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Improvement on Extracellular Production of Recombinant *Burkholderia cepacia* Lu10-1 Lipase by *Escherichia coli*

[1,2]Yao Zhang, [1,2]Yuanda Song, [1,2]Lu Wang and [1,2]Qing Liu

[1]*Colin Ratledge Center for Microbial Lipids, School of Agricultural Engineering and Food Science, Shandong University of Technology, 266 Xincun West Road, Zibo 255000, People's Republic of China*
[2]*Key Laboratory of Shandong Provincial Universities for Technologies in Functional Agricultural Products, Shandong University of Technology, 266 Xincun West Road, Zibo 255000, People's Republic of China*

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Corresponding Author:
Yao Zhang
Colin Ratledge Center for Microbial Lipids, School of Agricultural Engineering and Food Science, Shandong University of Technology, 266 Xincun West Road, Zibo 255000, People's Republic of China
Email: lz20011022@126.com

Abstract: The purpose of this study was to investigate several strategies on enhancing extracellular production of recombinant lipase from *Burkholderia cepacia* Lu10-1 in recombinant *Escherichia coli* BL21(DE3). In the present study, a fed-batch fermentation strategy for the excellular production of lipase by *E. coli* has been established. First of all, different induction methods (including selection of inducers, inducer concentration, induction temperature and induction time) were investigated and the results indicated that these factors played an important role in lipase production. When induced by 0.8 g L^{-1} h^{-1} lactose at 30°C and at a OD_{600} of 30, the lipase activity in the culture medium could achieve 58 U mL^{-1}. Moreover, addition of glycine and calcium ions can increase the extracellular yield of lipase. With supplementation of the culture with 0.5% (w/v) glycine and 2.5 mM Ca^{2+}, the maximum extracellular activity of lipase could reach 85 U mL^{-1}, which was 2.1 fold higher than that of the control. This study might provide fermentation strategy for the extracellular production of other heterogonous proteins expressed in *E. coli*.

Keywords: *Escherichia coli*, *Burkholderia cepacia*, Lipase, Extracellular Production, Induction

Introduction

Lipase (triacylglycerol acylhydrolase, E.C. 3.1.1.3) is a type of hydrolase which catalyze the hydrolysis of triglycerides to glycerol and free fatty acids. Furthermore, lipase is capable of catalysis of ester synthesis and transesterification reaction. Due to these diverse properties of lipase, it has been widely utilized in biotechnical applications including biofuel, food, detergent, chemical, textile and agricultural industry (Fickers *et al.*, 2011; Malekabadi *et al.*, 2018; Rogalska *et al.*, 1997). *Burkholderia* (previously *Pseudomonas*) *cepacia* lipase has high transesterification capacity and broad substrates adaptability and these distinct properties give the lipase great potential for basic research and various industrial applications (Mello Bueno *et al.*, 2015; Sasso *et al.*, 2016; Yang *et al.*, 2007). However, some limitations with respect to the low level of expression and high cost of production are becoming obstacles that restricted the use of *B. cepacia* lipase on a large scale.

In order to overcome the low yields of lipase produced by wild strains, the most effective approach has been considered to be overexpression of a lipase gene in *Escherichia coli* through genetically engineering. Heterologous proteins expression in *E. coli* has been gradually increased in recent years. However, there are two prominent problems during the experiment: (1) Low soluble expression level; (2) poor secretion efficiency. Researchers have tried various strategies to increase soluble expression and secretion efficiency and fermentation process parameter control and medium optimization are the most commonly used strateges. For example, Chen *et al*. (2014) controlled the fermentation temperature resulting in a significant increase in the expression and secretion of the target protein in *E. coli*. The addition of media additives such as metal ions, glycine, surfactants and penetrants can increase the permeability of the cell membrane and help improve protein secretion. For example, Nie *et al*. (2013) found that more than 90% of the target protein could be secreted to the extracellular by adding 0.6% (w/v) glycine in the later fermentation medium.

Previously, we have performed the expression of lipase from *B. cepacia* Lu10-1 in recombinant *E. coli*

BL21(DE3) with secreted PelB signal peptide in the vector pET20b(+). During the cultivation process, it is observed that most of the PelB-fused lipase was accumulated in the perplasmic space and small amount of lipase was secreted to the extracellular (Zhang *et al.*, 2017). However, targeting the recombinant lipase to the culture medium has several advantages over intracellular production, such as simplifying downstream processing, achieving high-level expression and limiting inclusion body formation (Makrides, 1996; Yang *et al.*, 2007). Therefore, in the present study, several strategies have been carried out to enhance the extracellular production of recombinant *B. cepacia* lipase by *E. coli*. Initially, we have attempted to investigate the cell growth and lipase activity through comparing different inducers and inducing concentrations. Subsequently, different induction methods (including induction temperature and induction time) were investigated to further promote the extracellular production of lipase into the culture medium. Additionally, the effect of two chemical additives (glycine and Ca^{2+}) as well as their interaction on the extracellular secretion of lipase during the culture period were also discussed in detail.

Materials and Methods

Baterial Strain, Plasmids and Chemicals

The recombinant *E. coli* BL21(DE3) carrying lipase gene of *B.* cepacia Lu 10-1 (constructed in our previously work) was used for all fermentation experiments (Zhang *et al.*, 2017). The recombinant plasmid included lipase gene, T7 promoter, PelB signal peptide, His-tag encoding sequence and so on.

Peptone and yeast powder were purchased from Oxiod Ltd. *p*-Nitrophenyl Palmitate (*p*-NPP) and Isopropyl-1-Thio-β-D-Galactopyranoside (IPTG) were purchased from Sigma. Glycine (AR) and other chemical reagents of analytical grade unless indicated were obtained from Sinopharm Chemical Reagent Co. Ltd.

Medium and Culture Methods

Luria-Bertani (LB) medium, containing peptone 10 g L^{-1}, yeast powder 5 g L^{-1}, NaCl 10 g L^{-1}, pH 7.1, was used for seed medium. The culture medium contained glycerol 6 g L^{-1}, peptone 12 g L^{-1}, yeast powder 24 g L^{-1}, $K_2HPO_4 \cdot 3H_2O$ 16.43 g L^{-1}, KH_2PO_4 2.31 g L^{-1}, pH 7.1. The feeding solution was consisted of 500 g L^{-1} glycerol. The IPTG (0.5 mmol mL^{-1}) and lactose (200 g L^{-1}) were prepared as inducers.

E. coli BL21(DE3) harboring pET20b/lip recombinant plasmid was inoculated into 50 mL of seed medium with 100 μg mL^{-1} ampicillin in a 250 mL shake flask at 37°C and 200 rpm for 8 h. A 10% (v/v) cell concentration of inoculum was inoculated into fermentation medium (containing 100 μg mL^{-1} ampicillin) for fed-batch cultivation. Fed-batch cultivation consisting of three phases were performed in a 3 L fermentor (BioFlo 110, New Brunswick Scientific Co., Edison, NJ). The first batch phase with an initial glycerol concentration of 6 g L^{-1} was carried out at the temperature of 37°C. After inoculation, the Dissolved Oxygen (DO) value decreased suddenly with an accompanied decrease in glycerol and pH. The exhausted consumption of glycerol was monitored by a prompt rise in both DO and pH value and afterwards followed by the pre-induction phase of fed-batch cultivation. During the second fed-batch phase, 1.5 g $L^{-1}h^{-1}$ glycerol was added at a sudden rise in DO. Ca^{2+} were added at the beginning of cultivation and glycine was added after cultivation of 6 h. When OD_{600} was reached at 15, 30 or 45 (namely the culture time at 15 h, 20 h or 25 h), respectively, the lactose with different concentrations was fed at 0.8 g L^{-1} h^{-1} (or IPTG added every four hours at final concentration of 0.5 mol L^{-1}) and temperature decreased to 25°C, 30°C or maintained at 37°C for inducing lipase production and then the post-induction phase started. During the whole cultivation process, the pH was kept at 7.1 by adding 20% (w/v) ammonia solution. The DO level was retained at 20-30% (v/v) of air saturation by cascading impeller speed and supplementation of air with pure oxygen for maintaining the final oxygen concentration of 20-30% (v/v). Polydimethylsiloxane as antifoam was added manually only when it was necessary. Temperature, pH, DO concentration and impeller speed were recorded using Advanced Fermentation Software (AFS) from New Brunswick Scientific Co. Inc. At certain time intervals (almost 3 to 5 h intervals), samples were collected and analyzed for biomass and lipase activity. Each value represents the mean of three independent measurements.

Determination of Bacteria Biomass

Cell growth was detected during cultivation by measuring the Dry Cell Weight (DCW), which was performed as follows. After centrifugation of a 5 mL culture broth at 12000 rpm for 10 min, the pellet was washed with 0.9% (w/v) NaCl, recentrifuged and then dried to a constant weight at 105°C.

Lipase Activity Assay

Culture supernatants were obtained by centrifugation with 12000 rpm for 10 min at 4°C and the supernatant was collected as extracellular fraction to measure activity. Lipase activity was estimated as described previously (Gricajeva *et al.*, 2016; Zhang *et al.*, 2017), utilizing *p*-Nitrophenyl Palmitate (*p*NPP) as the substrate. The hydrolysis of *p*NPP was monitored for the formation of *p*NP using spectrophotometry at 410 nm. One unit of lipase activity was defined as the amount of enzyme that produced 1 μmol of *p*-Nitrophernol (*p*NP)

per min at 40°C and pH 8.0. Each value represents the mean of three independent measurements.

Determination of Glycerol and Lactose

Culture supernatants in fed-batch cultivation were analyzed for glycerol and lactose by using an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA) with a differential Refractive Index Detector (RID). The analytical conditions were performed as follows: Shodex SUGAR SH1011 column (Showa Denko, Japan), 8.0 mm ID×300 mm; mobie phase, 5 mM H_2SO_4; column temperature, 50°C; injection volume, 5 μL; detector temperature, 30°C; flow rate, 0.8 mL min^{-1}.

Results and Discussion

Effect of Inducers on Cell Growth and Lipase Production

IPTG is a strong and stable inducer that was commonly used in laboratory fermentation and it cannot be easily metabolized by bacteria (Sorensen and Mortensen, 2005). However, IPTG is expensive and potentially toxic, limited to large-scale fermentation in industry. Many studies have focused on lactose instead of IPTG as a inducer to start the T7 promoter in the pET vector and start the transcription of the target protein gene due to its non-toxic and inexpensive features. Meanwhile, lactose itself as a carbon source, can be metabolized and utilized by bacteria (Ammar *et al.*, 2018; Huang *et al.*, 2016; Zou *et al.*, 2014). In this study, we have compared the effects of IPTG and lactose on cell growth and lipase production (Fig. 1) and found that lactose has a similar induction to that of IPTG, conversely, IPTG slightly suppressed the cell growth. In view of the cell damage and high industrial cost of IPTG, lactose was chosen as the inducer for the following study. According to report, high or low lactose concentration would greatly affect the enzyme production (Wang *et al.*, 2009), therefore, different flow rate control of lactose in a range of 0-2 g L^{-1} h^{-1} were investigated. As shown in Fig. 2, with increasing flow rate control of lactose, the cell density decreased significantly, indicating that high concentration of lactose certainly inhibited the cell growth which was consistent with previous study (Cheng *et al.*, 2011; Wang *et al.*, 2009). When the culture was induced with 0.8 g L^{-1} h^{-1} lactose, the optimum extracellular enzyme activity was 40 U m L^{-1}, which was increased by 14.3% and 66.7% compared to the activity induced with 0.4 g L^{-1} h^{-1} and 2 g L^{-1} h^{-1}, respectively.

Effect of Induction Temperature on Cell Growth and Lipase Production

Temperature can significantly influence the bacterial growth and protein expression, in this study, we developed a two-stage temperature fermentation strategy, in which three different induction temperatures (25°C, 30°C and 37°C) were investigated. As depicted in Fig. 3A, the final biomass reached 44 g L^{-1} at an induction temperature of 37°C, which was 1.1- and 1.6-fold that of 30°C and 25°C, respectively. However, a high level of biomass did not lead to high protein yield. The extracellular lipase activity obtained at 37°C was far lower than that of other temperatures (Fig. 3B) and this experiment indicated that induction at 37°C had a negative impact on lipase production. The reason might be that high temperature might cause excessive speed of lipase synthesis, resulting in large amounts of inactive protein aggregates, i.e., inclusion bodies (data not shown). Lower induction temperature decreased protein synthesis and fluidity of the cytoplasmic membrane (Cheng *et al.*, 2011; Schumann, 2000). Thus, induction at 25°C resulted in a lower extracellular enzyme activity than that at 30°C but higher than that at 37°C. Although high temperature improves the fluidity of cell membrane and the speed of protein synthesis, overexpression of proteins normally leads to the intracellular accumulation of inclusion bodies, which might influence the cell physiology and protein transport. According to our results, 30°C was best for lipase production, at which the extracellular lipase activity reached 46 U mL^{-1}.

Effect of Induction Time on Cell Growth and Lipase Production

It was known that the overexpression of recombinant protein generally imposes certain metabolic burden on the host cell, which might reduce the protein expression, cell biomass and plasmid stability (Cheng *et al.*, 2011; Donovan *et al.*, 1996). For the purpose of lowering the degree of metabolic burden imposed on the cell, in the present study, the best induction time for lipase was investigated, in which lactose was fed at a OD_{600} of 15, 30 and 45, respectively. As a result, cell growth was severely suppressed when induced at low cell density (OD_{600} of 15), under which the maximum DCW was only 15 g L^{-1} (Fig. 4A). Consequently, this serious suppression of cell growth resulted in decreased extracellular lipase activity of only 18.6 U mL^{-1} (Fig. 4B). In spite of no significant inhibition at high cell concentration induction (OD_{600} of 45), the extracellular lipase activity was also extremely low, only 23.8 U mL^{-1}. Therefore, induction at intermediate cell density (OD_{600} of 30) became the best condition and the maximum lipase activity in the medium achieved 58 U mL^{-1} (Fig. 4B). The above results demonstrated that the extracellular production of recombinant lipase was seriously influenced by the induction time, which was similar to the expression of CGTase (Cheng *et al.*, 2011) and glycine oxidase (Martínez-Martínez *et al.*, 2007), etc. In previous researches, it was reported that when

induced at high cell density (namely during the stationary phase), the cell membrane becoming more rigid was not suitable for nonspecific periplasmic leakage (Cheng *et al*., 2011; Shokri *et al*., 2003), which might lead to the reduced secretion of periplasmic proteins into the culture medium.

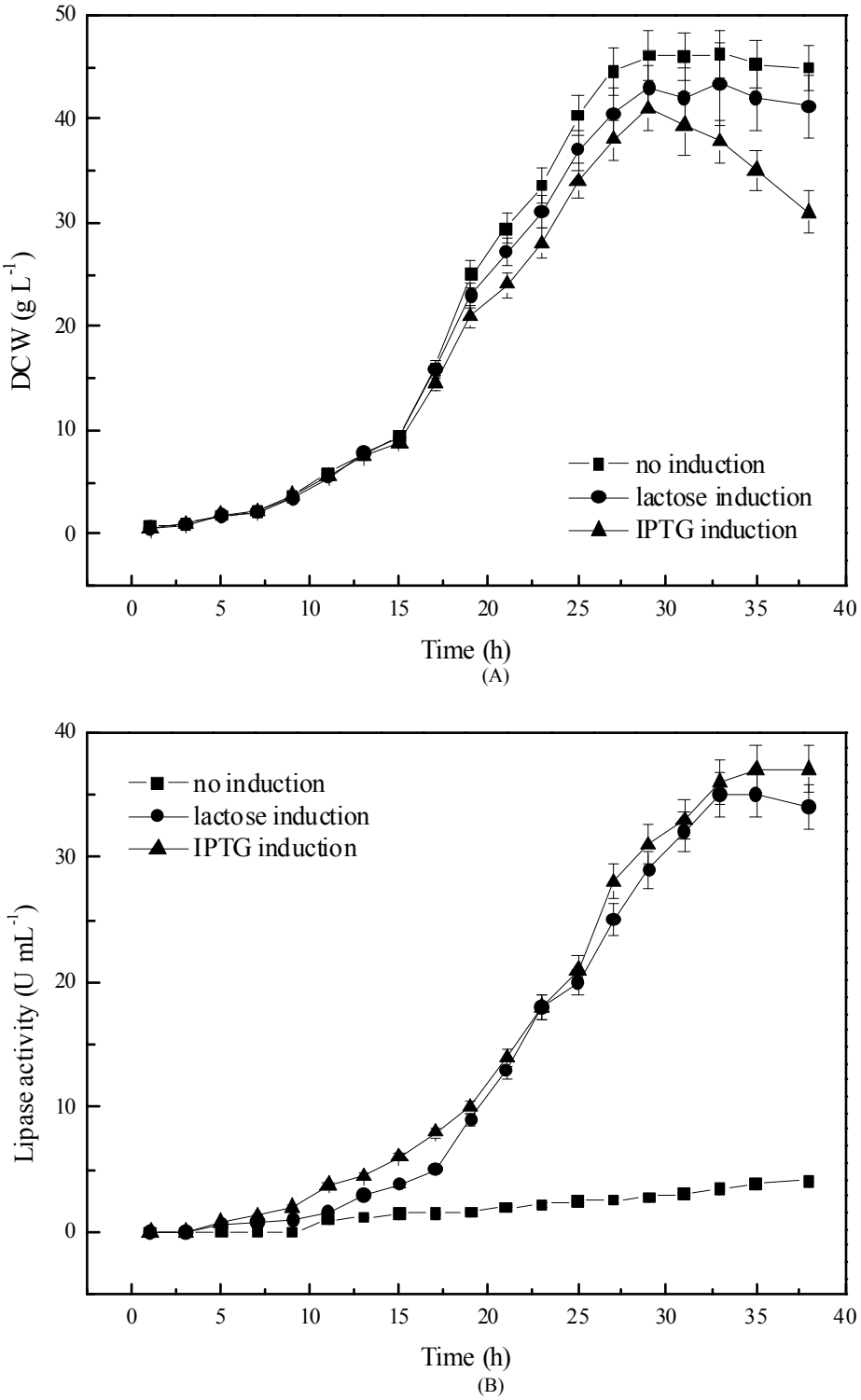

Fig. 1: Comparison of the effect of IPTG and lactose induction on cell growth (A) and lipase activity (B) in the culture medium. ■ no induction, ● lactose induction, ▲ IPTG induction

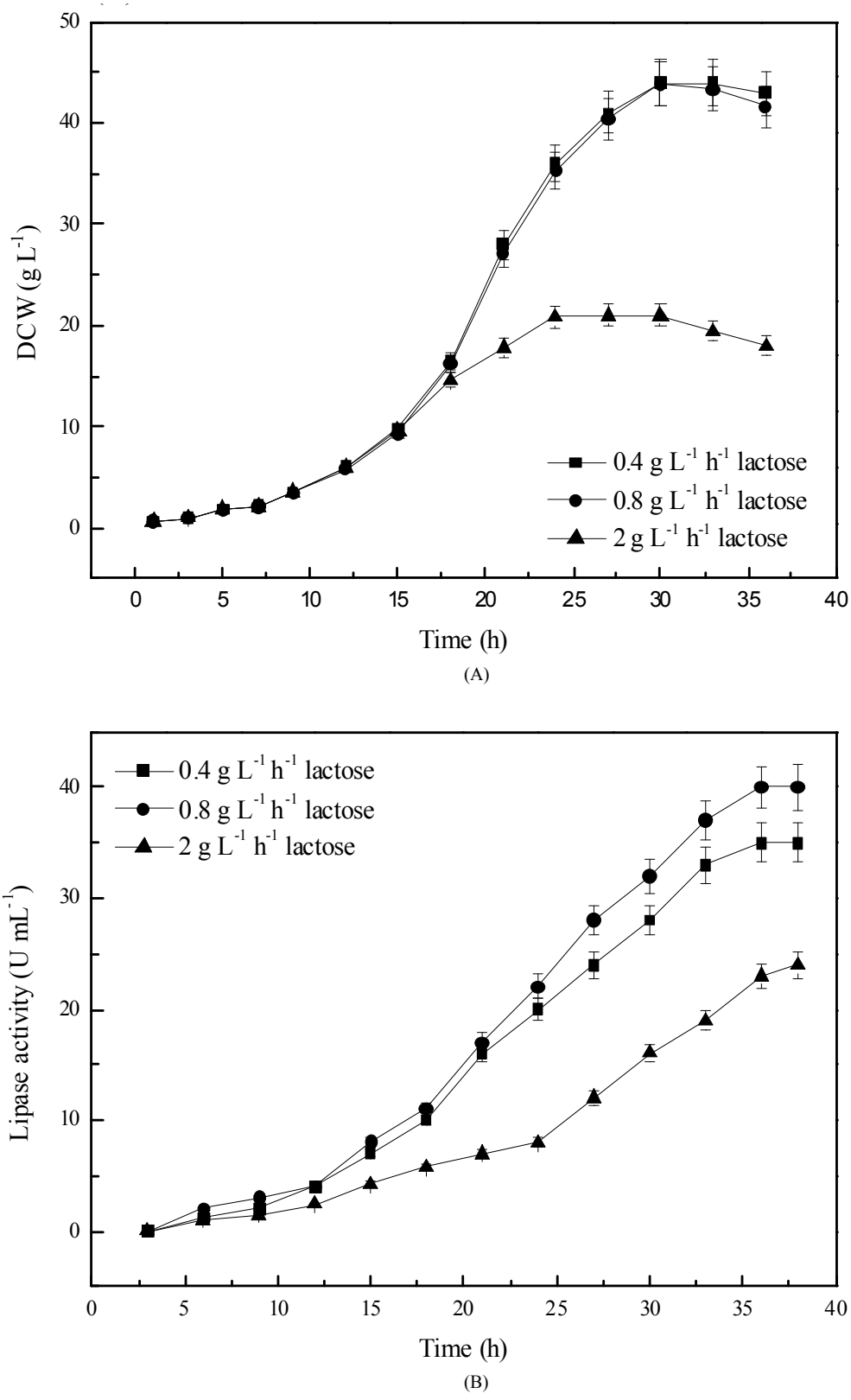

Fig. 2: Comparison of the time profiles for cell growth (A) and lipase activity (B) in the culture medium obtained from cultivations when induced with lactose at different flow rates. ■ 0.4 g L^{-1} h^{-1}, ● 0.8 g L^{-1} h^{-1}, ▲ 2 g L^{-1} h^{-1}

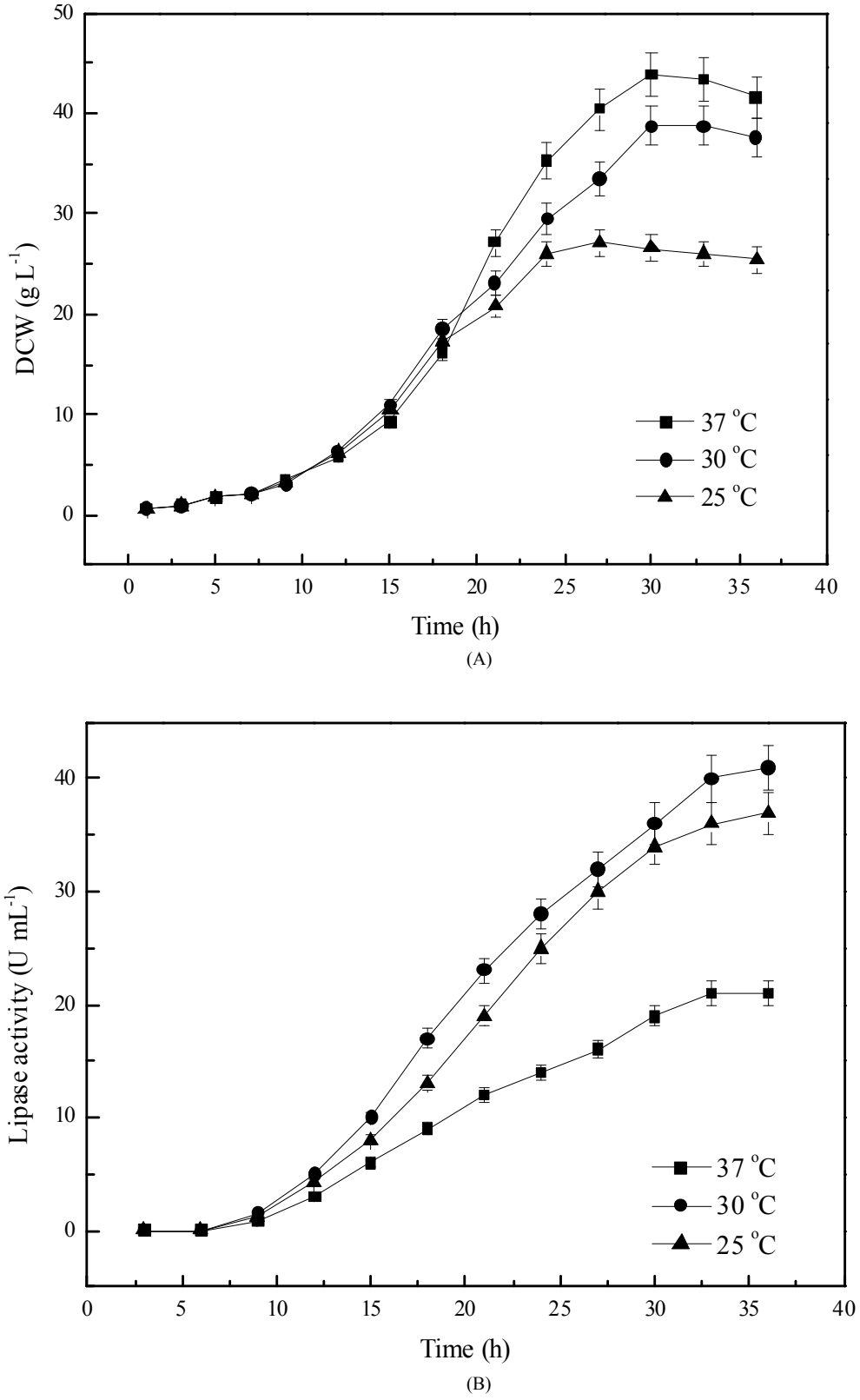

Fig. 3: Comparison of the time profiles for cell growth (A) and lipase activity (B) in the culture medium obtained from cultivations at different induction temperatures. ■37°C, ● 30°C, ▲ 25°C

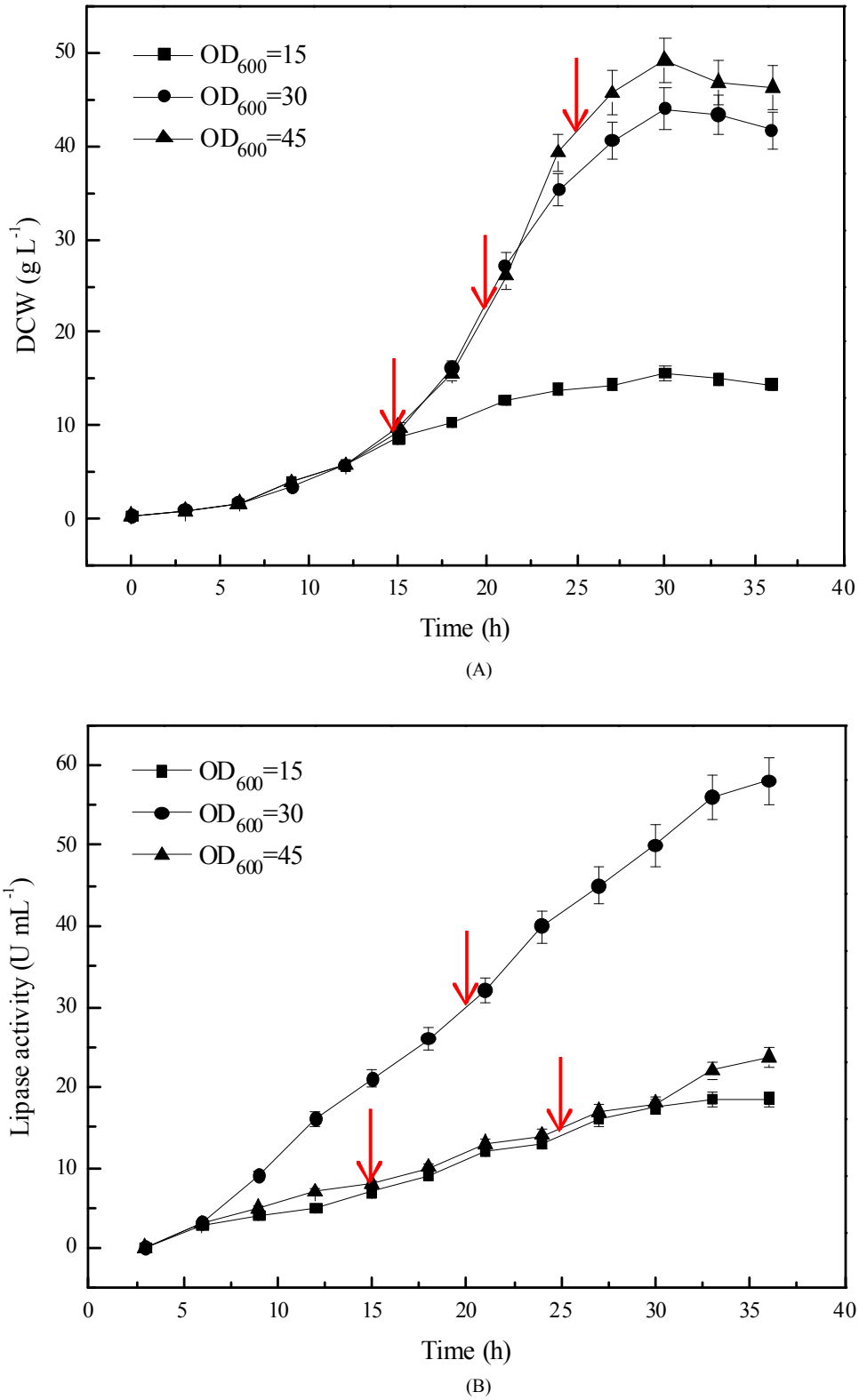

Fig. 4: Comparison of the time profiles for cell growth (A) and lipase activity (B) in the culture medium obtained from cultivations when induced at low cell density (■ OD_{600} = 15), intermediate cell density (● OD_{600} = 30) and high cell density (▲ OD_{600} = 45). Red arrows mark the induction start cell concentration

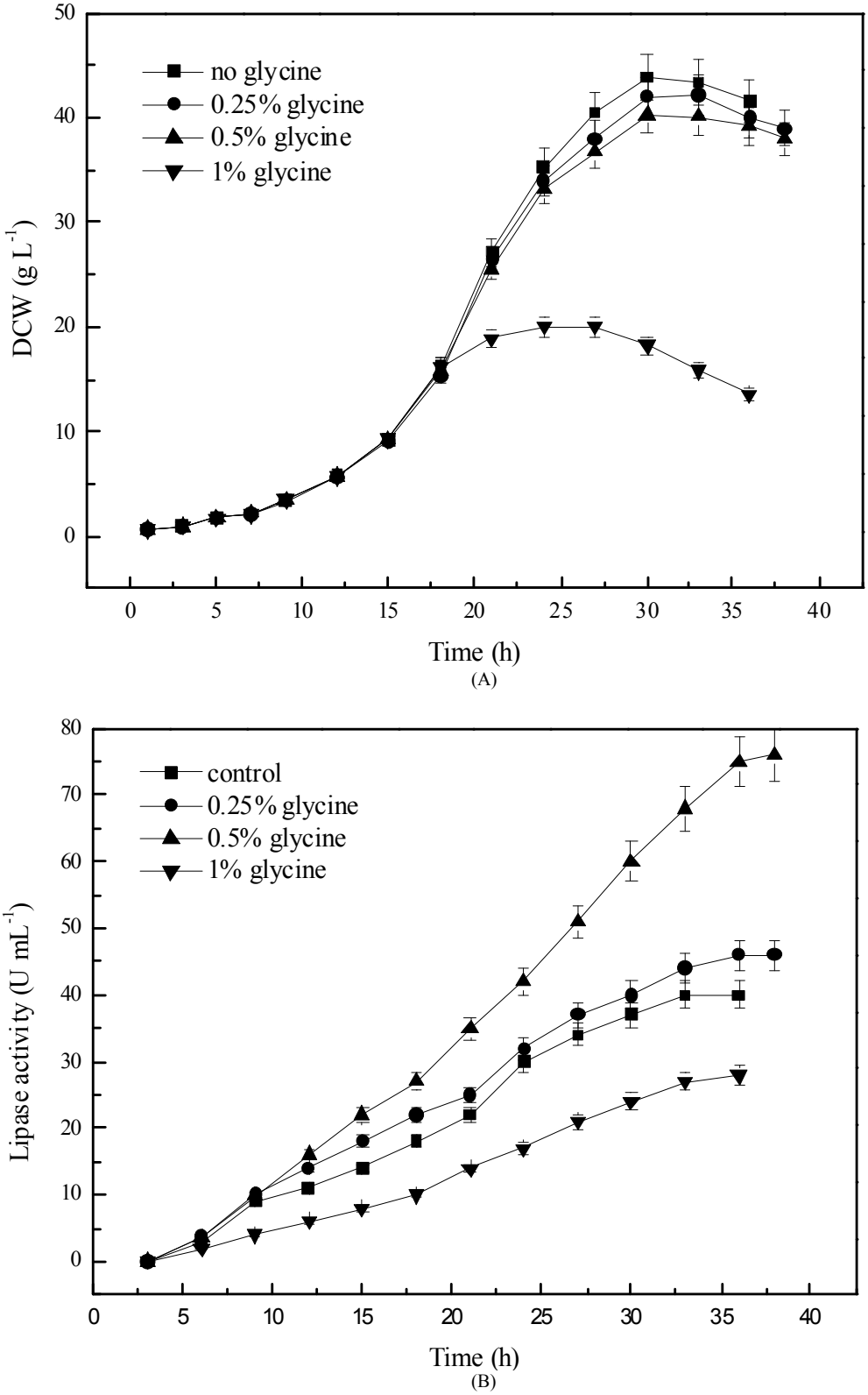

Fig. 5: Comparison of the time profiles for cell growth (A) and lipase activity (B) in the culture medium obtained from cultivations with supplementation of glycine at different concentrations. ■ control, ● 0.25% (w/v) glycine, ▲ 0.5% (w/v) glycine, ▼1% (w/v) glycine

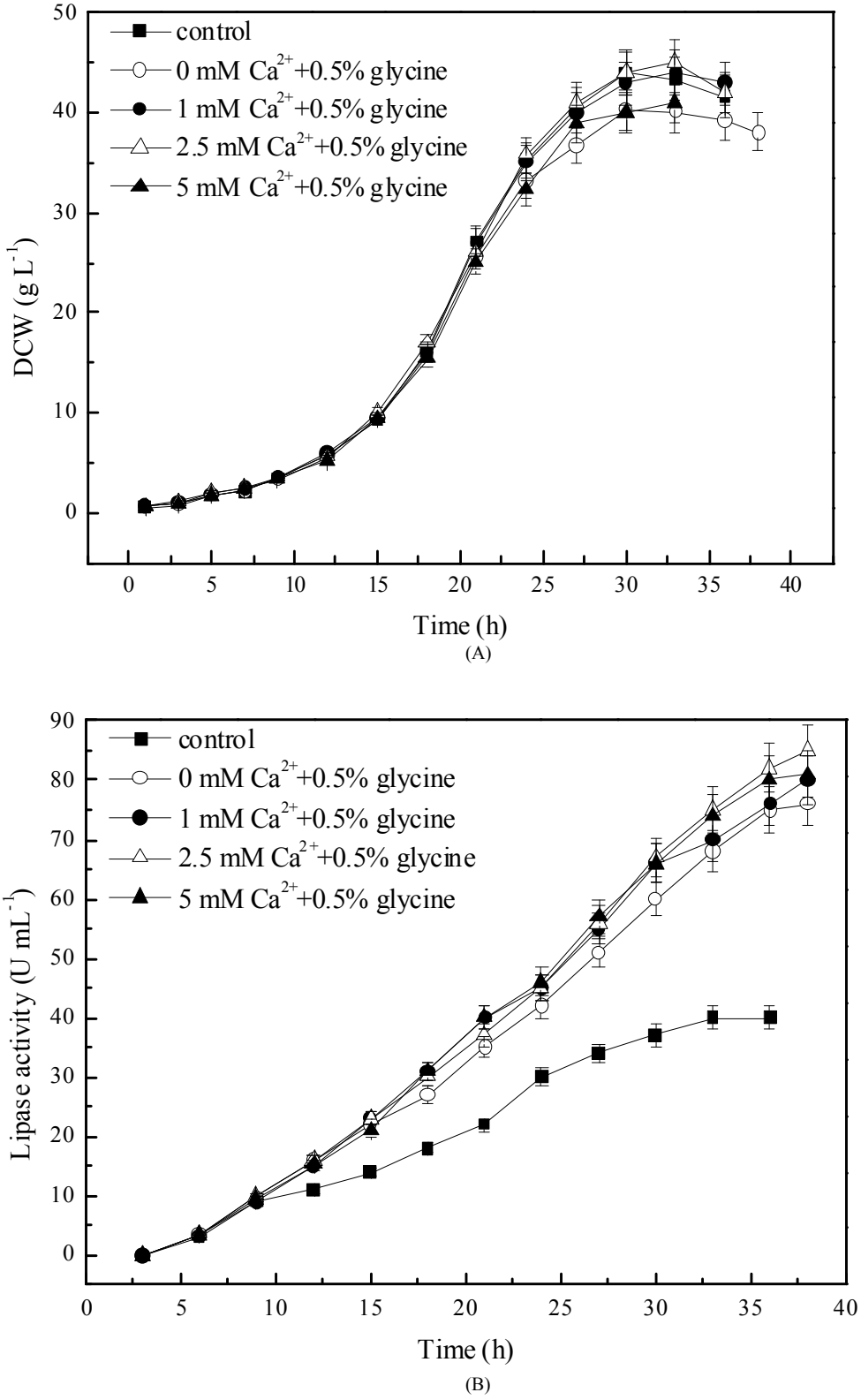

Fig. 6: Comparison of the time profiles for cell growth (A) and lipase activity (B) in the culture medium obtained from cultivations with supplementation of Ca^{2+} at different concentrations and 0.5% (w/v) glycine. ■ control, ○ 0 mM Ca^{2+} +0.5% (w/v) glycine, ● 1 mM Ca^{2+} +0.5% (w/v) glycine, ∆ 2.5 mM Ca^{2+} +0.5% (w/v) glycine, ▲ 5 mM Ca^{2+} +0.5% (w/v) glycine

Effect of Glycine on Cell Growth and Lipase Production

Previous studies indicated that the recombinant protein initially congested in periplasm to a maximum density and then gradually released to the extracellular medium (Chen *et al.*, 2011; Ding *et al.*, 2010). Therefore, the improvement of the membrane permeability would be an efficient approach to accelerate release of the accumulated protein. Previous studies have reported that glycine could improve the permeability of the outer membrane by *E. coli* and then increase the extracellular release of recombinant proteins (Ding *et al.*, 2010; Li *et al.*, 2010; Yang *et al.*, 2017; Zou *et al.*, 2014) In the present study, glycine at the concentration range of 0-1% (w/v) was supplied into the medium after culturing for 8 h (OD_{600} reached about 5.0) and consequently the cell growth and extracellular lipase production were explored (Fig. 5). The experimental results demonstrated that in spite of slight inhibition of cell growth (Fig. 5A), the addition of glycine at a concentration of less than 1% (w/v) greatly promoted the enzyme activity (Fig. 5B). With the supplementation of 0.5% (w/v) glycine into the culture medium, the lipase activity reached to 76 U mL^{-1}, which was 1.9 fold higher than in the control without glycine. Consistent with previous study, appropriate supplementation of the culture with glycine could enhance the secretion of recombinant enzymes from *E. coli* (Ding *et al.*, 2010; Li *et al.*, 2010). The potential mechanism was that glycine induced the modification of peptidoglycan structure in the cell wall, which led to a significant increase of cell membrane permeability (Hammes *et al.*, 1973; Li *et al.*, 2009). However, while more than 1% (w/v) glycine was supplied, bacterial growth was seriously inhibited with corresponding reduced lipase production remarkably. Therefore, 0.5% (w/v) glycine was selected as best concentration to study the glycine addition on the effect of cell growth and extracellular lipase production.

Effect of Ca^{2+} on Cell Growth and Lipase Production

Previous research showed that Ca^{2+} could promote the growth and survival of *E. coli* cells when was grown in the culture medium rich in glycine, which resulted in a further improve in the glycine-induced extracellular secretion of the recombinant protein (Ding *et al.*, 2010; Li *et al.*, 2009). In the present study, the effect of Ca^{2+} along with 0.5% (w/v) glycine on cell growth and extracellular lipase activity was determined in detail. The present results demonstrated that the cell growth was slightly increased when Ca^{2+} (1-5 mM) was supplied at the beginning of the culture (Fig. 6). The possible explanation for this promotion has been deduced to that Ca^{2+} could stabilize the lipopolysaccharide in the cell membrane aiding to preserve the orderly nature of the cell membrane (Smith, 1995; Thomas *et al.*, 2014). Moreover, Ca^{2+} can decrease the hydrolization of peptidoglycan by peptidoglycan hydrolase and so it has usually been used to control the process of cell autolysis (Smith, 1995; Thomas *et al.*, 2014). Beside the promotion of cell growth, the supplementation of Ca^{2+} in the culture medium also slightly enhanced the extracellular lipase activity. When 2.5 mM Ca^{2+} was added in the cultivation, the extracellular lipase activity reached 85 U mL^{-1}, which was 2.1 fold of that in the control. Simultaneously, it was the maximum activity of lipase we have achieved in this study.

Conclusion

In summary, we developed a fed-batch fermentation strategy for the extracellular production of lipase by *E. coli*. It is concluded that, the induction control and the increase of the cell membrane permeability could improve the extracellular lipase production. Under the optimum condition, the maximum lipase activity in the culture medium could reach 85 U mL^{-1}, which was 2.1 fold higher than that of the control. This study might provide fermentation strategy for the extracellular production of other heterogonous proteins expressed in *E. coli*.

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Author's Contributions

Yao Zhang: Has contributed in a whole experiment, data analysis, paper writing and publication.

Yuanda Song: Has reviewed and revised the manuscript.

Lu Wang and Qing Liu: Have assisted in the experiment.

Ethics

All authors read and approved the final version of this manuscript. There are not any ethical issues to declare that could arise after the publication of this manuscript.

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