be the potential substitute of GL.

Biocatalysis and biotransformation have many advantages, such as high substrate specificity, mild reaction conditions [12], which is a good way of biosynthesizing GAMG. The reaction was illustrated in Fig. 1. GAMG was produced after the distal glucuronide of GL was hydrolyzed.

Akao [13] and Kim and Lee [14] have screened bacteria from human intestinal, which could catalyze the GL hydrolyzing into GAMG, but with low enzyme specialty. In this paper, we described the screening for a highly directed biosynthesizing GAMG strain and its kinetics of enzyme production.

2. Materials and methods

2.1. Microorganism and media

Strains were isolated from *G. glabra* planting field in Shihizi Xinjiang. The medium of culture consisted of the following components (per liter): 3.0 g glycyrrhizic acid ammonium, 2.2 g KH_2PO_4, 3 g NH_4NO_3, 50 mg Bacto-yeast extract, 1 ml 1 M $MgSO_4$, and 1 ml trace element solution, pH 6. Trace element solution consisted of the following components (per liter): 55.8 g EDTA disodium salt dihydrate, 14.7 g $CaCl_2{\cdot}2H_2O$, 13.9 g

Abbreviations: GL, glycyrrhizin; GAMG, β-D-mono-glucuronide-glycyrrhizin; GA, glycyrrhetic acid

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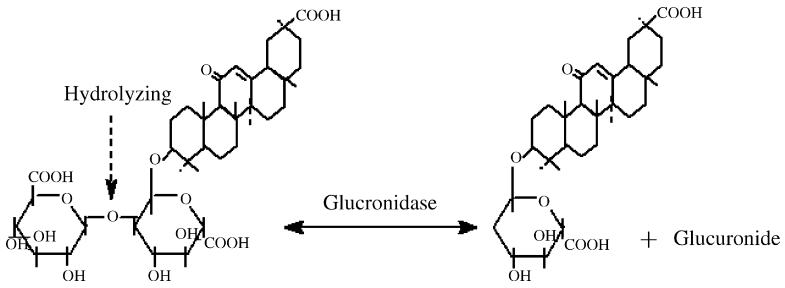

Fig. 1. Principle of biosynthesizing GAMG.

$FeSO_4{\cdot}7H_2O$, 2.9 g $ZnSO_4{\cdot}7H_2O$, 2.0 g $MnCl_2{\cdot}4H_2O$, 0.25 g $CuSO_4{\cdot}5H_2O$, 0.24 g $CoCl_2{\cdot}6H_2O$, 0.24 g $Na_2MoO_4{\cdot}2H_2O$, 0.03 g H_3BO_3, adjusted to pH 4.0 with KOH [15].

2.2. Chemicals

GL monoammonium salt and glycyrrhetic acid (GA) were purchased from Sigma Chemical Co. (USA). Methanol was chromatographic grade.

All other chemicals were of analytical reagent grade and used without further purification.

2.3. Screening strain

The samples of soil were diluted with sterile distilled water. Aliquots (200 μl) of the suspension were inoculated onto agar plates using glycyrrhizin monoammonium salt as a sole carbon and energy source, which were incubated at 28–30 °C for three days. Each colony was cultured in broth, and the fermentation was mounted directly onto a TLC plate for detection of metabolites. Fermentation, 0.5 ml, was taken to determine the molecular weight of metabolites. Mixing thoroughly with 1 ml methanol, fermentation was filtrated through disposable filter to remove insoluble materials and then applied to the LC–MS. The amount of GAMG was determined from the peak area of the chromatograms by HPLC.

2.4. Analytical methods

TLC was performed on pre-coated silica gel plate (Merck, silica gel 60F-254). Development solvents used were BuOH/$CHCl_3$/AcOH/H_2O (4/l/4/l). The plates were visualized under UV light (254 nm) [14].

The liquid chromatograph was a Hewlett-Packard (Palo Alto, CA, USA) 1100 Series system with Chemstation software. The analytical column was a ODS column (Shim-pack,VP-ODS,4.6 mm × 150 mm, Shimadzu corporation, Kyoto, Japan), and UV detector operating 254 nm. Mobile phases were water (pH 2.85 with acetic acid)/methonal (19:81). Flow-rate was 1 ml/min. Column department temperature was 25 °C. Injection volume was 20 μl. The quadrupole mass spectrometric detector was a Hewlett-Packard 1100 LC–MSD system equipped with an atmospheric pressure ionization electrospray interface. Selected ion monitoring (SIM) was performed in negative mode using *m/z* 645–649. Drying gas temperature was 320 °C the corona discharge was 3 mA.

2.5. Enzyme prepariations and assay

At the stationary phase, cells were removed by centrifugation at 10,000 rpm for 10 min. The harvested cells were washed and resuspended in acetic acid-ammonium acetate buffer (pH 4.5), and constant volume to 100 ml. Cells were broken by ultrasonic in ice-water bath, and then mixed solution was crude enzyme solution. Activities of β-D-glucuronidase were measured with glycyrrhizin monoammonium salt as substrate. The reaction medium (1 ml volume) was buffered with acetic acid-ammonium acetate buffer, pH 4.5. The reaction mixture, consisting of 200 μl of crude enzyme solution, 800 μl of 0.2% glycyrrhizin monoammonium salt, was incubated at 40 °C. The reaction was halted by boiling water bath and centrifuging for 10 min at 10,000 rpm. The supernatant was used for the determination of amount of β-D-mono-glucuronide-glycyrrhizin by HPLC. One unit (U) enzyme activity of β-D-glucuronidase was defined as the amount of enzyme that capable of converting glycyrrhizin to 1 μg β-D-mono-glucuronide-glycyrrhizin per hour under the certain conditions.

3. Results and discussion

3.1. Selection of strains

The sole carbon source (GL) in limited medium is a kind of terpenoid, which is difficult to be utilized by microorganisms. If one strain could expresses glucuronidase, it would grow well in this kind of limited medium, and vice versa. There were two kinds of productions, GAMG and GA, in the hydrolyzing reaction of GL, which lost the distal glucuronic acid and total two glucuronic acids, respectively. In this research, the strain which can biosynthesize GAMG directly was screened and the production was determined by TLC and LC–MS.

Limited medium was designed on the base of cited references [14–16], in which 65 strains, including 38 fungi and 27 bacteria, were found growing. Through the detection of fermentation with TLC, 12 strains among them were selected, which were found having another production except GAMG. And one fungus, tentatively named *Penicillium* sp. Li-3, had the largest quantity of unknown production (the clearest spot in TLC) and the fewest GA comparing with other strains (Fig. 2).

In order to determine the unknown production, LC–MS was used to analyze the fermentation of *Penicillium* sp. Li-3(Fig. 3). The molecular ion peak of this unknown production was at 645.3

Fig. 2. TLC result from fermentation of *Penicillium* sp. Li-3 (*Note*: (1) GL standard; (2) fermentation form *Penicillium* sp. Li-3; (3) glycyrrhetic acid standard).

while the molecular weight of GAMG was 648. The difference between them may be caused by the lost of hydrogen ion during ionization. From Fig. 3, it could be clearly known that the main metabolism production of this strain using GL as substrate was GAMG.

One mol GL could be inversed into GAMG 0.8845 mol, using HPLC to determine in quantity, which shows that the enzyme from this strain had high substrate specialty, and could hydrolyze GL to GAMG directly. From screening, one good strain was obtained to biosynthesize GAMG.

3.2. The expression mode of enzyme

The enzyme activity of supernatant and cell from fermentation were detected, respectively, in the whole fermentation process. No enzyme activity was found in the supernatant, while which is high in cell free. Considering the absence of enzyme activity in supernatant could be due to hydrolysis by proteinase from strain secreted, the cells were resuspended and disrupted, the cell homogenate was centrifuged, the enzyme activity of the pellets and supernatant were detected, respectively, there were higher enzyme activity in the pellets than supernatant, so this β-D-glucuronidase was one intracellular enzyme, and maybe lied in the cytolemma.

Table 1
Effect of carbon source on producing enzyme

Carbon source	Concentration (g/l)	Enzyme activity (U/ml)	Concentration of GAMG in fermentation (g/l)
GL	3	87.13	2.13
Source	10	0	0
Glucose	10	0	0
Maltpowder	5	0	0
Starch	5	0	0
Fluor	5	0	0

3.3. Influences of carbon sources and nitrogen on enzyme production

Five carbon sources (sucrose, glucose, malt, starch, flour) with GL as elicitor and GL as sole carbon source were chose, respectively, in the medium. The enzyme activity and the production of GAMG were determined with the results showing in Table 1. The enzyme expressed by *Penicillium* sp. Li-3 is a kind of inducing enzyme, which was repressed by other carbon sources and could not be expressed by *Penicillium* sp. Li-3 even with GL as elicitor, once other carbon sources were used. While this stain could produce the enzyme very well using GL as sole carbon source.

At the same time, to avoid carbon repression, different inorganic nitrogen sources were experimented to choose the best one, adding with the same mol quantity. Fig. 4 indicated that ammonium nitrate was the optimum nitrogen source among them.

Maybe β-D-glucuronidase gene from *Penicillium* sp. Li-3 was similar to *lac* operon, it was also a sort of operon. The β-D-

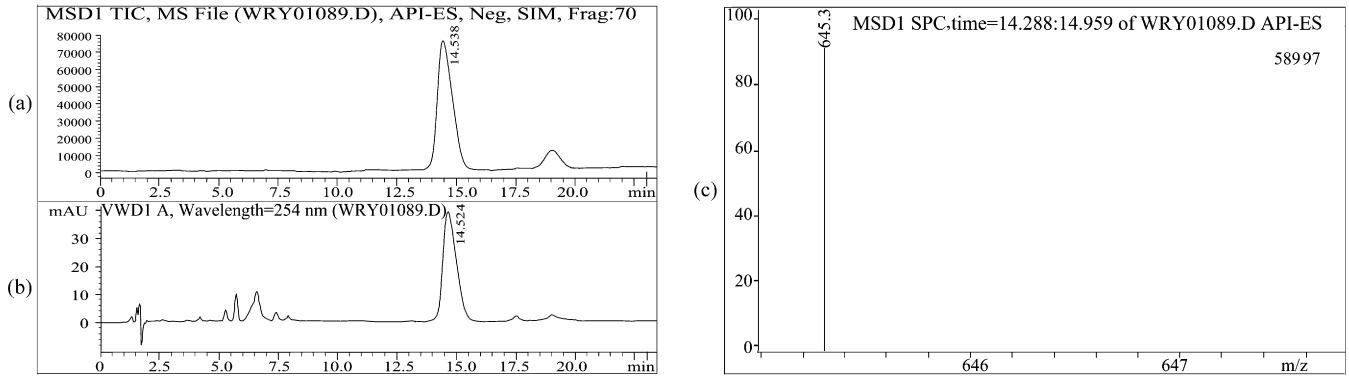

Fig. 3. LC–MS result from fermentation of *Penicillium* sp. Li-3 (*Note*: (a) responsible peak under performing in negative mode using *m/z* 645–649; (b) peaks of HPCL; (c) the corresponding peak of *m/z*).

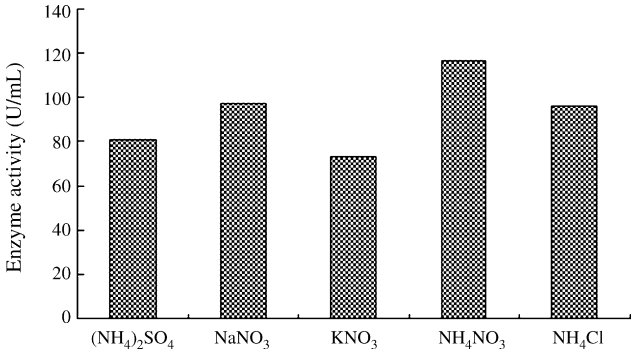

Fig. 4. Effect of nitrogen source on producing enzyme.

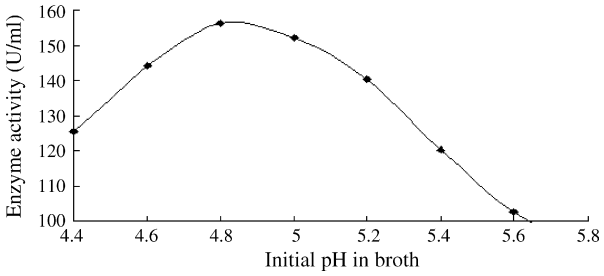

Fig. 5. Effect of initial pH in broth on producing enzyme.

glucuronidase gene had its own promoter to which RNA polymerase and initiates transcription. In the absence of an inducer (such as glycyrrhizin), β-D-glucuronidase gene was transcribed, producing repressor protein. These protein binded to the operator site and prevented transcription of the operon. In the presence of an inducer (glycyrrhizin), the inducer binded to the repressor and changed its conformation, reducing its affinity for the operator. Thus, the repressor now dissociated and allowed RNA polymerase to transcribe the operon. Then β-D-glucuronidase was expressed.

3.4. The influence of pH and culture temperature on enzyme production

The pH of growth environment is very important for the metabolism of microorganism. From Fig. 5, it can be found that the initial pH from 4.8 to 5.0 was good to make enzyme for this strain, which drop dramatically after pH up to 5.2. So optimum initial pH 4.8 was chose in the broth.

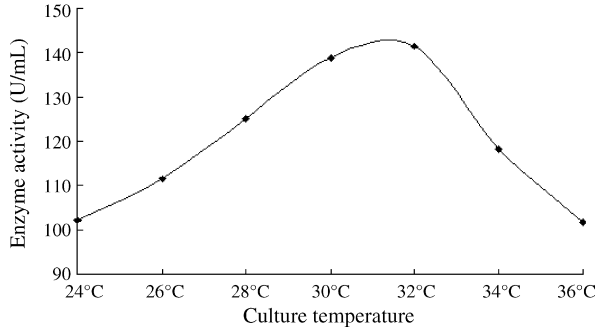

Fig. 6. Effect of culture temperature on producing enzyme.

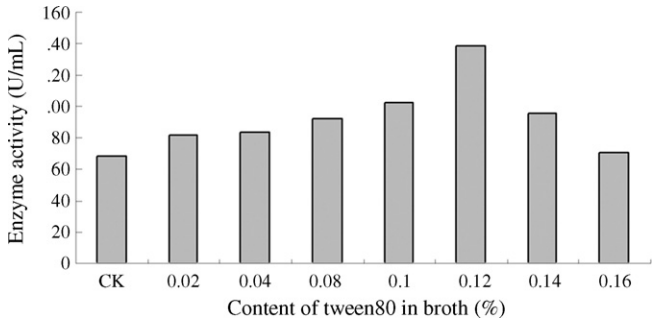

Fig. 7. Effect of Tween 80 in broth on producing enzyme.

Enzyme production experiments were carried on under different temperature (Fig. 6). Temperature range from 30 to 32 °C was good for the production of enzyme, and the highest enzyme activity was found when culture at 32 °C. When temperature was lower than 24 °C, only some mycelium were found growing with low enzyme activity. It is also not suitable for enzyme production when temperature was up to 32 °C. So, the optimum culture temperature was set at 32 °C.

3.5. The influence of surfactant on enzyme production

It was found that GL has high molecular weight and low polarity, which is not easy to be consumed by microorganisms, whose growth was limited and the ability of enzyme production was low. So surfactants with different concentration were added into the medium to increase the permeability of cell membrane. The results in Fig. 7 showed that strain cultured with surfactant had high enzyme activity comparing with the control, and the increase rate was proportional to the quantity of surfactant. Adding Tween 80 with the concentration 0.12 of the total medium, the enzyme activity reached the highest point, but dropped suddenly after adding more, which was estimated by the poison function of surfactant for the normal growth and enzyme production of this strain.

3.6. Relation of cell growth and enzyme production

In view of Fig. 8, there was obvious hysteresis between the enzyme production and cell growth. The strain expressed the

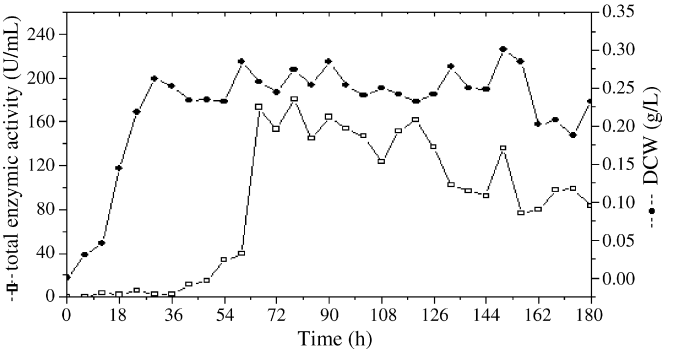

Fig. 8. Relation curve of cell growth and enzyme production (*Note*: (–●–) curve of cell growth; (–□–) curve of enzyme production).

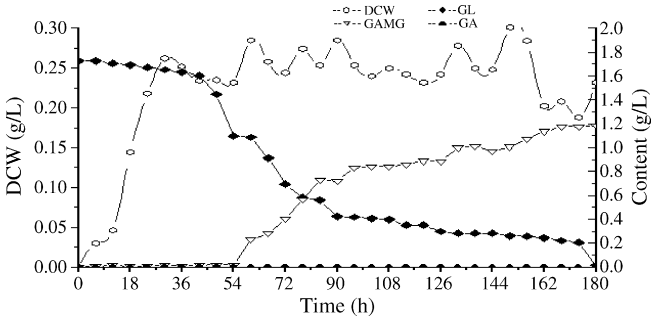

Fig. 9. Metabolism of GL during cell growth (*Note*: (–◯–) curve of cell growth; (–◆–) metabolism of GL; (–▽–) formation of GAMG; (–●–) formation of glycyrrhetic acid).

enzyme massively after 64th hour, although the change trends of enzyme production and cell growth was roughly proportional. So, the growth of *Penicillium* sp. Li-3 was partial coupled with enzyme production. The β-D-glucuronidase by *Penicillium* sp. Li-3 was a kind of induced enzyme, after the strain was induced, the active β-D-glucuronidase was expressed through transcription, translation and modification, this physiological process lagged cell growth. Massive cells could be seen at inoculating 12th hour, but no enzyme activity was determined. At the end of exponential phase, enzyme activity was increasing quickly. So the enzyme production curve could be described as following: the first 36 h was growing phase of strain without enzyme production, then the strain growth was entering stable phase, and the enzyme was expressed in large quantity. At 72 h after culture, the enzyme activity reached the peak point.

3.7. Metabolism of GL during cell growth

It can be seen that GL(Fig. 9), as the sole carbon source and energy, was utilized for strain growth at the beginning, but the utilizing ratio was rather low, which could not provide enough strain to producing enzyme. At the end of stationary phase the enzyme production, the GAMG production increased fast and continuously, which denoted that strains reaching the dead phase started to solve and set free intracellular enzyme in large quantity. In the biosynthesis of GAMG, the by-production GA was very few, indicating that this enzyme was extremely high special, directly hydrolyzing GL into GAMG.

4. Conclusion

One method for screening strains was built up in this research. Using glycyrrhizin as elicitor, one fungus, *Penicillium* sp. Li-3, expressing high substrate special β-D-glucuronidase was obtained, which could directly hydrolyze GL into GAMG. Many glucuronidase have been reported, originating from bacteria to mammals [17]. The substrate specificity of these enzymes is different. Scarce previous studies has been found on biosynthesis of GAMG by fungi. Since *Penicillium* sp. Li-3 were capable of expressing target enzyme, it could be potential candidates for biosynthesis of GAMG.

Research about kinetics of β-D-glucuronidase production by this strain showed that the growth and enzyme production of this strain was partial coupled. Different with other strains reported, carbon catabolite repression existed during the expressing of target enzyme in this strain, where glycyrrhizin was the elicitor as well as the best and sole carbon source. To our knowledge, this is first report of this phenomenon, which is helpful to the systemic research of β-D-glucuronidase by fungi.

The maximum enzyme activity of 181.53 U/ml was obtained, choosing initial pH 4.8 of the medium, the culture temperature 32 °C and the surfactant Tween 80 0.12%, which can improve the ability of enzyme production markedly. More properities of β-D-glucuronidase production by *Penicillium* sp. Li-3 will be further studies in the future.

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