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Enhanced production of glycyrrhetic acid 3-O-mono-β-D-glucuronide by fed-batch fermentation using pH and dissolved oxygen as feedback parameters☆

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ABSTRACT

Glycyrrhetic acid 3-O-mono-β-D-glucuronide (GAMG), the major functional ingredient in licorice, has widespread applications in food, pharmacy and cosmetics industry. The production of GAMG through *Penicillium purpurogenum* Li-3 cultivation was for the first time performed through both batch and fed-batch processes in bioreactors. In batch process, under optimal conditions (pH 5.0, temperature 32 °C, agitation speed 100 $r \cdot min^{-1}$), 3.55 $g \cdot L^{-1}$ GAMG was obtained in a 2.5 L fermentor. To further enhance GAMG production, a fine fed-batch process was developed by using pH and DO as feedback parameters. Starting from 48 h, 100 ml 90 $g \cdot L^{-1}$ substrate Glycyrrhizin (GL) was fed each time when pH increased to above 5.0 and DO was increased to above 80%. This strategy can significantly enhance GAMG production: the achieved GL conversion was 95.34% with GAMG yield of 95.15%, and GAMG concentration was 16.62 $g \cdot L^{-1}$ which was 5 times higher than that of batch. Then, a two-step separation strategy was established to separate GAMG from fermentation broth by crude extraction of 15 ml column packed with D101 resin followed by fine purification with preparative C18 chromatography. The obtained GAMG purity was 95.79%. This study provides a new insight into the industrial bioprocess of high-level GAMG production.

1. Introduction

Glycyrrhizin (GL), which is a major natural compound extracted from the traditional Chinese medicine licorice root [1], harbors a broad spectra of beneficial pharmacological activities, such as antiviral [2], antiulcerative [3], antitumor [4] and anti-inflammatory activities [5]. Due to its sweet taste and pleasant smell, GL is also widely used in many food products. Glycyrrhetic acid 3-O-mono-β-D-glucuronide (GAMG) is an important derivative of GL through hydrolysis of one molecule of distal glucuronic acid (Fig. 1) [6]. Compared with GL, GAMG has stronger biological activities due to its proper polarity and solubility [7]. In addition, GAMG possesses a higher sweetness with an extremely low caloric value (5 times higher than GL, 950 times higher than sucrose) [8]. Moreover, the LD_{50} value of GAMG (5000 $mg \cdot kg^{-1}$) is much higher than that of GL (805 $mg \cdot kg^{-1}$), demonstrating its higher safeness [9]. Given these properties, GAMG which is considered to be a potential and better substitute of GL has inspired global interest in pharmacy and food industry [10].

Currently, most commercial GAMG is obtained through chemical synthesis with heavy metals as catalysts, and the overall yield was only 2.2%, which has largely limited its bulk industrial production [11]. Recently, biosynthesis of GAMG has drawn much attention due to high chemical bond selectivity, high reaction rate, mild reaction conditions and eco-friendliness. β-Glucuronidase (GUS, EC 3.2.1.31) which can hydrolyze glycosidic bonds has been applied to produce GAMG with particular concern to enzyme activity and substrate selectivity [12], widely ranging from bacterial and fungi to plants and animals [13–15]. However, most of them suffer from very low hydrolytic selectivity, which show preference for hydrolyzing GL to glycyrrhetic acid (GA) [16,17]. To date, only two β-glucuronidases from yeast *Cryptococcus magnus* MG 27 and liver of domestic duck have been used for the biotransformation of GL [18,19]. However, such enzymes exhibit low activity, high cost and unsustainability. Therefore, an efficient biotransformation process for GAMG is still highly desirable.

Within this context, we previously isolated a filamentous fungal strain named *P. purpurogenum* Li-3, and it can directly hydrolyze GL into GAMG by its β-glucuronidase (*pgus*, GenBank Accession NO. EU095019) with very few by-products GA [6]. However, the amount of enzyme produced by *P. purpurogenum* Li-3 was quite low and it was unable to meet the high requirement of GAMG production. In order to decrease GAMG production costs, optimizing fermentation conditions is another effective strategy, such as suitable pH, temperature and other environmental tolerances. Besides, GL functions as the sole carbon source and inducer due to the catabolite repression, simultaneously achieving high biomass and relatively high β-glucuronidase production. Normally, most researches mainly investigate the effects

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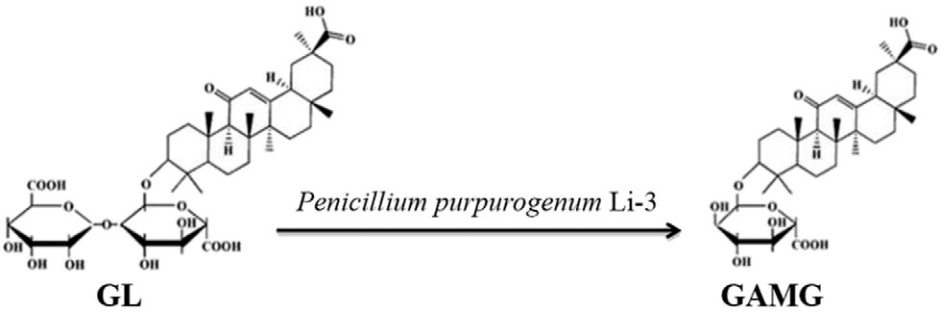

Fig. 1. The hydrolysis of GL into GAMG by *Penicillium purpurogenum* Li-3 [6].

of initial pH on biomass [20–22], but little attention has been paid to develop a pH control strategy by feeding GL to obtain both high cell growth rate and GAMG production rate. Therefore, it is quite necessary to establish a suitable substrate feeding strategy during fermentation to maintain a high-yield GAMG production.

In this article, we aim to evaluate the production of GAMG through *P. purpurogenum* Li-3 cultivation performed through both batch and fed-batch processes in bioreactors. In batch process, the effect of pH, temperature, and agitation intensity on biomass and GAMG yield was thoroughly investigated. Then, a fine substrate feeding strategy for fed-batch process was established by using pH and DO as feedback parameters to develop an efficient and high-yield process for GAMG production. Finally, a separation process based on D101 resin absorption and preparative HPLC was developed to purify GAMG from fermentation broth. This study provides a new insight into the industrial bioprocess of GAMG production.

2. Materials and Methods

2.1. Materials

GL monoammonium salt and glycyrrhetic acid (GA) as standards were purchased from Sigma Chemical Co. (USA). GL monoammonium salt with 75% purity for fermentation was purchased from Xinjiang Tianshan Pharmaceutical Ind. Co., Ltd. Glycyrrhetic acid 3-O-mono-β-D-glucuronide (GAMG) was generously donated by Nanjing University of Technology (China). D101 macroporous resins were purchased from Nankai Hecheng S & T Co., Ltd. (Tianjin, China). All other reagents were of analytical grade and used without any pretreatment.

2.2. Microorganism and media culture medium

The GAMG-producing strain *P. purpurogenum* Li-3 was screened and isolated from soil of licorice plantation in Xinjiang, China and was preserved by the lab of biotransformation and microecology (Beijing Institute of Technology, China). Optimization studies on media components for maximum production of GAMG have been performed [6,23]. The activation medium used for seed activation contained 6 $g \cdot L^{-1}$ glucose, 3 $g \cdot L^{-1}$ $NaNO_3$, 1 $g \cdot L^{-1}$ K_2HPO_4, 0.5 $g \cdot L^{-1}$ KCl, 0.5 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.01 $g \cdot L^{-1}$ $FeSO_4 \cdot 7H_2O$. The fermentation medium used for GAMG production contained 6 $g \cdot L^{-1}$ GL monoammonium salt, 3 $g \cdot L^{-1}$ $NaNO_3$, 1 $g \cdot L^{-1}$ K_2HPO_4, 0.5 $g \cdot L^{-1}$ KCl, 0.5 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.01 $g \cdot L^{-1}$ $FeSO_4 \cdot 7H_2O$. All the media were finally adjusted to pH 4.8–5.0, and then sterilized at 121 °C for 15 min.

2.3. Preparation of seed culture

Conditions of seed culture like plate age, inoculum age and percentage of inoculum were optimized previously in our laboratory. The strain from PDA agar plates pre-cultured for 5–7 d in an incubator at 30 °C, was transferred to 100 ml sterile seed medium in each 250 ml Erlenmeyer flask. The pH of the medium was adjusted to 5.0 and the culture was incubated on a rotary shaker at 170 $r \cdot min^{-1}$ and 32 °C. The seed culture was allowed to grow for 72 h and then 5% of the seed culture was transferred to 200 ml sterile seed medium again in each 500 ml Erlenmeyer flask, followed by incubation at 170 $r \cdot min^{-1}$ and 32 °C for 24 h. At least two generations of activation cultures were required before fermentation. The resultant culture was used as the seed culture.

2.4. Batch and fed-batch fermentation of P. purpurogenum Li-3

A 150 ml aliquot of seed culture was inoculated into a 2.5 L stirred bioreactor (Infors, Switzerland) with 1.5 L working volume. The bioreactor was equipped with a DO analyzer and an automatic pH controller controlled by the software. During the fermentation, samples were collected every 12 h for the determination of biomass, GL conversion and GAMG yield. Various parameters like pH (4.4–5.8), temperature (28–38 °C), agitation (100–300 $r \cdot min^{-1}$) and aeration (0.5–1.5 vvm) were studied in order to achieve the optimal operation. Fed-batch was performed by the addition of 100 ml 90 $g \cdot L^{-1}$ GL each time as carbon source and substrate to the medium at specific fermentation time. Samples were withdrawn after specified time intervals (6 h) from the bioreactor for the routine assay.

2.5. Analytical methods

Biomass was estimated based on dry cell mass. The mycelia from samples were collected by centrifugation at 4 °C and 8000 $r \cdot min^{-1}$, and washed twice with 10 ml distilled water under the same conditions. The resulting mycelia were dried under vacuum at 80 °C to constant mass. For GAMG analysis, the sample of fermentation broth was centrifuged at 4 °C and 8000 $r \cdot min^{-1}$, and the supernatant was filtered (0.22 μm pore size membrane) for further analysis. The concentrations of GL, GAMG and GA were determined by HPLC equipped with SHIMADZU Shim-pack VP-ODS (4.6 mm × 150 mm, 5 μm). The isocratic elution was used with methanol (solvent A) and water/0.6% acetic acid (solvent B) at 1 $ml \cdot min^{-1}$ and A/B ratio of 81:19. GL, GAMG and GA were detected at 254 nm and 40 °C.

2.6. Kinetic parameters calculation

The specific growth rate (μ) and specific GAMG production rate (q_p) can be calculated according to Eqs. (1–2), respectively.

$$\mu = \frac{1}{X}\frac{dX}{dt} = \frac{1}{X}\lim_{\Delta t \to 0}\frac{\Delta X}{\Delta t} \tag{1}$$

$$q_p = \frac{1}{X}\frac{dP}{dt} = \frac{1}{X}\lim_{\Delta t \to 0}\frac{\Delta P}{\Delta t} \tag{2}$$

where X is the dry cell mass concentration ($g \cdot L^{-1}$).