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RESEARCH ARTICLE

Enhanced production of β-glucuronidase from *Penicillium purpurogenum* Li-3 by optimizing fermentation and downstream processes

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Abstract β-Glucuronidase from *Penicillium purpurogenum* Li-3 (PGUS) can efficiently hydrolyze glycyrrhizin into the more valuable glycyrrhetic acid monoglucuronide. However, a low productivity of PGUS and the lack of an effective separation strategy have significantly limited its industrial applications. Therefore, the production of PGUS has been improved by optimizing both the fermentation and purification strategies. A two-stage fermentation strategy was developed where PGUS was first grown with glucose and then PGUS was produced in the presence of glycyrrhizin as an inducer. By using this strategy, the biomass was increased 1.5 times and the PGUS activity increased 5.4 times compared to that when glycyrrhizin was used as the sole carbon source. The amount of PGUS produced was increased another 16.6% when the fermentation was expanded to a 15-L fermenter. An effective protocol was also established to purify the PGUS using a sequential combination of hydrophobic, strong anion-exchange and gel filtration chromatography. This protocol had a recovery yield of 6% and gave PGUS that was 39 times purer than the crude PGUS. The purified PGUS had a specific activity of 350 $U \cdot mg^{-1}$.

Keywords β-glucuronidase, glycyrrhetic acid monoglucuronide, cell disruption, purification, chromatography

1 Introduction

Glycyrrhizin (GL) is the principal active ingredient in licorice, and it has been widely used as a herbal medicine and a sweetener [1,2]. Several studies have demonstrated that GL has anti-inflammatory [3,4], anti-ulcer [5], anti-viral [6] and anti-cancer [7] activities. However, GL contains two hydrophilic glucuronide groups (Scheme 1) which make it difficult for GL to penetrate cell membranes. As a result, the bioavailability of GL is low [8]. GL can be transformed into the more active glycyrrhetic acid monoglucuronide (GAMG), by hydrolyzing one of the glucuronidic acid moieties. GAMG has a medium polarity, which gives it a better bioavailability [9–12]. In addition, the sweetness of GAMG is five times higher than that of GL [13]. Thus GAMG is a promising substitute for GL in the pharmaceutical, cosmetic and food industries [14,15]. Currently, the use of enzymatic catalysts is the most popular route for the biotransformation of GL into GAMG. However, the lack of efficient enzymes with substrate specificity for GL (i.e., only one glucuronic acid group is hydrolyzed to render GAMG as the sole product) is a problem.

β-Glucuronidase (GUS, EC 3.2.1.31) can hydrolyze glucuronide groups in many natural products to yield high valued-added derivatives [16,17]. GUS which can hydrolyze GL has been found in many organisms including bacteria [18,19], fungi [20,21] and animals [22] . Recently, our group used *Penicillium purpurogenum* Li-3 which can produce β-glucuronidase (PGUS) to hydrolyze GL into GAMG with very high substrate specificity [9] (Scheme 1). However, the amount of PGUS produced was very low, even though the fermentation conditions were optimized. This is because GL was used as the carbon source to induce the secretion of PGUS, but GL does not provide enough carbon nutrition for the propagation of biomass and a low dry cell weight (DCW) was obtained [9]. In addition, the amount of the PGUS protein in the system is quite low, which causes difficulties in the downstream separation and purification steps. Further, the crude extract had a specific activity of only 9 $U \cdot mg^{-1}$.

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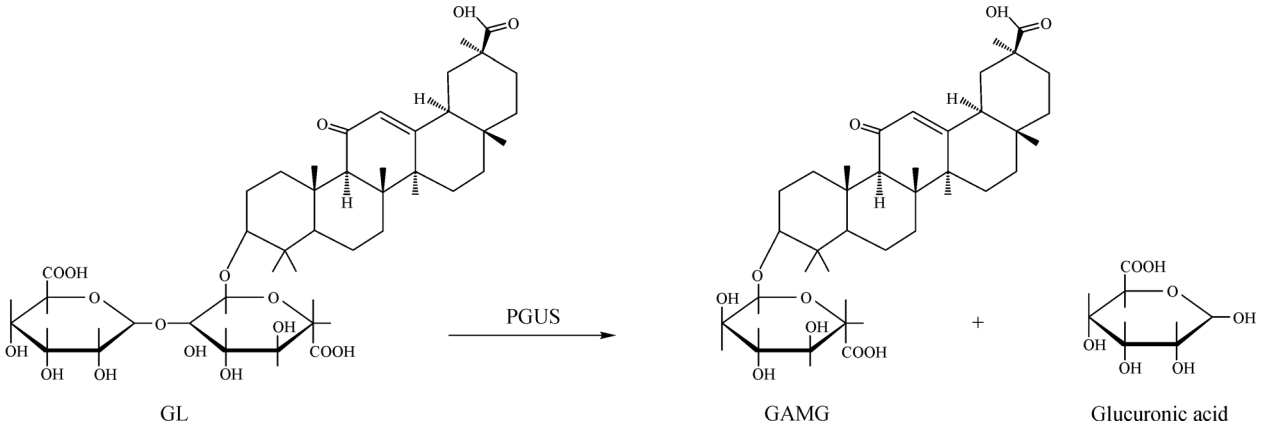

Scheme 1 Hydrolysis of GL into GAMG and glucuronic acid catalyzed by PGUS

In an attempt to improve the amount of PGUS, *β*-glucuronidase was heterologously expressed in *Escherichia coli*, *Pichia pastoris* and *Aspergillus niger* (unpublished data). However, this changed the substrate specificity of the recombinant *β*-glucuronidase so that the product was mainly (more than 90%) GA (one glucuronide less than GAMG) instead of GAMG (less than 10%). A purification protocol for PGUS has been developed which gave a specific activity of 273 $U \cdot mg^{-1}$ [11]. However this protocol suffers from a number of problems. The method involves using $(NH_4)_2SO_4$ to extract the PGUS from the cell disruptate. The extraction yield was low and the product was contaminated with a large amount of cytochromes, which damaged the chromatographic columns in the subsequent purification steps. A DEAE-cellulose ion exchange column was used in one of the purification steps, but the efficiency was low due to weak electrostatic adsorption and a poor resolution [11]. A stronger ion exchange column may be useful for improving the resolution and increasing the purity.

The main objective of this study was to enhance PGUS production by optimizing the fermentation strategy and by establishing an effective protocol for the separation and purification of PGUS. A two-stage fermentation strategy was adopted where glucose was initially used as the carbon source to support rapid growth and then, when the glucose was completely consumed GL was added to the medium to induce the production of PGUS. The PGUS was then separated and purified using a sequential combination of hydrophobic chromatography, strong anion-exchange chromatography and gel filtration chromatography.

2 Materials and methods

2.1 Materials

P. purpurogenum Li-3 was previously isolated from soil from Xinjiang (China) and stored in our laboratory at the Beijing Institute of Technology, China. Glucose and ammonium sulfate were purchased from Dingsheng (Beijing, China). The ion exchange chromatography column (Source 15Q 4.6/100), the gel filtration chromatography column (Superdex™ 200 (2.6/60)) and the hydrophobic interaction chromatography column (HiTrap HIC Selection Kit 7×1) were obtained from GE Healthcare (Uppsala, Sweden). The Bradford reagent was obtained from Sigma-Aldrich (St. Louis, MO, USA). Butyl sepharose for hydrophobic chromatography was a gift from the Institute of Process Engineering, Chinese Academy of Science (Beijing, China). The 15-L BIO-TECH-5BG-2 fermenter was purchased from Baoxing (Shanghai, China).

2.2 Medium and cultivation

P. purpurogenum Li-3 was maintained on an agar slant (4 °C) which contained 18 $g \cdot L^{-1}$ agar, 5 $g \cdot L^{-1}$ glucose, 3 $g \cdot L^{-1}$ $NaNO_3$, 1 $g \cdot L^{-1}$ K_2HPO_4, 0.5 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.5 $g \cdot L^{-1}$ KCl, and 0.01 $g \cdot L^{-1}$ $FeSO_4 \cdot 7H_2O$. The seed culture medium contained 5 $g \cdot L^{-1}$ glucose, 3 $g \cdot L^{-1}$ $NaNO_3$, 1 $g \cdot L^{-1}$ K_2HPO_4, 0.5 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.5 $g \cdot L^{-1}$ KCl, and 0.01 $g \cdot L^{-1}$ $FeSO_4 \cdot 7H_2O$. The glycyrrhizin medium that was used for fermentation contained 6 $g \cdot L^{-1}$ glycyrrhizin, 3 $g \cdot L^{-1}$ $NaNO_3$, 1 $g \cdot L^{-1}$ K_2HPO_4, 0.5 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.5 $g \cdot L^{-1}$ KCl, and 0.01 $g \cdot L^{-1}$ $FeSO_4 \cdot 7H_2O$ [9,11].

The seed culture was prepared in two steps. *P. purpurogenum* Li-3 from an agar slant was inoculated into a 250-mL flask with 80 mL of seed culture medium, and grown at 30 °C for 72 h (step 1). Then the culture was inoculated into another 80 mL of seed culture medium with 1% inoculum, and incubated at 30 °C and 170 $r \cdot min^{-1}$ for 24 h (step 2).

2.3 Two-stage fermentation for PGUS production in a flask

The seed culture was inoculated (10% inoculum, v/v) into

a 1000-mL flask with 300 mL glycyrrhizin medium, which contained glucose at the stated concentration. The temperature was kept at 30 °C with a shaking speed of 170 $r \cdot min^{-1}$ (orbital shaker). The culture time was 96 h, and samples were taken every 12 h to determine the dry cell weight and enzyme activity.

2.4 Determination of biomass

The broth was centrifuged at 6000 $r \cdot min^{-1}$ for 10 min and the resultant cells were washed with distilled water and centrifuged again. This washing process was repeated three times. Then the wet cells were dried at 105 °C until the weight was constant.

2.5 PGUS activity assay

The PGUS activity was determined by GL hydrolysis [11]. Briefly, 100 mg GL was dissolved in 200 μL acetate buffer (30 $mmol \cdot L^{-1}$, pH 6.0) and then 5 mg purified enzyme was added to start the reaction. The tube was incubated for 10 min at 40 °C. After the reaction, the concentrations of GL, GAMG and GA were determined by HPLC (Shimadzu LC10AVP) equipped with a vacuum degasser, quaternary pump, autosampler, thermostated column compartment, and UV detector. The separation was performed on a Shimadzu ODS column (4.6 mm × 250 mm, diameter of packing material of 5 μm). The injection volume was 10 μL and the flow rate was 1 $mL \cdot min^{-1}$. The column temperature was constant at 40 °C, and the detection wavelength was 254 nm. The mobile phase was composed of 19% water (pH 2.85, with 0.6% acetic acid) and 89% methanol.

2.6 Preparation of crude enzyme

PGUS is an intracellular enzyme and no activity was detected in the broth during the fermentation (data not shown). So the first step was to disrupt the cells to release the product. Three physical methods were tried to disrupt the cells including sonication, freeze milling and high pressure homogenization (HPP), and the HPP showed the highest PGUS activity: 25% higher than freeze milling and 80% higher than sonication. Therefore, HPP was chosen as the method for cell disruption. After centrifugation at 12000 $r \cdot min^{-1}$ for 30 min (4 °C), acetone pre-chilled at −20 °C was added to the supernatant and incubated for 15 min at 4 °C to allow for the precipitation of protein. After centrifugation at 12000 $r \cdot min^{-1}$ for 30 min (4 °C), the precipitate was suspended with 30 $mmol \cdot L^{-1}$ Tris-HCl buffer (pH 7.3, containing 100 $mmol \cdot L^{-1}$ NaCl).

2.7 Purification of PGUS by chromatography

First, the crude enzyme solution was purified by hydrophobic interaction chromatography (HIC) using a butyl sepharose column. The column was equilibrated with buffer A (1.5 $mol \cdot L^{-1}$ $(NH_4)_2SO_4$, 50 $mmol \cdot L^{-1}$ Tris-HCl, pH 7.3) and then loaded with the crude enzyme. Then the column was washed with buffer A until the UV_{280} baseline was stable. Then buffer B (50 $mmol \cdot L^{-1}$ Tris-HCl, pH 7.3) was used to elute the absorbed protein. The collected protein was concentrated by ultrafiltration with a cut-off of 10 kDa and then dialyzed in buffer B until the conductivity was lower than 4 $mS \cdot cm^{-1}$.

The resulting sample was further purified by anion-exchange chromatography with a Source 15Q 4.6/100 column, pre-equilibrated with buffer B. The absorbed protein was eluted with a linear gradient of buffer B and buffer C (50 $mmol \cdot L^{-1}$ Tris-HCl, pH 7.3, 1 $mol \cdot L^{-1}$ NaCl) from 0 to 100%. The fractions with the highest PGUS activity were concentrated by ultrafiltration and further purified by gel filtration chromatography with a Superdex™ 200 10/300 column, which was equilibrated with buffer D (50 $mmol \cdot L^{-1}$ Tris-HCl, pH 7.3,0.1 $mol \cdot L^{-1}$ NaCl). The absorbed protein was eluted with buffer D and the peak was collected. The chromatographic processes were all performed using an AKTA purifier 10 system (USA, GE Healthcare). The purified enzyme was frozen with liquid nitrogen and stored at −80 °C.

2.8 Fermentation in a 15-L fermenter

The seed culture prepared as described in Section 2.2 was inoculated (10% inoculum, v/v) into a 15-L BIOTECH-5BG-2 fermenter filled with 9 L glycyrrhizin medium. The agitation speed was 200 $r \cdot min^{-1}$, and the aeration rate was 1 vvm. The temperature was controlled at 32 °C. A pH electrode and DO (dissolved oxygen) probe (Mettler Toledo, German) were used online to monitor the pH and DO, respectively. Samples were taken every 12 h. All the fermentation experiments were performed in triplicate.

2.9 Other assays

The total protein concentration was determined by the Bradford method at 595 nm using bovine serum albumin as the standard [23]. The PGUS concentration was determined by correlating it with the activity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a 12% polyacrylamide gel using the tris (hydroxymethyl) amino-methane-glycine buffer system.

3 Results and discussion

3.1 Enhanced PGUS production by two-stage fermentation

A two-stage fermentation strategy was developed for PGUS production: (i) accumulation of biomass by adding glucose, and (ii) PGUS production induced by GL. The effect of the concentration of glucose on fermentation in a

1000-mL flask was tested and the results are shown in Fig. 1. The accumulated biomass increased as the concentration of glucose increased from 0 to 4 g·L^{-1} (e.g., a DCW of 2 g·L^{-1} was obtained with 4 g·L^{-1} glucose whereas the DCW was 0.3 g·L^{-1} with no glucose present, Fig. 1(a)). In addition, with 4 g·L^{-1} of glucose the maximum biomass level was obtained in 36 h whereas in the absence of glucose it took 72 h to obtain the maximum biomass level. These results indicate that glucose is more suitable than GL for the growth of *P. purpurogenum* Li-3.

PGUS activity was first detected at 24 h for all the glucose concentrations (Fig. 1(b)). For 0, 1 and 2 g·L^{-1} of glucose, the highest activities were reached after 60 h. For 3 g·L^{-1} glucose, the highest activities were reached after 50 h. For 4 g·L^{-1} glucose, the activities were kept increasing with culture time until 84 h. The expressed activity does not strictly correlate with the amount of *P. purpurogenum* Li-3 biomass that was produced. The activity increased when the glucose concentration was increased from 0 to 2 g·L^{-1} reaching a maximum of 12 U·mL^{-1}. However, for higher levels of glucose (3 and 4 g·L^{-1}), the highest activities were smaller. This may be because at higher glucose concentrations there is extra glucose in the medium (i.e., more than the cells need for growth) which impairs the GL induction effect since *P. purpurogenum* Li-3 preferentially utilizes glucose as the carbon source. These results indicate that a two-stage fermentation strategy with 2 g·L^{-1} glucose is quite effective for PGUS production, especially compared to fermentation with no glucose added.

3.2 Scale-up of PGUS production in fermenter

The fermentation was then scaled up to 9 L of GL medium with 2 g·L^{-1} glucose in a 15-L fermenter and the results are shown in Fig. 2. The PGUS activity increased rapidly after 24 h, peaking at 14 U·mL^{-1} at 60 h. This result is similar to those for the fermentation in the flask (Fig. 1), but the peak activity was 16.6% higher in the 15-L fermenter. Oxygen is often a limiting component in fermentations involving filamentous fungi, since the hyphae cause a high viscosity that limits oxygen transfer [24,25]. Therefore, the higher activity in the 15-L fermenter can be attributed to better mixing and enhanced oxygen mass transfer.

The profiles of GL and GAMG were also monitored (Fig. 2). After 72 h, 90% of the GL was transformed and the GAMG concentration was 3.5 g·L^{-1}. Moreover, the increase in PGUS activity corresponded closely with the consumption of GL. Glucose severely repressed the production of PGUS and no enzymatic activity was detected when there was glucose in the medium. Therefore, the inducer does not affect the fermentation before the glucose is depleted. For easy operation, the inducer was added at the beginning of the fermentation. After 24 h, the glucose was mostly depleted and the cell biomass became relatively stable with a DCW of 0.5 g·L^{-1}. At this time, the cells began to utilize the GL and secret large amount of GPUS, resulting in a rapid increase in the enzymatic activity.

3.3 Disruption of *P. purpurogenum* Li-3 cells

High pressure homogenization (HPH) was used to disrupt the *P. purpurogenum* Li-3 cells and the operational parameters including pH, pressure and the number of breaking cycles were optimized. As shown in Fig. 3, the total protein concentration increased significantly as the pH used for cell disruption increased. The highest activity (around 11 U·mL^{-1}) was obtained in the pH range of 5 to 7. A pH of 5 was selected for cell disruption since it had the

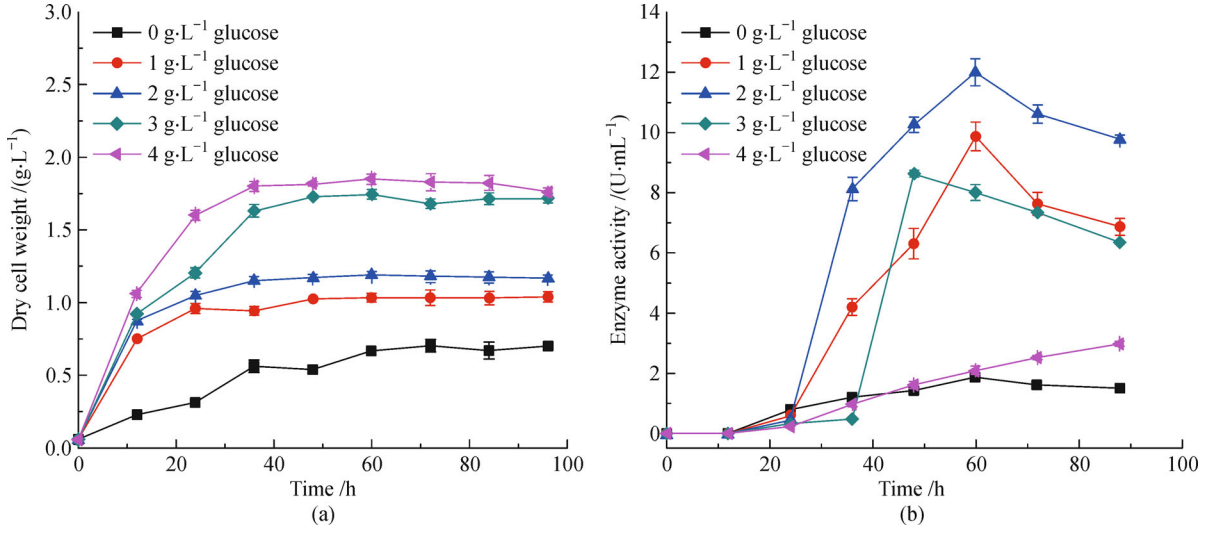

Fig. 1 Effect of glucose concentration on (a) dry cell weight and (b) enzyme activity. Results are from triplicate fermentation experiments and the error bars represent the average±one standard deviation

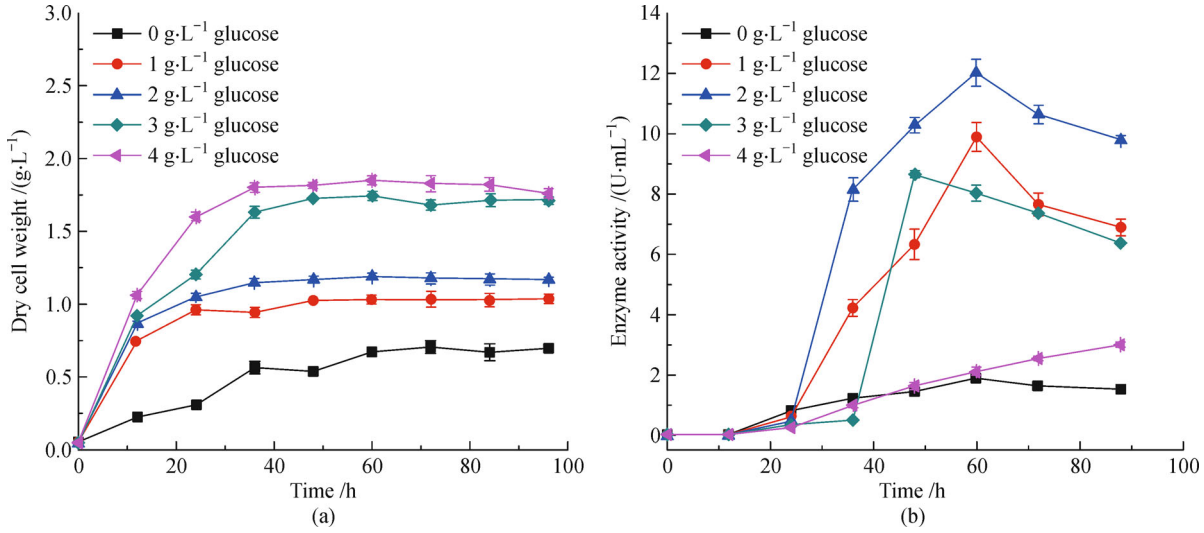

Fig. 2 Fermentation performance of *P. purpurogenum* Li-3 in a 15-L fermenter. The initial glucose concentration was 2 g·L^{-1}

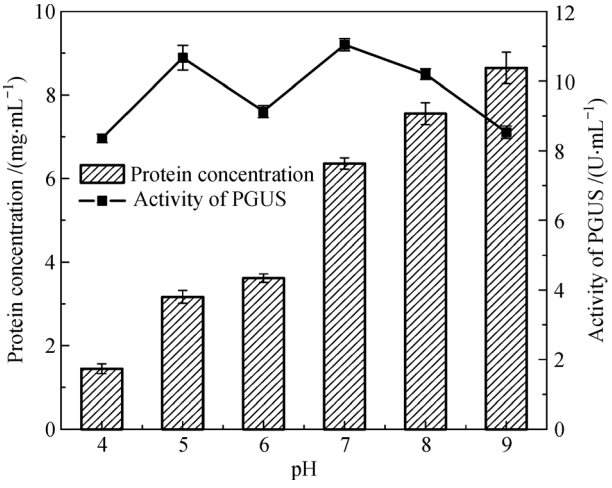

Fig. 3 Effect of pH on protein concentration and PGUS activity during the disruption of cells by HPH. Results are from independent triplicate measurements and error bars represent the average±one standard deviation

lower total protein concentration.

Next the effects of pressure (1500 and 1800 bar) and the number of breaking cycles were investigated. As shown in Figs. 4(a) and (b), the cells were only partially disrupted with a pressure of 1500 bar, and filamentous mycelium still can be seen compared to the control. When higher pressure of 1800 bar was applied, as shown in Fig. 4(c), all the cells were completely disrupted and no obvious filamentous morphologies are observed (Fig. 4(c)). The number of breaking cycles (one, two, or three) did not affect the cell morphology (Figs. 4(c)–4(e)).

Finally, the effect of the number of breaking cycles on the released protein and activity was studied (Fig. 5). The total protein concentration increased by 49.1% when the number of breaking cycles increased from one to two, but it only increased another 5.9% for three breaking cycles. However the PGUS activity was smaller after three breaking cycles than that for two cycles. This may be attributed to the mechanical damage to PGUS. Therefore, two breaking cycles most effectively disrupts the cells while maintaining the highest enzyme activity. This result is comparable to those of Choonia et al. who used HPH to break *Lactobacillus acidophilus* and *Pectobacterium caratovorum* at a pressure of around 550–700 bar [26]. These results indicated that filamentous fungi were more difficult to break than bacterial cells. The optimized parameters for the HPH operation are pH 5, a pressure of 1800 bar, and two breaking cycles.

3.4 Extraction of PGUS from the cell lysate

Organic solvents were then used to precipitate PGUS from the cell lysates (Fig. 6) since they have relatively high precipitation efficiencies [27]. Four organic solvents (methanol, ethanol, acetone and chloroform) were tested and acetone gave the best precipitation yield. The amount of acetone and the extraction time were then optimized and the results are shown in Fig. 7. As shown in Fig. 7(a), the yield of PGUS was highest for an extraction time of 30 min. The optimal acetone to crude lysate ratio (v/v) was 0.5 : 1 which gave a yield of 81% (Fig. 7(b)).

3.5 Purification of PGUS by chromatography

Initially hydrophobic interaction chromatography was used to purify the PGUS from the resuspended precipitate. A Hitrap HIC Selection Kit 7 × 1 was used to determine the best packing medium for the separation which was butyl sephorose (data not shown). The chromatogram for the separation of PGUS is shown in Fig. 8. The peak that was eluted at 147 mL with 20% buffer B was identified as

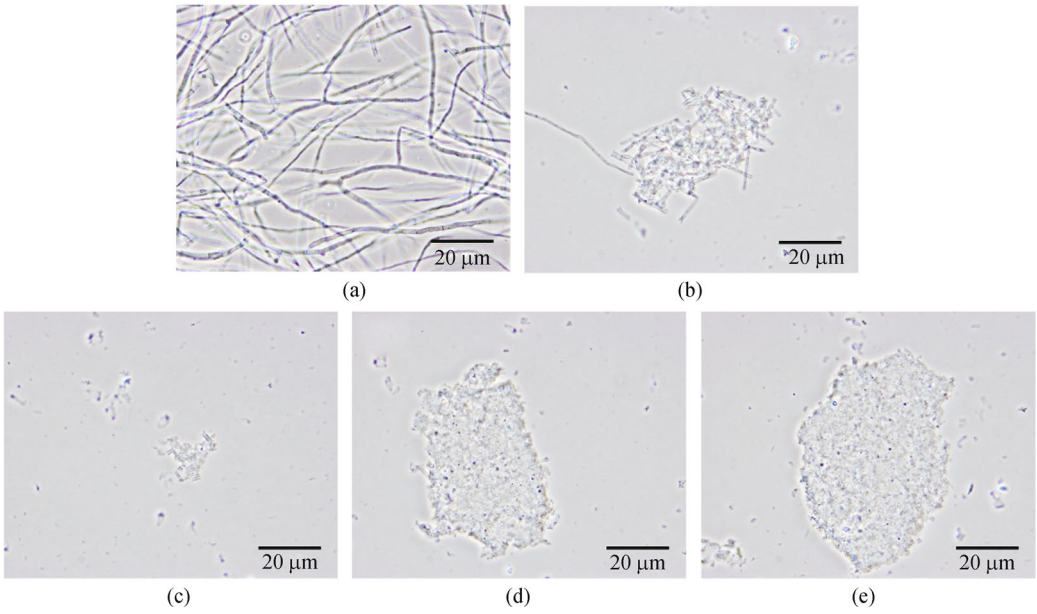

Fig. 4 The optical images of *P. purpurogenum* Li-3 cells disrupted under different conditions: (a) before disruption; (b) disrupted once under pressure of 1500 bar; (c) disrupted once under pressure of 1800 bar; (d) disrupted twice under pressure of 1800 bar; (e) disrupted three times under pressure of 1800 bar

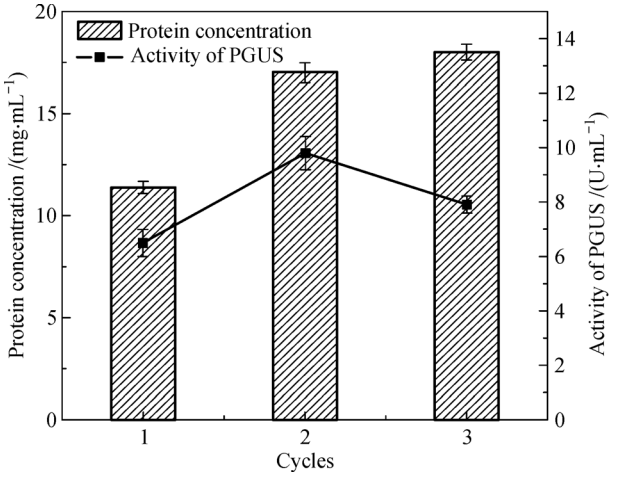

Fig. 5 Effect of the number of HPH cell disruption breaking cycles. Results are from independent triplicate measurements and error bars represent the average±one standard deviation

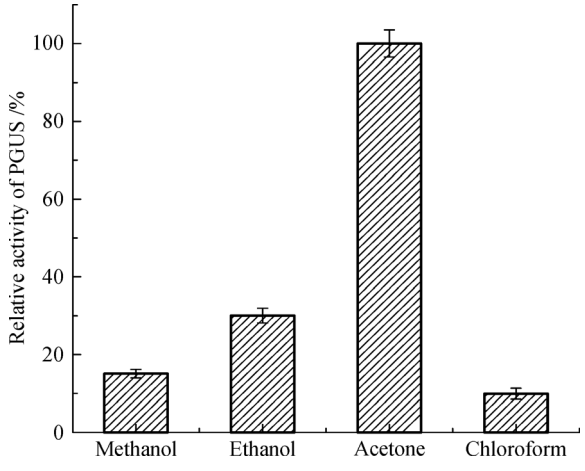

Fig. 6 Effect of organic solvents on the activity of PGUS during precipitation. Results are from independent triplicate measurements and error bars represent the average±one standard deviation

PGUS using an enzyme activity assay and SDS-PAGE analysis. Table 1 shows the PGUS yields and purification factor in each purification step. As this data shows, the HIC improved the specific activity of the purified PGUS to 188 U·mg^{-1} which is 21 times higher than that of crude enzyme. The SDS-PAGE analysis (Fig. 9) also indicated that HIC substantially purified the PGUS. There is no PGUS band for the crude enzyme due to its low-abundance (Fig. 9, lane 1), but after purification by HIC, the PGUS band (at around 69 kDa) is evident (lane 2) and the amount of contaminating protein is significantly reduced. These results show that HIC is a suitable technique for the preliminary purification of PGUS.

The isoelectric point of PGUS is 5.65, so anion exchange chromatography (AEC) with a Source 15 Q column was used to further purify the PGUS. The sample purified by HIC was first dialyzed to remove any salts until the conductivity was below 4 mS·cm^{-1}, and then the sample was loaded onto the AEC column. As the amount of NaCl in the eluent was increased, several peaks were

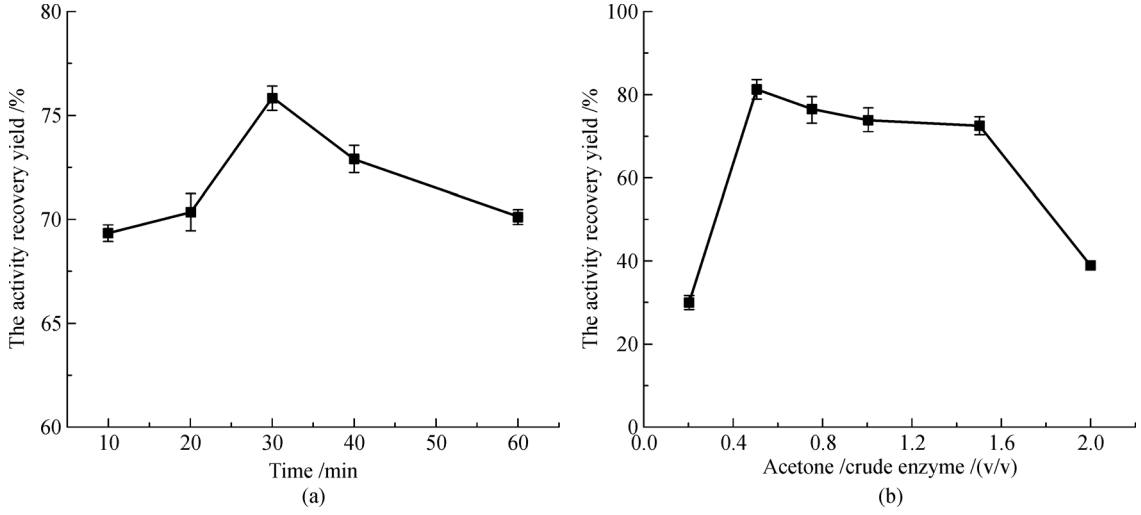

Fig. 7 Optimization of operational parameters for the precipitation of PGUS by acetone: (a) time; (b) volume ratio of acetone to crude enzyme. Results are from independent triplicate measurements and error bars represent the average±one standard deviation

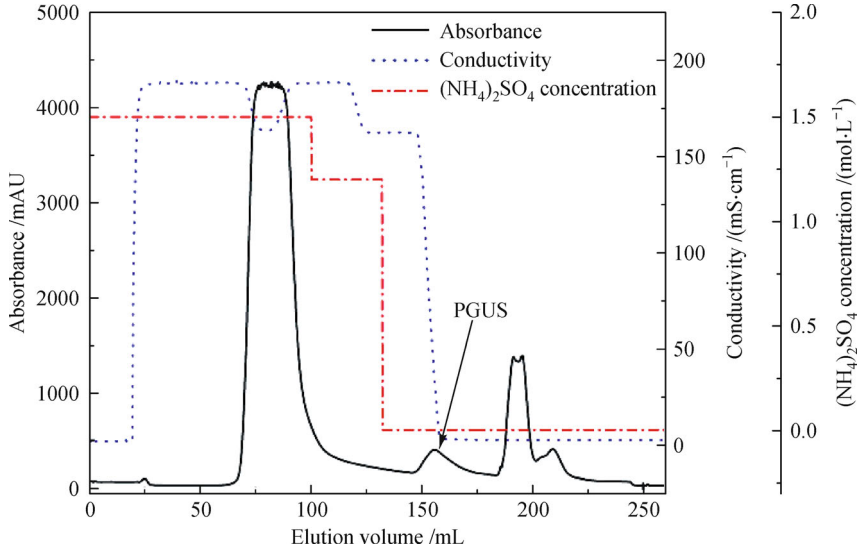

Fig. 8 Purification of PGUS by butyl sepharose hydrophobic interaction chromatography

observed in the chromatogram (Fig. 10). The peak eluted with 20 mS·cm^{-1} buffer (at 44 mL) showed high activity and was identified as PGUS. The SDS-PAGE analysis (Fig. 9, lane 3) shows that even more of the impurities were removed by this step and the specific activity increased to 308 U·mg^{-1} which is 64% higher than that for the PGUS purified by HIC alone.

Generally, gel filtration chromatography is applied as the final step for removing contaminates and assaying the aggregation state of a target protein. The theoretical molecular weight of the PGUS monomer is around 69 kDa, and a Superdex™ 200 (2.6/60) column was selected to further purify PGUS. As shown in Fig. 11, the peak with an elution volume of 12 mL was identified as PGUS. This was also confirmed by SDS-PAGE, where a single band was observed, indicating the high purity of PGUS (Fig. 9, lane 4).

As shown in Table 1, after the three-step purification, the specific activity was 350 U·mg^{-1}, with a recovery yield of 6%. The purified PGUS was 39 times purer than the crude PGUS. The specific activity of the PGUS achieved here is higher than that of two previously reported *β*-glucuronidases isolated from *Streptococcus* LJ-22 [28]. They also can convert GL into GAMG and had specific activities of 137–190 U·mg^{-1}. Specific activities that have been obtained for *β*-glucuronidases purified from other bacteria, namely *Escherichia coli* HGU-3, *Aspergillus niger* and *Lactobacillum brevis*, are considerably lower, ranging

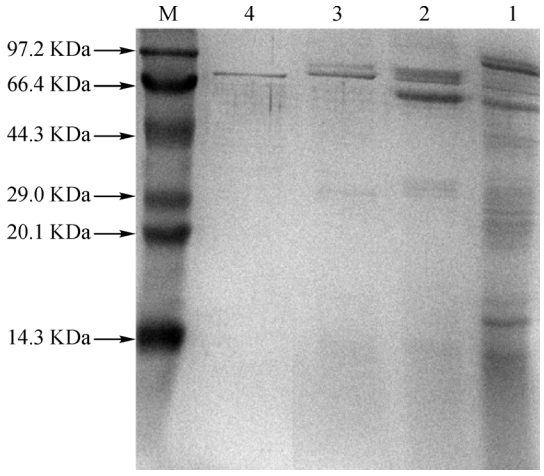

Fig. 9 SDS-PAGE analysis of purified PGUS. Lane M: marker; lane 1: crude enzyme; lane 2: PGUS purified by butyl sepharose hydrophobic interaction chromatography; lane 3: PGUS purified by anion exchange chromatography; lane 4: PGUS purified by gel filtration chromatography

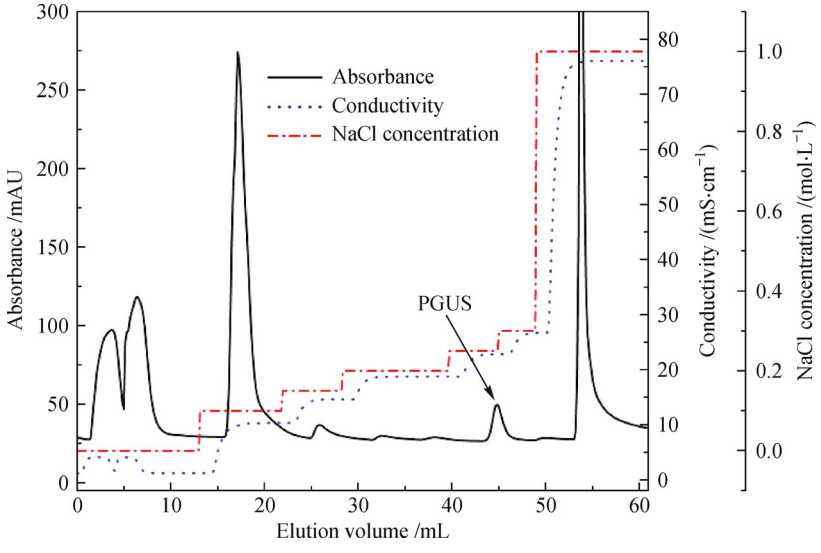

Fig. 10 Purification of PGUS by anion exchange chromatography

from only 8 to 28 U·mg^{-1} [18,29,30]. The specific activity reported here is also higher than those for other enzymes from filamentous fungus, for example, purified β-fructofuranosidase from *Aspergillus niger* had a specific activity of 52 U·mg^{-1} [31]. Therefore, this two-stage fermentation and downstream purification process effectively enhances the production of PGUS and also provides new insight into the production of other low-abundance proteins from filamentous fungus.

4 Conclusions

The production of PGUS by *Penicillium purpurogenum* Li-3 was significantly enhanced by optimizing both the fermentation and the downstream separation and purification processes. Compared to a one-stage fermentation, the

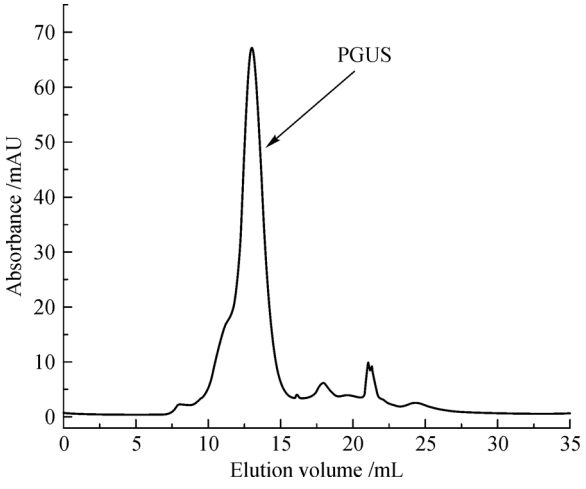

Fig. 11 Purification of PGUS by gel filtration chromatography

Table 1 PGUS yields and purification data

Purification steps	Total protein /mg	Total activity /U	Specific activity /(U·mg^{-1})	Recovery yield/%	Purification factor
Supernatant	266	2634	9	100	1
Acetone precipitation	25	2007	80	76	9
Hydrophobic interaction	5	967	188	38	21
Strong anion exchange	3	483	308	18	34
Gel filtration	0.4	140	350	6	39

two-stage fermentation strategy increased the biomass by 1.5 times and the PGUS activity by 5.4 times. When the PGUS fermentation was performed in a 15-L fermenter, the activity increased by 16.6% compared to fermentation in a flask. In the downstream processing, high pressure homogenization was used to disrupt the cells, with the optimized conditions being pH 5, a pressure of 1800 bar and two breaking cycles. Crude PGUS was then extracted from the cell disruptate by acetone precipitation using an acetone to crude enzyme volume ratio of 0.5 : 1, which gave a yield of 81.2%. The PGUS was then purified using a sequential combination of hydrophobic chromatography, strong anion-exchange chromatography and gel filtration chromatography.

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