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Enhanced a novel β-agarase production in recombinant *Escherichia coli* BL21 (DE3) through induction mode optimization and glycerol feeding strategy

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Abstract

Agarases are hydrolytic enzymes that act on the hydrolysis of agar and have a broad range of applications in food, cosmetics and pharmaceutical industries. In this study, a glycerol feeding strategy based on induction mode optimization for high cell density and β-agarase production was established, which could effectively control acetate yield. First, exponential feeding strategy of glycerol with different overall specific growth rates (μ) was applied in the pre-induction phase. The results showed that the low μ (μ=0.2) was suggested to be the optimal for cell growth and β-agarase production. Second, the effects of induction temperature and the inducer concentration on cell growth and β-agarase production were investigated in the post-induction phase. When induced by isopropyl-β-d-thiogalactoside (IPTG), the strategy of 0.8 mmol/L IPTG induction at 20°C was found to be optimal for β-agarase production. When cultivation was induced by continuous lactose feeding strategy of 1.0 g/(L·h), the β-agarase activity reached 112.5 U/mL, which represented the highest β-agarase production to date. Furthermore, the β-agarase was capable of degrading *G. lemaneiformis* powder directly to produce neoagarooligosaccharide, and the hydrolysates were neoagarotetraose (NA4) and neoagarohexaose (NA6). The overall research may be useful for the industrial production and application of β-agarase.

Key words: β-agarase, *Escherichia coli*, process optimization, glycerol feeding strategy, neoagarooligosaccharide

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1 Introduction

β-agarase (EC 3.2.1.81) hydrolyses the β-D-(1, 4) linkages of agarose to produce neoagarooligosaccharides with D-galactopyranose residues at the reducing ends (Hassairi et al., 2001) . The hydrolysis products of β-agarases are reported to possess diverse physiological and biological functions and have potential applications in food, cosmetics and medical industries (Kobayashi et al., 1997; Jang et al., 2009; Yoshizawa et al., 1995). In addition, β-agarases are useful tools for isolation of protoplasts from algae (Araki et al., 1998) and recovery of deoxyribonucleic acid (DNA) from agarose gels (Sugano et al., 1993).

However, the conventional production method of oligosaccharides is chemical degradation, which is limited by many disadvantages such as unstable products, high pollution and high cost. Given the advantages of β-agarases, many β-agarases have been isolated from several microorganisms, particularly, from bacteria, such as *Pseudomonas* (Lee et al., 2000), *Pseudoalteromonas* (Oh et al., 2010; Chi et al., 2014; Li and Sha 2015), *Alteromonas* (Wang et al., 2006; Seo et al., 2014), *Acinetobacter* (Roseline and Sachindra 2016), *Catenovulum* (Xie et al., 2013), *Bacillus* (Suzuki et al., 2003), *Micrococcus* (Choi et al., 2011), *Agarivorans* (Long et al., 2010), *Vibrio* (Dong et al., 2007), *Strenotrophomonas* (Zhu et al., 2016) and *Flammeovirga* (Xu et al., 2011; Han et al., 2012; Liu et al., 2015; Dong et al., 2016). The optimization of β-agarase production from these wild-type strains has been widely studied (Choi et al., 2011; Lakshmikanth et al., 2006a, b), but none of them have been commercially used. In spite of the β-agarases isolated from wild-type microorganisms so far have been found to be secreted enzymes, the expression of recombinant proteins has the advantages of improved product productivity and quality. To date, the highest β-agarases yield reported, 11 U/mL, was from *Micrococcus* sp. GNUM-08124 (Choi et al., 2011), which was obtained using Box–Behnken design in shaking-flask. In a word, low yield, low productivity and high cost are the major limiting factors for large-scale production and widespread application of this enzyme.

With the development of recombinant DNA technology, a substantial number of genetically engineered microorganisms

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have been constructed to express β-agarases in *Escherichia coli* (*E. coli*) (Oh et al., 2010; Yang et al., 2011; Lin et al., 2012; Hou et al., 2015; Chen et al., 2016). However, none process optimization of recombinant β-agarases has been reported. In our previous study, an agar-degrading bacterial strain of *Flammeovirga pacifica* (*F. pacifica*) was isolated from the deep-sea sediment of the western Pacific Ocean (Xu et al., 2011). The genome of *F. pacifica* (GenBank under accession number JRYR00000000) was sequenced using the Illumina/Solexa MiSeq technology and ten agarases gene were found (Chan et al., 2015). In addition, a novel β-agarase gene *aga4383* (GenBank under accession number KJ573600.1) from *F. pacifica* was expressed in *E. coli*, which exhibited a notable thermostability and a broad range of pH stability (Hou et al., 2015). Through the optimization of medium components by response surface methodology and the optimization of induction conditions by orthogonal experiment, β-agarase activity was enhanced from 7.3 to 18.5 U/mL.

To enhance the heterologous production of recombinant protein in *E. coli*, different fed-batch fermentation strategies were attempted, such as indirect feedback methods (e.g., pH-stat and DO-stat) (Goyal et al., 2009), predetermined feeding methods (e.g., exponential feeding and linearly increased feeding) (Ramalingam et al., 2007) and step-wise feeding strategies (Fang et al., 2007; Cheng et al., 2011). In the present study, we have attempted to promote the β-agarase production by fed-batch fermentation. A glycerol feeding strategy based on induction mode optimization was developed to achieve high cell density and β-agarase production in *E. coli*, in which different specific growth rates of exponential feeding method were used in pre-induction phase and appropriate induction conditions were established by examining major influential factors (e.g., induction temperature, inducer concentration and feeding strategy) in the post-induction phase. The results showed that the specific growth rate of 0.2 h^{-1} was suitable for high cell growth and high β-agarase production. When induced at 20°C with 1.0 g/(L·h) lactose feeding rate, the yield of β-agarase in the fermentation reached 112.5 U/mL, which was the highest production of β-agarase reported to date.

2 Materials and methods

2.1 *Bacterial strains and plasmids*

The ORF without signal peptide encoding the *aga4383* gene was amplified from the genomic DNA of *F. pacifica* (preserved in our laboratory, isolated from the deep-sea sediment of the west Pacific Ocean, Accession Number: MCCC 1A06425) by PCR with specific primers. The *aga4383* gene was amplified from the genomic DNA of *F. pacifica* by using primers *aga4383*-F(5′-TCATCATATGCAAGATTGGGCACAAATTCC-3′) and *aga4383*-R (5′-CGACAAGCTTTTATTCTTTGATAATCCTCTG-3′). The *aga4383* gene was inserted into the plasmid pColdII. Subsequently, the resulting expression plasmid pColdII-*aga4383* was transformed into *E. coli* BL21 (DE3) for recombinant β-agarase expression (Hou et al., 2015). The recombinant strain was used in all fermentation experiments.

2.2 *Culture media and feeding solutions*

For seed cultivation, Luria-Bertani (LB) broth medium supplemented with 100 μg/mL ampicillin was used (pH 7.0), which contained 10 g/L NaCl, 10 g/L peptone and 5 g/L yeast extract.

For fed-batch fermentation, the fermentation medium for β-agarase production contained 10 g/L glycerol, 5 g/L yeast extract, 6 g/L $(NH_4)_2HPO_4$, 10.5 g/L KH_2PO_4, 3.4 g/L $MgSO_4{\cdot}7H_2O$, 1.7 g/L citric acid, 100 μg/mL ampicillin and pH 7.0.

The feeding solution used for β-agarase production in *E. coli* contained 500 g/L glycerol, 30 g/L $MgSO_4{\cdot}7H_2O$, 15 g/L peptone and 30 g/L yeast extract and pH 7.0. IPTG, an inducer for protein expression, was filter-sterilized and added into the fermenter with the final concentration of 1 mmol/L. In addition, lactose was used for protein expression induction as a substitute for IPTG.

2.3 *Culture conditions*

The seed culture was started by inoculating 100 μL of frozen glycerol stock (maintained at –75°C) in 50 mL Luria–Bertani medium (containing 100 μg/mL ampicillin) in a 250 mL shake flask and cultured for 10 h at 37°C and 200 r/min. Then, a 3% (v/v) concentration of inoculum was inoculated into fermentation medium for batch cultivation. Batch fermentation was performed in a 7.0 L stirred fermenter (Biostat 5, Sartorius, Germany) with 5 L working volume. The recombinant cells were grown at 37°C in LB medium supplemented with 100 μg/mL ampicillin. Recombinant cells were induced at 0.4 to 0.6 OD_{600} with 1 mmol/L (final concentration) IPTG.

Fed-batch fermentation consisted of three phases. In the first phase, i.e., the batch phase, the cultivation started with 3.0 L fermentation medium which was incubated at 37°C. The initial glycerol concentration of the first phase was 10 g/L. When the initial glycerol was exhausted with a sudden increase in both dissolved oxygen (DO) and pH values approximately 9 h after cultivation. The second phase, i.e., the pre-induction phase, the fed-batch fermentation was started by feeding 500 g/L glycerol solution. To select the optimal induction point, the feeding procedure of exponential feeding was performed with the different predetermined specific growth rates (μ, h^{-1}). In this study, the feeding rate was adjusted every half an hour through the exponential feeding method. When the second phase was maintained for approximately 10 h, the fermentation entered the third phase, i.e., the post-induction phase, and maintained at a constant feeding rate of 6 g/(L·h) glycerol until the cell growth reached the maximum cell density, and IPTG or lactose was subsequently added or continuously fed at a certain rate into the cultivation broth. At the same time, the temperature was adjusted to a set value (e.g., induced at 16°C, 20°C, 25°C, 30°C or 35°C) by re-circulating water to start β-agarase production.

During the entire process, the pH was adjusted and controlled at 7.0±0.1 by automatic addition of 25% (v/v) ammonia solution. The pH value was detected by a pH electrode (Mettler Toledo) during cultivation. Agitation was provided by two Rushton impellers and varied from 200 to 800 r/min and was adjusted by a ring sparger with a range of 1.5–4 L/min, rendering DO, which was monitored with a DO electrode (Mettler Toledo), constantly at approximately (20±5)%.

2.4 *Cell fractionation, protein determination and β-agarase purification*

The cultivation broth was centrifuged at 8 000 *g* for 10 min at 4°C. The precipitate was re-suspended in 20 mmol/L phosphate buffer saline (PBS) buffer (pH 7.4) and then disrupted by ultrasonic (290 W, 2.5 s bursts and 7.5 s pulses for 10 min). The mixture was centrifuged at 15 000 *g* for 30 min at 4°C to separate soluble from insoluble protein fraction. The soluble and insoluble proteins were examined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE). Same volume of fractions were analysed by a 12% (w/v) SDS-PAGE to estimate both the overexpression level and solubility of the protein.

The soluble fraction was subjected to purification under non-denaturing conditions with Ni^{2+}-NTA resin according to the

manufacturer's protocol (Thermo Scientific, Rockford, USA), and the purified proteins were examined by SDS-PAGE. The concentration of purified proteins was measured using the BCA Protein Assay Kit (Thermo Scientific, USA), employing bovine serum album (BSA) as a standard.

2.5 *Enzyme activity assay*

The activity of agarase was assayed by detecting the release of the reducing sugar using the 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959) with slight modification. The standard reaction contained 100 μL enzyme solution (concentration of 500 μg/mL) and 900 μL PBS buffer (pH 7.4) containing 0.2% (w/v) melted agar (BIOSHARP, Japan). After incubation at 40°C for 5 min, the reaction was halted by immersion in boiling water for 5 min. The heat-inactivated purified β-agarase was used as a negative control. About 250 μL of the reaction solution was mixed with 750 μL DNS reagent and heated for 10 min in a boiling water bath and then cooled to room temperature. The absorbance of reducing sugar was measured at 550 nm wavelength and was compared with the standard curve of D-galactose. Enzyme activity (U/mL) was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per minute under the assay conditions.

2.6 *Analytical methods*

Samples were withdrawn from fermenter for analyses at regular intervals. The biomass was determined in triplicate for 50 mL cell suspensions that were harvested by centrifugation (6 000 *g*, 5 min), washed with distilled water and dried at 60°C for 24 h to a constant weight (dry cell weight, DCW).

Fermentation supernatant was analysed for glycerol and acetic acid contents. Glycerol was measured using a Waters HPLC equipped with a differential refractive index detector (RID) and a 300 mm×7.8 mm HPX-87H ion exclusion column (Aminex). A 5 mmol/L H_2SO_4 was used as the mobile phase. Acetic acid concentration was measured with a BioProfile 300A biochemical analyser (Nova Biomedical, Waltham, MA).

Lactose was determined by HPLC equipped with a refractive index detector using a Rezex ROA-organic acid H^+ (8%) column (Phenomenex Inc., Torrance, CA). The column was eluted with 0.005 mol/L of H_2SO_4 at a flow rate of 0.6 mL/min at 50°C.

2.7 *Hydrolysis of Gracilaria lemaneiformis powder and products identification*

The recombinant agarase was incubated with 1% *G. lemaneiformis* powder in a 250 mL shake flask at 40°C, 100 r/min for 8 h. The enzyme activity was stopped by heating a boiling water bath for 5 min. In order to determine the components of the enzymatic hydrolysates, the hydrolysis products were analyzed by the ion exchange chromatography (IC). For IC analysis, the hydrolysis products were analyzed under the same conditions with an anion exchange chromatography (DIONEX, Sunnyvale, CA, USA) equipped with a 250 mm×4 mm IonPac column (ASII-HC). After the sample was loaded, the column was washed with the mobile phase (100 mmol/L NaOH, 150 mmol/L NaAc) at a flow rate of 0.25 mL/min for 50 min. The liquid chromatography (LC) plot was acquired by plotting the electrical conductivity of the eluent against the retention time. Neoagarobiose (NA2), neoagarotetraose (NA4), neoagarohexaose (NA6), neoagarooctaose (NA8), neoagarodecaose (NA10), and neoagarododecaose (NA12) were used as standards.

3 Results and discussion

3.1 *Fed-batch fermentation with different specific growth rates (μ)*

The production of recombinant proteins is mainly determined by both the cell density and specific cell productivity in the fermentation process (Wang et al., 2015). To achieve high cell density and β-agarase activity, applying appropriate feeding strategies is necessary. Exponential feeding is a simple method that allows cells to grow at a constant growth rate below the critical value for acetic acid formation. In the fermentation process, over-accumulation of acetic acid (>2 g/L) will result in inhibition of cell growth and formation of recombinant protein and thus should be avoided. Based on the above knowledge, in this study, exponential feeding strategy with different overall μ (0.2, 0.4 and 0.6 h^{-1}) was applied in the pre-induction phase (Table 1, Fig. 1). In the post-induction phase, 1 mmol/L IPTG induction was employed to examine its influence on β-agarase production and cell growth. When the overall μ was set at 0.6 h^{-1}, the cell growth reached 37.78 g/L before the post-induction phase started. When induced at a high cell density, the cell growth was seriously inhibited and the final DCW decreased to 31.88 g/L, and the β-agarase activity was only 13.2 U/mL. When the overall μ was set at 0.2 and 0.4 h^{-1}, the cell growth reached 16.24 and 27.11 g/L, respectively. Similarly, the cell growth was inhibited when the fermentation was induced with IPTG after growth at a low μ (0.2 h^{-1}) or an intermediate μ (0.4 h^{-1}) in pre-induction phase, and the final DCW reached 36.53 and 34.89 g/L, respectively. The maximum β-agarase activity reached 63.7 and 37.5 U/mL. During the post-induction phase, the overall μ was 0.15 h^{-1} for inductions at a low μ and 0.025 h^{-1} for inductions at an intermediate μ in pre-induction phase. However, this inhibitory effect was insignificantly observed in the pre-induction phase. Comparing the three different μ in the pre-induction phase, we found that the cells probably suffered a low level of metabolic burdens and accordingly showed a high cell growth rate, when the inductions started

Table 1. Comparison of the cultivation methods for recombinant β-agarase production

Parameter[1)]	Cultivation method		
	μ=0.2	μ=0.4	μ=0.6
DCW[2)]/g·L^{-1}	16.24±0.59	27.11±0.67	37.78±0.96
Final DCW[3)]/g·L^{-1}	36.53±0.50	34.89±0.79	31.88±0.41
β-agarase activity/U·mL^{-1}	63.7±1.08	37.5±1.15	13.2±0.95
Maximum specific β-agarase production rate/h^{-1}	0.476±0.03	0.456±0.02	0.444±0.02
Maximum acetic acid/g·L^{-1}	0.87±0.08	2.94±0.40	4.73±0.18
Maximum specific rate of acetic acid/h^{-1}	0.085±0.003	0.243±0.01	0.341±0.02
Glycerol consumption/g·L^{-1}	72.38±3.09	76.46±3.67	87.55±2.97
Induction time[4)]/h	15	15	12.5

Note: [1)] Statistical analysis by *t*-test, $p<0.05$; [2)] cell growth before the post-induction phase started; [3)] cell growth at the end of post-induction phase; [4)] time of post-induction phase.

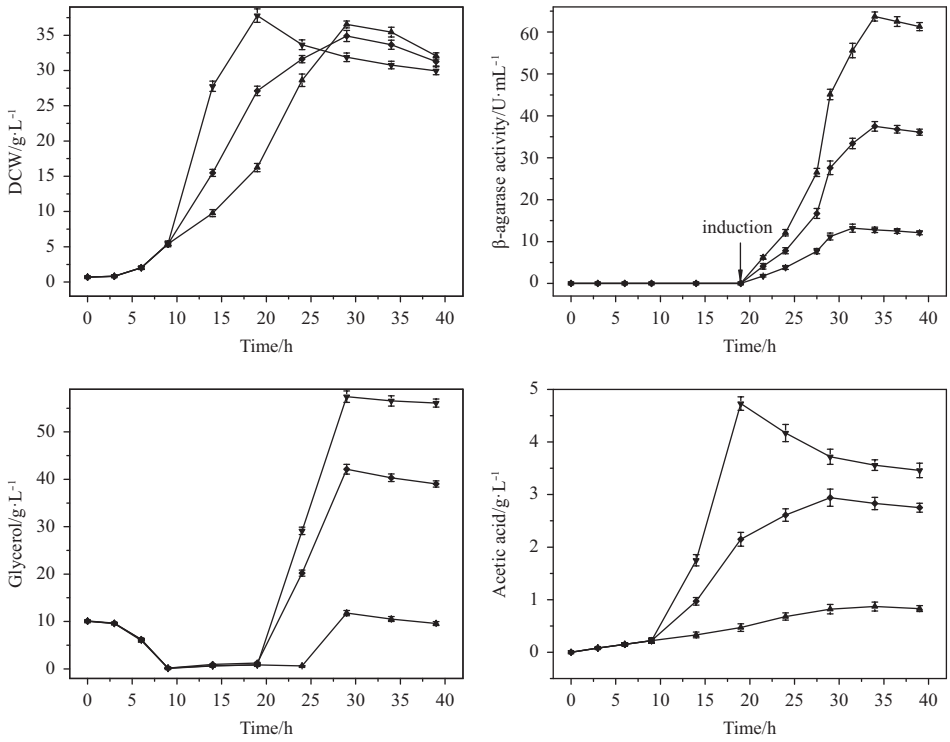

Fig. 1. Time profiles of cell growth, β-agarase activity, glycerol and acetic acid for fed-batch fermentation based on different μ in pre-induction phase (▲ μ=0.2, ◆ μ=0.4, ▼ μ=0.6). Values are expressed as average of triplicate independent experiments with standard deviation.

at a low μ. Cell density also had an important part in the over-production of heterologous protein, but immoderate cell density in the pre-induction phase can influence protein expression and cell growth in the post-induction phase. When the fermentation was induced at immoderate cell density, this phenomenon may result in exhaustion of aerobic conditions, such as the DO-limitation and metabolic burdens, which may lead to serious inhibition of cell growth (as previously described) and thus inhibition of recombinant protein production.

In Fig. 1, with the change of the set μ in the pre-induction phase, the acetic acid concentrations were also varied. When μ was 0.2 h^{-1} in the pre-induction phase, the acetic acid concentration was at a low level (0.87 g/L) during the whole process. However, when μ were 0.4 and 0.6 h^{-1} in the pre-induction phase, the maximum acetic acid concentrations were 2.94 and 4.73 g/L, respectively. Furthermore, the maximum specific rates of acetic acid were 0.085, 0.243 and 0.341 (Table 1). Cells usually produce acetate when they encounter the conditions of high cell specific growth rate, DO-limitation or high-level carbon source concentration. When high acetic acid concentration (>2 g/L) was maintained at fed-batch culture stages, the inhibition of acetic acid on cell growth and protein production were found in the fermentation. Therefore, induction at a low μ (μ=0.2) was suggested to be the optimal for cell growth and β-agarase production.

3.2 *Effect of induction temperature on cell growth and β-agarase production*

In the post-induction phase, the production of recombinant protein depends on the process variables (e.g., temperature and pH) and induction methods (e.g., IPTG or lactose concentration) (Cheng et al., 2011). Induction temperature contributes in the recombinant protein production in *E. coli* (Xu et al., 2000; Shokri et al., 2003). To select a suitable induction temperature for β-agarase production, the influence of induction temperature (16°C, 20°C, 25°C and 30°C) was investigated (1 mmol/L IPTG was used). In Fig. 2, a high level of biomass was obtained at the induction temperatures of 25°C and 30°C, which produced the highest DCW of 36.53 and 41.32 g/L, respectively. However, when the cultivations were induced at a low temperature (16°C), the growth of the cells was limited because of low metabolic level. The maximum β-agarase activity reached 84.2 U/mL after 15 h of induction when cultivations were induced at the induction temperature of 20°C. According to the results, the induction at 25°C and 30°C had a negative effect on β-agarase production.

Low temperature was unfavourable to cell growth but was believed to reduce protein synthesis and to decrease fluidity of the cytoplasmic membrane, which consequently would influence membrane-associated cellular functions such as periplasmic transport and nonspecific periplasmic leakage (Cheng et al., 2011). Moreover, high temperature may cause excessive synthesis of β-agarase at a high speed, leading to the production of large amounts of inactive proteins, i.e., inclusion bodies, because many studies (Fang et al., 2011; Jhamb and Sahoo, 2012) have demonstrated that the formation of inclusion body could be reduced by decreasing the induction temperature. Therefore, to obtain high β-agarase yield, a medium induction temperature of 20°C was selected to further investigate the effect of IPTG concentrations on β-agarase production.

In Figs 1 and 2, with the different μ in the pre-induction phase and different induction temperature in the post-induction phase, the consumption of glycerol was different. With the increase of glycerol consumption, cell growth and acetic acid also increased. However, β-agarase production did always increase. Meanwhile, high substrate concentration may affect the expression of recom-

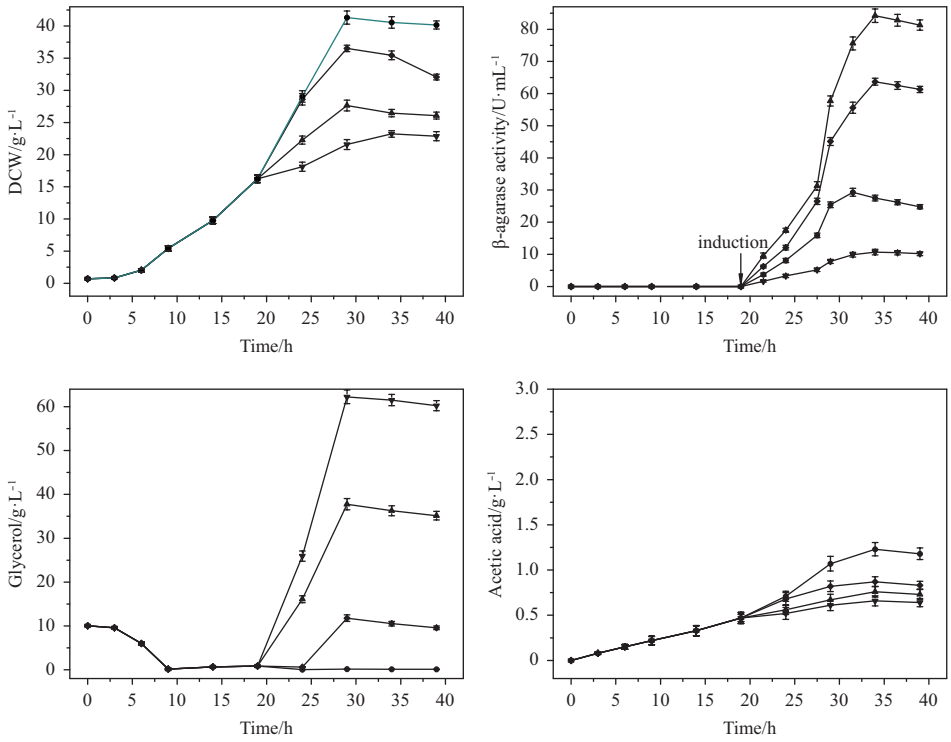

Fig. 2. Time profiles of cell growth, β-agarase activity, glycerol and acetic acid for fed-batch fermentation based on different induction temperatures in post-induction phase (▼ 16°C, ▲ 20 °C, ◆ 25°C, ● 30°C). Values are expressed as average of triplicate independent experiments with standard deviation.

binant protein, and the residual glycerol concentration was too high (>30 g/L); hence, the feeding rate of glycerol will be reduced from 6 to 3 g/(L·h).

3.3 *Effect of IPTG concentration on cell growth and β-agarase production*

The IPTG concentration in the cultivation is another important factor and determines the protein expression level when other conditions are identical. Although increase of the IPTG concentration to a suitable range can accelerate protein production rate, over-accumulation of proteins in the intercellular cytoplasm may yield inclusion bodies. In Fig. 3, different IPTG concentrations (0.5, 0.8, 1.0 and 1.5 mmol/L) were investigated for β-agarase production at the optimal induction temperature of 20°C.

Increase of the IPTG concentration had a significant negative effect on cell growth. At the IPTG concentration of 0.5 mmol/L, the maximum DCW reached 29.07 g/L, which was 1.03- and 1.04-fold higher than the maximum DCW obtained at 0.8 mmol/L (28.24 g/L) and 1.0 mmol/L (27.91 g/L) IPTG concentrations, respectively. However, the maximum β-agarase activity reached 105.7 U/mL at the IPTG concentration of 0.8 mmol/L, which was 1.50- and 1.24-fold higher than the IPTG concentration obtained using 0.5 mmol/L IPTG (70.7 U/mL) and 1.0 mmol/L IPTG (85.3 U/mL), respectively. However, when the cells were induced with a high IPTG concentration (1.5 mmol/L), the cell growth and β-agarase yield were severely inhibited, resulting in the lowest DCW and β-agarase activity (Fig. 3). In summary, the suitable IPTG concentration was beneficial to cell growth and enzyme expression. On the contrary, an excessive IPTG concentration seriously inhibited the cell growth and reduced the enzyme yield, which may be because of the increase of inactive inclusion bodies. Cultivations at optimal induction temperature (20°C) along with an optimal IPTG concentration (0.8 mmol/L) could achieve high β-agarase production.

3.4 *High-level production of β-agarase by using a continuous lactose feeding approach as a substitute for IPTG induction*

IPTG, a toxic substrate for cells, exhibits a negative effect on cell growth and viability (Malakar and Venkatesh, 2012; Xu et al., 2013). Therefore, the use of lactose for induction instead of IPTG can reduce the cost and decrease toxicity. Mild induction by lactose could reduce the metabolic burden and promote the accumulation of expressed proteins, when compared with a drastic induction by IPTG (Kilikian et al., 2000).

In Fig. 4, to select the suitable feeding rate of lactose solution in the post-induction phase, three feeding rates (0.5, 1.0 and 1.5 g/(L·h)) were used. At the lactose feeding rate of 1.5 g/(L·h), the maximum DCW reached 32.46 g/L, which was 1.11- and 1.06-fold higher than that obtained at 0.5 g/(L·h) (29.17 g/L) and 1.0 g/(L·h) (30.53 g/L) of lactose feeding rate, respectively. Compared with IPTG induction, the cell growth of the cultivations induced by continuous lactose feeding approach was not significantly affected. With the increase of lactose feeding rate, the DCW increased, but the boost effect was not evident. Lactose raised the DCW by serving as a carbon source. However, high lactose concentrations had some inhibitory effects on the cell growth.

The maximum β-agarase activity reached 112.5 U/mL at lactose concentration of 1.0 g/(L·h) (Fig. 4b), which was 1.28- and 2.03-fold higher than that obtained by using 0.5 g/(L·h) lactose (87.7 U/mL) and 1.5 g/(L·h) lactose (55.3 U/mL), respectively. In summary, the increase of the lactose feeding rate enhanced the rate of protein synthesis, but the overexpression of proteins often resulted in inclusion body accumulation when the capability for protein expression was limited. Induction at intermediate lactose

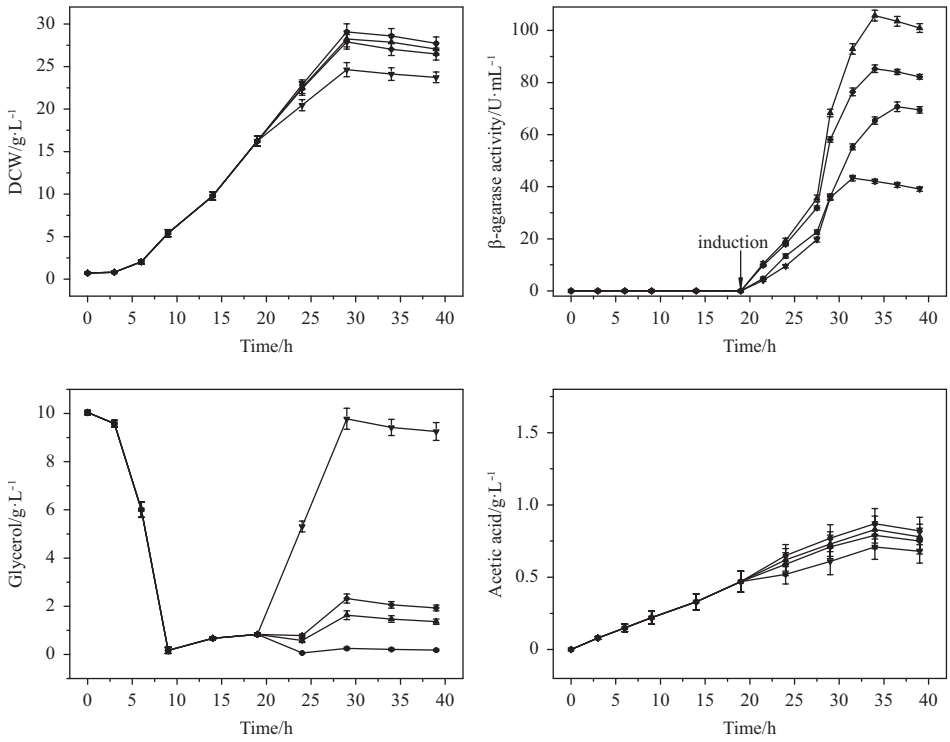

Fig. 3. Time profiles of cell growth, β-agarase activity, glycerol and acetic acid for fed-batch fermentation based on different IPTG concentrations in post-induction phase (● 0.5 mmol/L, ▲ 0.8 mmol/L, ◆ 1.0 mmol/L, ▼ 1.5 mmol/L). Values are expressed as average of triplicate independent experiments with standard deviation.

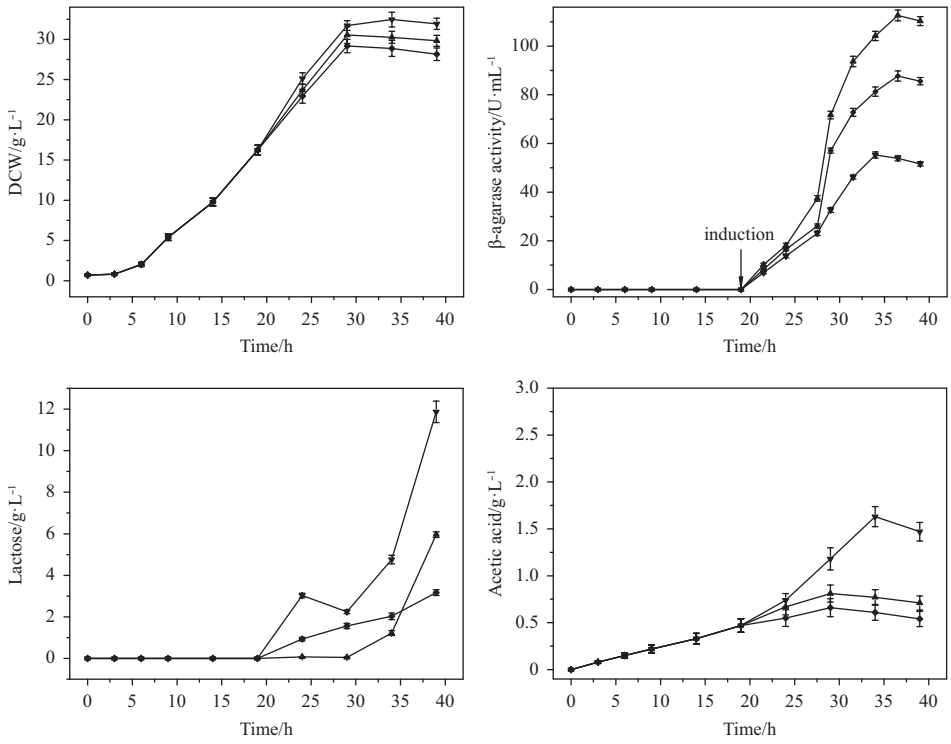

Fig. 4. Time profiles of cell growth, β-agarase activity, acetic acid and lactose for cultivations induced by using different continuous lactose feeding rates in post-induction phase (◆ 0.5 g/(L·h), ▲ 1.0 g/(L·h), ▼ 1.5 g/(L·h)). Time profiles of glycerol were the same, so data were not shown. Values are expressed as average of triplicate independent experiments with standard deviation.

feeding rates obtained the highest yield of β-agarase in the culture process. Although the fermentations induced by IPTG exhibited a high rate of protein synthesis and only took 15 h to achieve the maximum β-agarase production compared with 17.5 h in the

case of lactose feeding, the cells had undergone drastic induction with high metabolic burdens because of high IPTG toxicity, which could result in loss of cell activity and lower β-agarase activity. As a result, the recombinant β-agarase could easily move from the intracellular space to the cytoplasm or periplasm space. In this way, accumulation of expressed proteins in the cytoplasm and periplasm can become inactive proteins.

To explore whether lactose induction could reduce inclusion body production, the soluble proteins and insoluble proteins for lactose or IPTG induction were analysed by SDS-PAGE. In Fig. 5, compared with IPTG induction, the cells induced with lactose showed higher amount of soluble β-agarase and lower amount of insoluble β-agarase, which provided a reasonable explanation for the improved overall enzyme activity with lactose induction. Besides, lactose induction can lower costs and improve β-agarase production, as recently shown by Dvorak et al. (2015). Therefore, we inferred that lactose may be a better inducer than IPTG for the expression of heterologous proteins in *E. coli* BL21 (DE3).

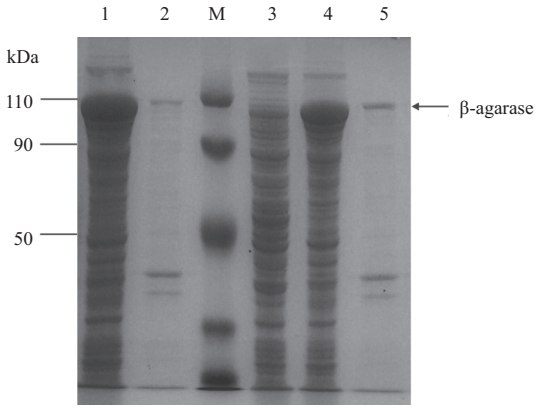

Fig. 5. SDS-PAGE analysis of β-agarase. Lane 1: soluble fragments, induced by lactose; Lane 2: insoluble fragments, induced by lactose; Lane 3: total proteins, not induced; Lane 4: soluble fragments, induced by IPTG; Lane 5: insoluble fragments, induced by IPTG.

Table 2 shows the various strategies and results for the β-agarase production of wild bacteria and recombinant *E. coli*. Although dozens of β-agarases have been well studied, process optimization was carried out in shake flask. Many researchers used one-factor-at-a-time design and Box–Behnken design for process optimization of β-agarases production, but the highest β-agarases activity was only 11 U/mL (Choi et al., 2011). Some β-agarase genes were heterologously expressed in *E. coli* (Chen et al., 2016; Lin et al., 2012; Yang et al., 2011; Hou et al., 2015; Seo et al., 2014), the process optimization of recombinant β-agarase has not been reported in fermenter. In the present study, we developed a multi-step glycerol feeding strategy based on induction mode optimization for β-agarase production in a 7.0-L fermenter for the first time and achieved the highest activity of recombinant β-agarase in *E. coli*.

3.5 *Hydrolysis products analysis of β-agarase by degrading G. lemaneiformis powder*

To identify the hydrolysis products of β-agarase by degrading *G. lemaneiformis* powder, the hydrolysis products of 8 h reaction sample were analyzed with IC. As a kind of red algae, the cell wall of *G. lemaneiformis* is mainly constituted by agar, xylan and cellulose. As shown in Fig. 6, the hydrolysates were NA4 and NA6

Table 2. Comparison of parameters for β-agarase production using various strategies

Strain	Bioreactor	Optimizing strategy	Culture time/h	β-agarase activity/U·mL^{-1}	Product	Reference
Agarivorans albus OAY02	shake flask	Box-Behnken design	48	2.65	NA2, NA4, NA6	Choi et al. (2011)
Pseudomonas aeruginosa AG LSL-11	shake flask	one-factor-at-a-time design	36	0.32	–	Lakshmikanth et al. (2006a)
Acinetobacter sp. AG LSL-1	shake flask	one-factor-at-a-time design	18	0.45	NA2, NA4	Lakshmikanth et al. (2006b)
Micrococcus sp. GNUM-08124	shake flask	one-factor-at-a-time design	72	11	–	Yang et al. (2014)
Stenotrophomonas sp. NTa	shake flask	one-factor-at-a-time design	48	1.03	NA2, NA4, NA6	Zhu et al. (2016)
E. coli	shake flask	optimization of culture conditions and induction strategy	11	6.7	NA2, NA4	Seo et al. (2014)
E. coli	7.0-L fermenter	induction mode optimization and glycerol feeding strategy	36.5	112.5	NA4, NA6	this work

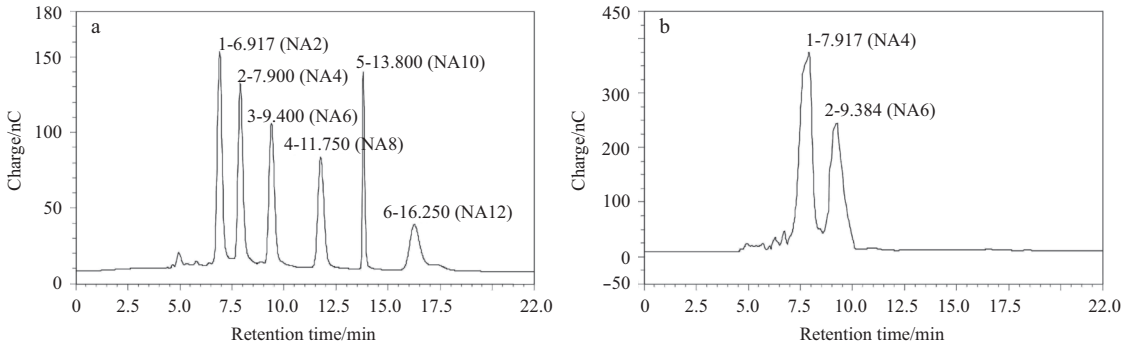

Fig. 6. Identification of hydrolysis products. IC analysis of oligosaccharide standards (a) and Hydrolysis Products of β-agarase by Degrading *G. lemaneiformis* Powder (b). Peaks were labelled with retention times.

after 8 h hydrolysis, which were the same products as degrading agar by Hou et al. (2015) and similar with other β-agarases (Table 2). As mentioned in our previous work, the β-agarase from *F. pacifica* was a newly agarase belonging to GH86 family, which should make it capable of degrading *G. lemaneiformis* powder directly to produce neoagarooligosaccharide. The outstanding properties enable β-agarase to produce oligosaccharides with low cost and high efficiency under industrial conditions.

4 Conclusions

In the present study, a multi-step glycerol feeding strategy based on induction mode optimization for high cell density and β-agarase production was developed in a 7.0-L fermenter. This strategy showed that low μ in the pre-induction phase was appropriate for cell growth and β-agarase production. A lactose continuous feeding strategy was established in the post-induction phase, and the maximum β-agarase activity of 112.5 U/mL was obtained, which represented 5.08-fold increase compared with increase observed under unoptimized conditions and represented the highest value reported to date. The β-agarase was capable of degrading *G. lemaneiformis* powder directly to produce neoagarooligosaccharide, the hydrolysates were NA4 and NA6.

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