

Full Length Research Paper

# Effects of fermentation conditions on the production of 4- $\alpha$ -glucanotransferase from recombinant *Escherichia coli*

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**4- $\alpha$ -Glucanotransferase (4- $\alpha$ -GTase, EC 2.4.1.25) simultaneously catalyzes amylose molecular intra- and inter- transglycosylation to produce cyclodextrins (CDs) with DP of 9 to several hundreds. In this study, the 4- $\alpha$ -glucanotransferase (4- $\alpha$ -GTase) from recombinant *Escherichia coli* DH-5 $\alpha$  was proved to produce large ring cyclodextrins (LR-CDs) with polymer degree of 20 to 47. To maximize the 4- $\alpha$ -GTase productivity effect, conditions for the production of 4- $\alpha$ -GTase were initially carried out in shake flasks to investigate the effects of carbon sources, nitrogen sources, trace elements, inoculum size, liquid volume, pH and temperature. The results showed that the production of 4- $\alpha$ -GTase and increase of biomass (*E. coli* growth) was out of sync. Glycerol of 20 g/l in medium, as the best carbon source, provided as high as 102.55 U/ml of 4- $\alpha$ -GTase, which was 0.59-fold compared with the minimal medium. Organic nitrogen source enhanced more enzyme activity than the inorganic source; beef extract was identified as the most effective source for enzyme activity enhancement. In order to get the highest enzyme activity, the optimum incubation conditions were initial pH of fermentation broth at 7.0, culture temperature of 37°C, inoculum size of 2.0% and liquid volume of 50 ml in 250 ml Erlenmeyer. Under these conditions, 4- $\alpha$ -GTase activity in 5 L fermenter reached as high as 198.7 U/ml.**

**Key words:** High cell density, 4- $\alpha$ -glucanotransferase, recombinant *Escherichia coli*, large-ring cyclodextrin, optimum incubation conditions.

## INTRODUCTION

4- $\alpha$ -Glucanotransferase (4- $\alpha$ -GTase, EC 2.4.1.25) belongs to the  $\alpha$ -amylase superfamily that includes about 20 different enzymes (Janecek et al., 1997; Kuriki and Imanaka, 1999) and is an enzyme capable of converting starch. 4- $\alpha$ -GTases catalyze not only intermolecular transglycosylation of maltooligosaccharides (disproportionation) to produce linear  $\alpha$ -1,4-glucans, but also intramolecular transglycosylation to produce cyclic  $\alpha$ -1,4-glucans (cyclodextrins, CDs) with DP of 6 to several hundred from amylose molecules (Cho et al., 2009; Euverink and Binnerma, 2005; Yanase et al., 2002). CDs have the ability of forming complexes with a wide

range of hydrophobic molecules, thus, CDs have currently been used in the food, pharmaceutical, cosmetic, agro-chemical, medical and plastic industrial as stabilizing agents, emulsifiers and antioxidants (Formming and Szejtli, 1994; Shieh and Hdeges, 1996; Szejtli, 1988). Compared with normal CDs, large-ring cyclodextrins (LR-CDs) composed of more than 9 glucopyranose units have more complex cavity geometries and convenient functions, which expand the range of macrocyclic host molecules available for molecular inclusion processes (Larsen, 2002). In addition, due to high conversions of 4- $\alpha$ -GTase, many researches have been conducted to use this enzyme as a modifier for starches (Cho et al., 2009; Euverink and Binnerma, 2005; Seo et al., 2007).

4- $\alpha$ -GTases widely exist in microorganisms (for example, *Escherichia coli*) and plant tissues such as

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potato tubers, germinating barely seeds, sweet potato and spinach (Lin and Preiss, 1988; Monod and Torriana, 1948; Peat et al., 1956). But the productivity is too low to meet industry amendment. To achieve high productivity, majority of methods are used to transfer 4- $\alpha$ -GTases gene into a host to express it efficiently, which makes the process more economical. *E. coli* is an important host for expression of a wide variety of commodity and high value-added recombinant proteins (Sharma et al., 2007). The culture of *E. coli* cells to high cell densities is an essential prerequisite for efficient recombinant protein formation. However, by-product, inclusion body formation, proteolytic degradation and disulfide bond generation have long impeded the production of complex heterologous proteins in a properly folded and biologically active form (Baneyx and Mujacic, 2004). Various strategies have been developed to increase the productivity of recombinant proteins. For instance, Tsai et al. (1988) studied the recombinant protein of human IGF-1 in rich and minimal medium and found that there was no recombinant protein produced when grown on minimal medium, but the yield was as high as 30 mg/g when yeast and bactotryptone were used in the rich medium. Baneyx and Georgiou, (1990) changed medium pH to 5.5 to 6.0, which resulted in obtaining 4-fold higher yield of recombinant protein A- $\beta$ -lactamase compared to the medium pH at 7.0. Harcum and Bentley, (1999) and Kanemori (1997) reported that recombinant protein production at elevated temperatures (42°C) has higher productivities than normal (37°C) culture temperatures. Other researchers interestingly found that low temperature (20°C) could induce recombinant protein production (Chesshyre and Hipkiss, 1989). Those reports showed that controlling fermentation conditions including temperature, pH, media composition, oxygen availability and cofactor were important for optimization recombinant protein. However, for 4- $\alpha$ -Glucanotransferase (with capacity of producing LR-CD) from recombinant *E. coli* production, no optimum medium or optimum fermentation conditions were reported, besides the production of 4- $\alpha$ -GTase in minimal medium. Therefore, the optimum medium and optimum fermentation conditions selection is of great significance and also very necessary for the production of 4- $\alpha$ -GTase effectively and timely.

In this study, we observed the effect of carbon sources, nitrogen sources, trace elements, inoculum size, liquid volume, pH and temperature on 4- $\alpha$ -GTase production and on the biomass of recombinant *E. coli* DH-5  $\alpha$  in shake flask fermentation and provided desired conditions for fermentation in 5 L ferment pot.

## MATERIALS AND METHODS

### Recombinant strain

Plasmid containing 4- $\alpha$ -GTase gene (GenBank: AY459352.1) was kindly donated by Professor Kwan-Hwa Park, Seoul National University, Korea. The recombinant plasmid was transformed into

*E. coli* DH-5 $\alpha$  and the recombinant *E. coli* DH-5 $\alpha$  has been deposited in CGMCC (China General Microbiological Culture Collection Center) and the deposition No. is 3093.

The *E. coli* DH-5 $\alpha$ -TA transformant carrying recombinant plasmid was cultivated in Luria-Bertani medium (LB medium: 10 g/l Peptone, 5 g/l yeast extract, and 5 g/l NaCl) containing ampicillin (100  $\mu$ g/ml) at 37°C. For solid agar medium preparation, LB medium with 2% (w/v) agar powder was used for the recombinant clone.

### Biomass detection

*E. coli* DH-5 $\alpha$ -TA was transferred into an Erlenmeyer flask (250 ml) containing 25 ml of fermentation medium and was cultured at 37°C on a rotary shaker incubator (FLY-2112B, Shanghai FengLing Experimental Facilities Co., Ltd, Shanghai) at 200 rpm until the visible light absorbance of the fermentation broth at 600 nm was 1.0. Inoculum was harvested by centrifugation at 3000 *g* for 10 min to collect wet cells and then washed three times by sterilized water. Wet cells were dried in air dry oven at 105°C for 6 h. Biomass was measured by the dry weight of cells.

### Preparation of crude enzyme

The preparation of crude enzyme was based on a slight modification method from Park et al. (2007). The cultivation wet cells were resuspended in lysis buffer [50 mM Tris-HCl buffer, (pH 7.5) containing 300 mM NaCl and 10 mM imidazole] and was sonicated over an ice bath (400 w, 30 min) using a sonicator (JYD-900, Zhisun Instrument Co., Ltd, Shanghai). The blends were then heated at 60°C for 10 min and centrifuged at 10 000 rpm for 15 min to remove thermolabile protein. The supernatant was used as crude enzyme solution for enzyme assay.

### Determination of protein concentration

Quantity of protein was measured using the method described by Bradford (Bradford, 1976). The Bradford store solution was prepared by mixing 100 mg of Coomassie brilliant blue G-250, 50 ml of ethanol, 100 ml of 85% phosphoric acid and 850 ml water. For protein concentration determination, 100  $\mu$ l of diluted enzyme solution were added into 4 900  $\mu$ l of Bradford solution and the mixed solution was measured at 595 nm using a spectrophotometer. A blank was prepared by adding buffer instead of enzyme solution. The protein concentration was calculated from bovine serum albumin standard curve.

### Assay for 4- $\alpha$ -GTase activity

The starch degrading activity of 4- $\alpha$ -GTase was determined by measuring the decrease in absorbance of a starch-iodine complex during the conversion of amylose by the enzyme. The assay mixture containing 250  $\mu$ l of 0.2% (w/v) amylose (potato type III, Sigma-Aldrich Co., Louis, MO, USA), 50  $\mu$ l of 1% (w/v) maltose, 600  $\mu$ l of 50 mM Tris-HCl buffer (pH 7.5) and 100  $\mu$ l of enzyme solution was incubated at 70°C for 10 min. The reaction was stopped by boiling the mixture for 10 min.

Samples (0.1 ml) withdrawn at 0 min and 10 min were mixed with 1 ml 0.02% iodine/potassium iodine solution (Lugol's solution, diluted 1:50 with 50 mM Tris-HCl, pH 7.5) and the absorbance at 620 nm was immediately measured using a UV-1800 spectrophotometer (Mapada Instrument Co., Ltd, Shanghai). One unit of 4- $\alpha$ -GTase activity was defined as the amount of enzyme that reduced the absorbance at 620 nm by 1.0 unit in 10 min under

the earlier mentioned conditions.

Relative activity was adopted for further experimental optimum, which was divided by protein concentration.

### Analysis of large ring cyclodextrin produced by 4- $\alpha$ -GTase

#### Large ring cyclodextrin production

Amylose solution was prepared by dissolving 1 g of potato amylose (type III, Sigma, USA) with average molecular weight of  $7 \times 10^6$  in 25 ml of DMSO (90%) and 25 ml of 0.05 M Tris-HCl buffer (pH 7.5). 40 units of 4- $\alpha$ -GTase were added to the amylose solution and reacted at 75 °C for 8 h. The reaction solution was treated at 100 °C for 15 min and centrifuged at 4 000 rpm for 10 min to remove the denatured enzyme protein.

Then, 30 units of  $\beta$ -amylase were added and the solution was treated at 30 °C for 4 h to remove linear glucan. Further, heat treatment (100 °C for 5 min) and centrifugation (4000 rpm for 10 min) were used to move the denatured enzyme protein. Thereafter, 5 fold of acetone were added to collect the precipitate. The precipitate was vacuum dried to obtain pure large ring cyclodextrin.

#### HPAEC and TOF-MS analysis

High performance anion exchange chromatography (HPAEC) with a pulsed amperometric detector (3000, DIONEX) and a CarboPac™ PA-200 column (0.3 x 25 cm, DIONEX, USA) was used. 5  $\mu$ l of the prepared LR-CD sample and standard (DP range: 22-50, Ezaki Glico Co. Ltd, Japan) was injected separately and eluted with a gradient of 1 M sodium acetate and sodium hydroxide (0 for 5 min, 70 mM of sodium acetate and 96.5 mM of sodium hydroxide; 6 to 45 min, 320 mM of sodium acetate and 72.5 mM of sodium hydroxide) with a flow rate of 0.5 ml/min. Molecular mass spectrum of large ring cyclodextrin was collected by time of flight (TOF) mass spectrometry (WATERS SYNAPT Q-TOF MS, USA) with a positive electrospray ionization mass spectrometry. The voltage of ionspray was 3.5 kV.

## RESULTS

### Identification of large ring cyclodextrin (LR-CD)

One direct way to identify the 4- $\alpha$ -GTase expression from recombinant *E. coli* was to check the ability of producing LR-CD. The expressed 4- $\alpha$ -GTase was reacted with amylose, and the reaction productions were detected by HPAEC (Figure 1a). We observed that the DP range of LR-CD sample was 20 to 47 by comparing with LR-CD standard. To identify these peaks, mass with ESI was used to detect the exact molecular mass of LR-CD sample. The results gave LR-CD sample double electricity charges (Figure 1b) with intervals of 81 Da. Mass information (2M+Na)<sup>+</sup> showed those of cyclic glucans with polymer degree of 20 to 35. The maximum intensity of peak in Figure 1b was the molecular mass of LR-CD with polymerization degree of 26 and minimal peak was the molecular mass with DP more than 35. From Figure 1b, linear glucans with polymer degree of 7 (1175), 8 (1337) and 9 (1499) (M+ Na)<sup>+</sup> were also found with single electricity charge, which probably was due to the impurity

of the LR-CD products.

### Effect of carbon sources on biomass and 4- $\alpha$ -GTase production

As shown in Figure 2a, the medium with glycerol, maltose, sucrose, cyclodextrin, soluble starch, corn starch and potato starch gave a generally increased activity compared to the minimal medium, in which, glycerol was the best carbon source for 4- $\alpha$ -GTase production. The highest specific activity obtained in the medium with glycerol was approximately 102.55 U/ml, which improved 0.59-fold higher than that achieved in the minimal medium after 10 h fermentation (64.5 U/ml). Decrease trends of relative enzyme activity were found with increasing carbon source concentration. Figure 2b shows that all of the biomass with the addition of carbon source was much larger than the control. Further, the increase of enzyme activity was out of sync with biomass increasing.

The largest increase in 4- $\alpha$ -GTase production was observed in the presence of 20 g/l glycerol, while 25 g/l of glycerol gave the largest biomass, suggesting that, too much glycerol showed inhibitory effect for the production of recombinant protein.

### Effect of nitrogen sources on biomass and 4- $\alpha$ -GTase production

Figure 3a shows that the organic nitrogen medium provided significantly higher ( $P \leq 0.05$ ) yield for 4- $\alpha$ -GTase than that in the inorganic nitrogen medium. The highest enzyme activity (140.7 U/ml) obtained by adding beef extract (20 g/l) in the medium was improved 1.18-fold compared to the control.

The initial increase and then decrease of 4- $\alpha$ -GTase activity were found with increasing concentrations of wort, beef extract, yeast extract and corn steep liquor. Inorganic nitrogen showed much lower facilitation for enzyme production, but also enhanced enzyme activity with increasing content. The biomass in beef extract, wort, yeast extract and corn steep extract showed higher values than that in the other nitrogen sources, but extreme high nitrogen content resulted in the decrease in biomass (Figure 3b). The maximum biomass was obtained in the beef extract of 25 g/l.

### Effect of trace elements on 4- $\alpha$ -GTase production

In this study, FeSO<sub>4</sub>·7H<sub>2</sub>O, MnSO<sub>4</sub>, ZnSO<sub>4</sub>·7H<sub>2</sub>O, CuCl<sub>2</sub>, MgSO<sub>4</sub>·7H<sub>2</sub>O and CaCl<sub>2</sub> with different concentration were added to improve the biomass and enzyme production and the results are presented in Figure 4.

The initial increase and then the decrease trends for enzyme activity and biomass were found with increasing

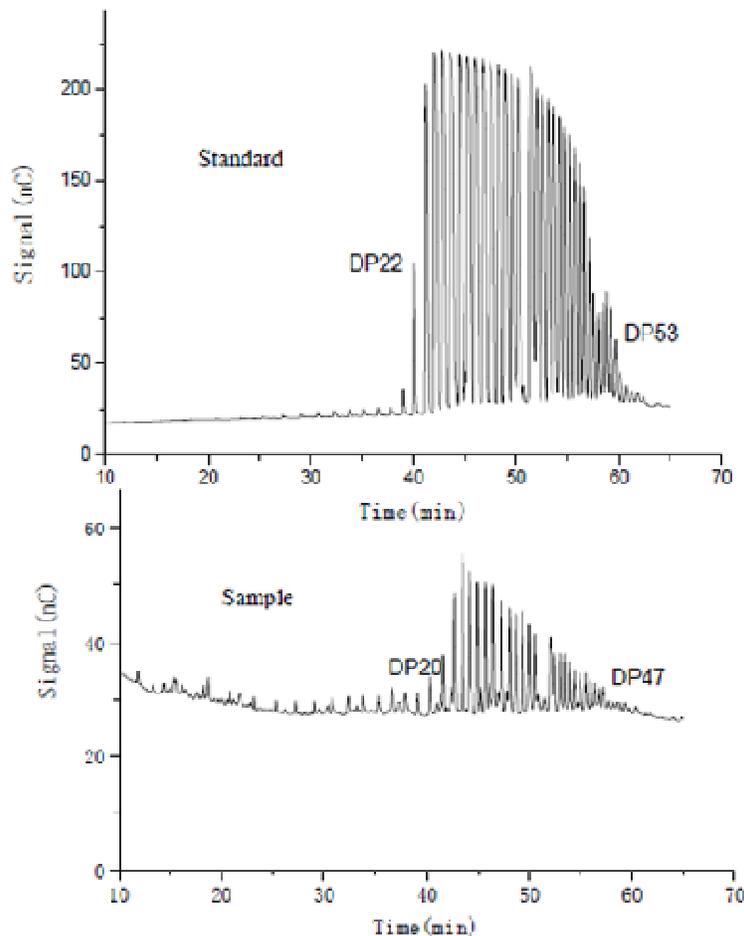


Fig.1 A

**Figure 1.** (A) HPAEC diagram of LR-CD produced by 4- $\alpha$ -GTase; (B) molecular mass of LR-CD produced by 4- $\alpha$ -GTase. The subscript number 'n' in CDn means the number of glucose in LR-CD product; 23 is mass of Na<sup>+</sup>.

concentration of Ca<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>, Fe<sup>2+</sup> and Cu<sup>2+</sup>. While Zn<sup>2+</sup> was found to negatively affect enzyme activity and biomass with the addition of more content in the medium, FeSO<sub>4</sub>·7H<sub>2</sub>O was found to be the most effective for 4- $\alpha$ -GTase production and biomass; caused increase than the other trace elements (Figure 4). The maximum enzyme activity (171.6 U/ml) was obtained in the medium with FeSO<sub>4</sub>·7H<sub>2</sub>O (0.4 g/l).

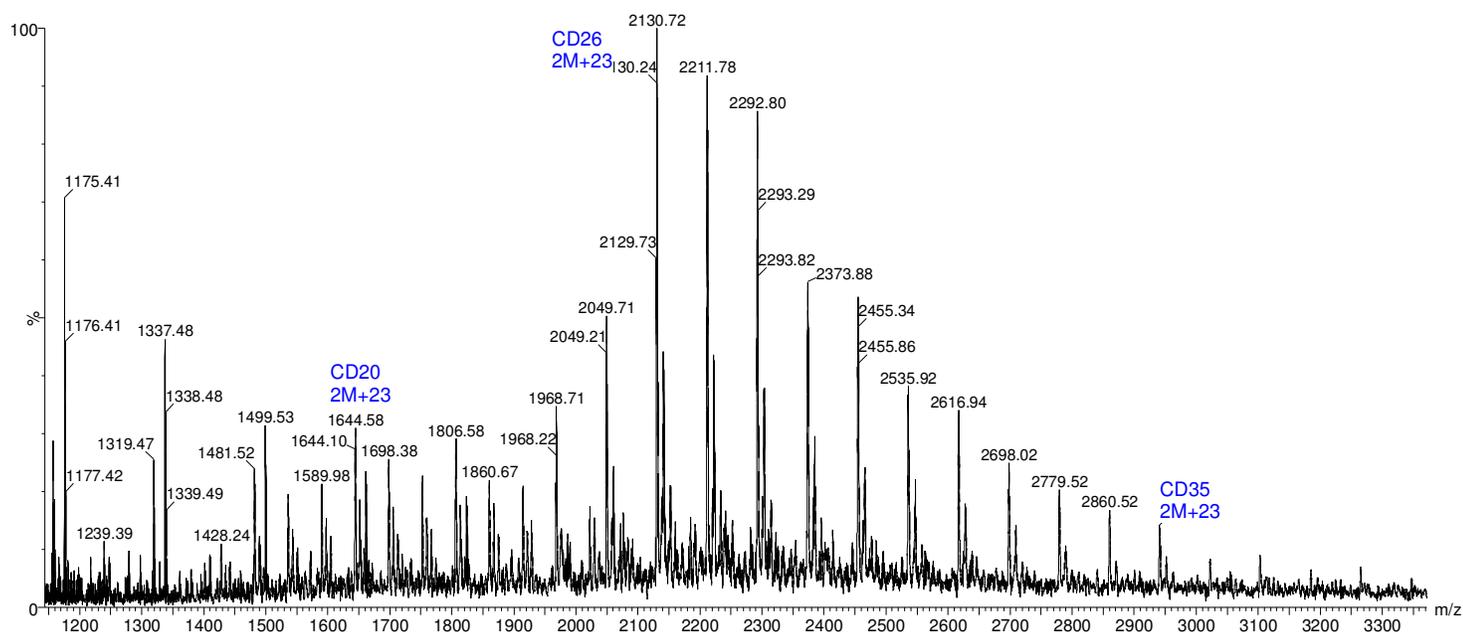
#### Effect of environmental conditions on 4- $\alpha$ -GTase production

In order to provide optimum conditions for 4- $\alpha$ -GTase production, liquid medium volume and inoculum size were also investigated for 4- $\alpha$ -GTase and biomass

production (Figure 5a). From the growth curve of *E. coli*, lag phases were shortened with increasing inoculum size, but the biomass increased during the stationary phase. As for enzyme activity, the highest yield of 4- $\alpha$ -GTase activity was found for 2.0% inoculum size.

Less liquid volume gave a much shorter lag phase and fewer biomasses; when the liquid volume in 250 ml Erlenmeyer was less than 50 ml (Figure 5b). However, the biomass in the 60 ml medium was less than 50 ml when the incubation time was more than 10 h. The increasing rate of enzyme activity in the 20, 30 and 40 ml medium was much larger than that in the 50 and 60 ml medium at the lag phase, but the highest enzyme activity was shown in the 250 ml Erlenmeyer flask containing 50 ml medium (Figure 5b).

As shown in Figure 6a, 4- $\alpha$ -GTase production and



B

Figure 1. Contd.

strain biomass was significantly affected by temperature. Low temperature (30°C) prolonged the lag phase and decreased the enzyme production and strain biomass, while high temperature (42°C) was also not feasible fermentation condition. The optimum incubation temperature for production of 4- $\alpha$ -GTase was 37°C and the highest biomass was also achieved at 37°C. From Figure 6b, with initial pH ranging from 6.0 to 8.0, the maximum enzyme activity was obtained at pH 7.0, while the maximum biomass was obtained at pH 8.0 (Figure 6b).

#### Time course of 4- $\alpha$ -GTase production profile in fermenter

Time course of 4- $\alpha$ -GTase production and the growth of recombinant *E. coli* DH-5 $\alpha$ -TA were further studied in 5 L fermenter under the selected conditions (Figure 7). 4- $\alpha$ -GTase activity obtained in the fermenter was 198.7 U/ml after 10 h fermentation and then a sharp decrease in 4- $\alpha$ -GTase activity was observed. Meanwhile, biomass of *E. coli* DH-5 $\alpha$ -TA reached the maximum after 8 h fermentation.

## DISCUSSION

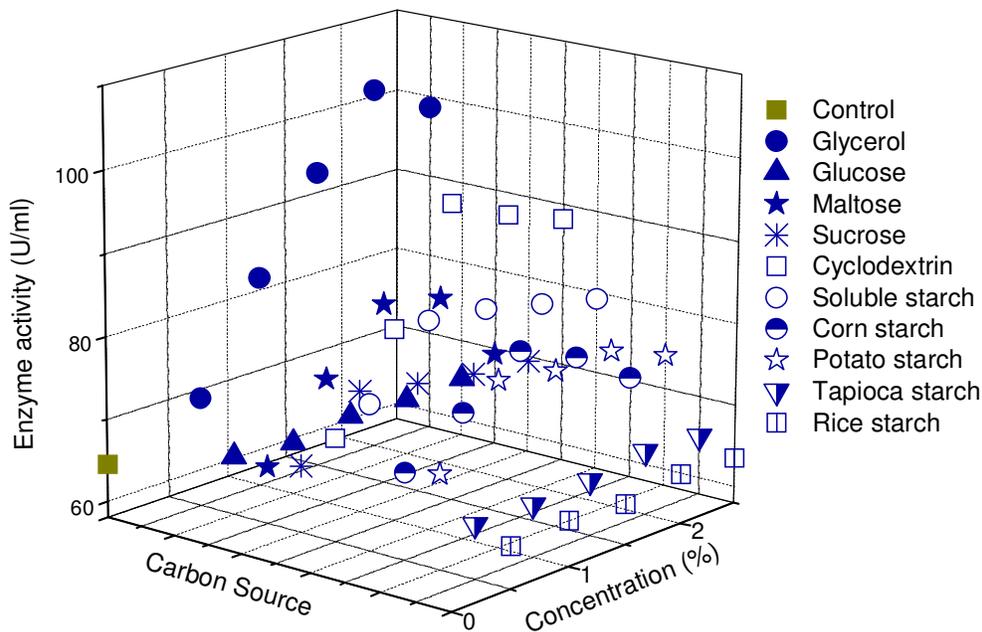
### The produced LR-CD by 4- $\alpha$ -GTase

We investigated the reaction of 4- $\alpha$ -GTase (GenBank:

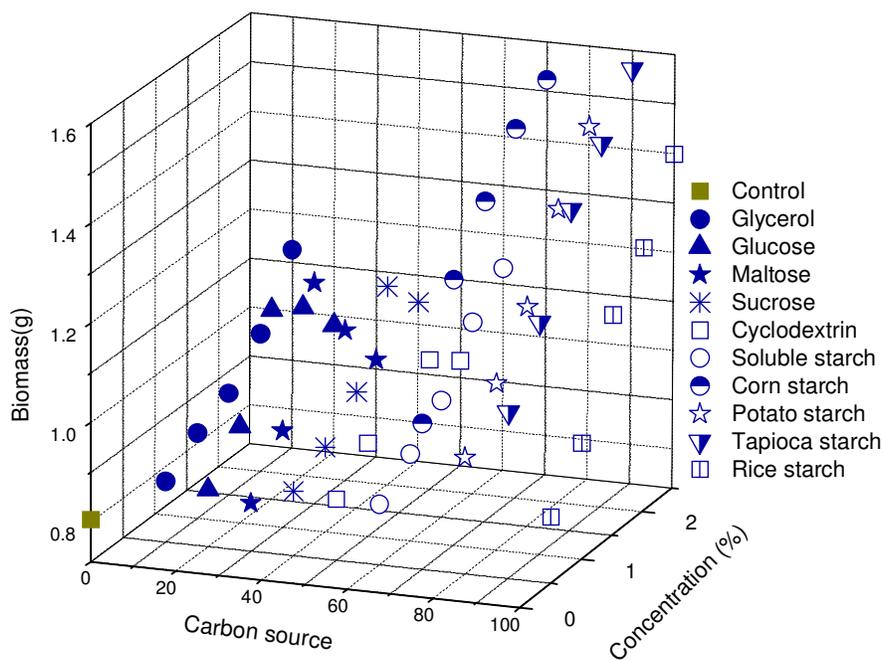
AY459352.1, *Thermus aquaticus* 4-alpha-glucano-transferase gene) in this study. The obtained results clearly indicated the possibility of altering the amylose into LR-CD with DP of 20 to 47. However, the DP range was a little different from the previous reports, which showed that 4- $\alpha$ -GTase (also named amyloamylase) from *Thermus aquaticus* YT-1 and *Thermus aquaticus* ATCC33923 has the ability of producing LR-CD with DP of 19 to 35 and 22 to 50, respectively (Park et al., 2007; Terada et al., 1999). A recent report also found that *Thermus aquaticus* YT-1 (ATCC 25104) could modify rice starch to produce CD with DP of 5 to 19 (Cho et al., 2009). Those DP differences were inferred depending on the reaction mechanism of the 4- $\alpha$ -GTase, including three transglycosylation reactions (cyclization reaction, disproportionation reaction and coupling reaction) and hydrolysis reaction (Terada et al., 1997; Terada et al., 2001; Zheng et al., 2002; Qi et al., 2004). Factors influencing the reaction mechanism, such as substrate resource, reaction conditions as well as selected enzyme would result in different size of LR-CD, not just enzyme as mentioned in previous study (Taira et al., 2006).

### The nutrition on the 4- $\alpha$ -GTase production

Glycerol, glucose, maltose, sucrose, cyclodextrin and soluble starch exhibited almost the same trend patterns for 4- $\alpha$ -GTase production and *E. coli* growth, in which, glycerol was the most effective carbon source. As carbon source, it has been widely accepted that glucose



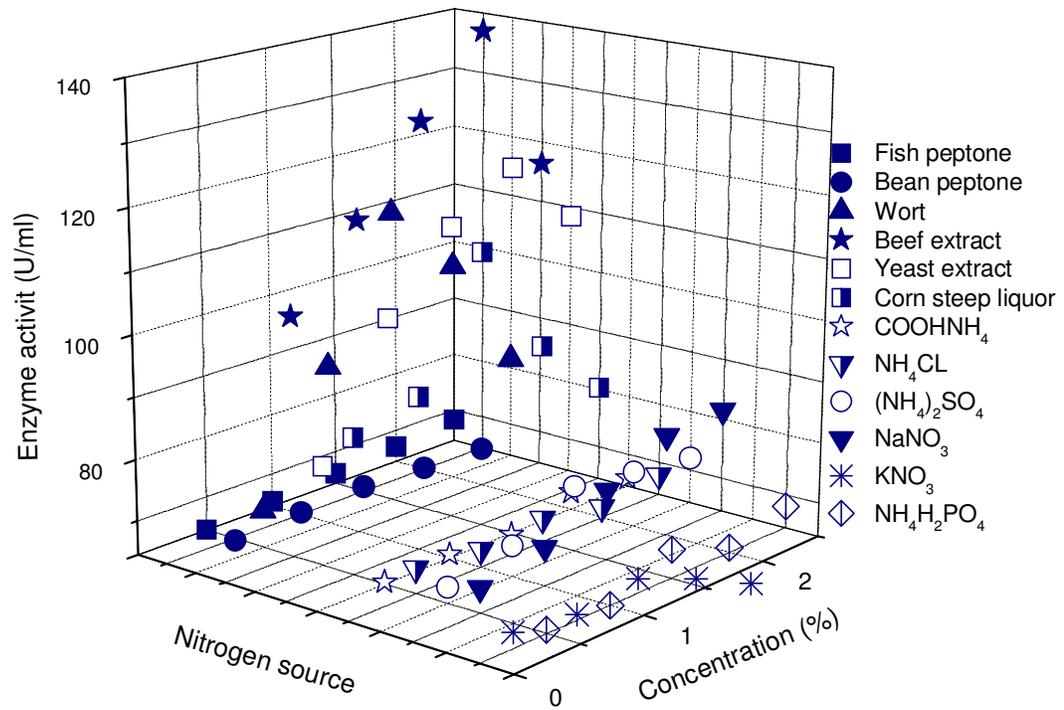
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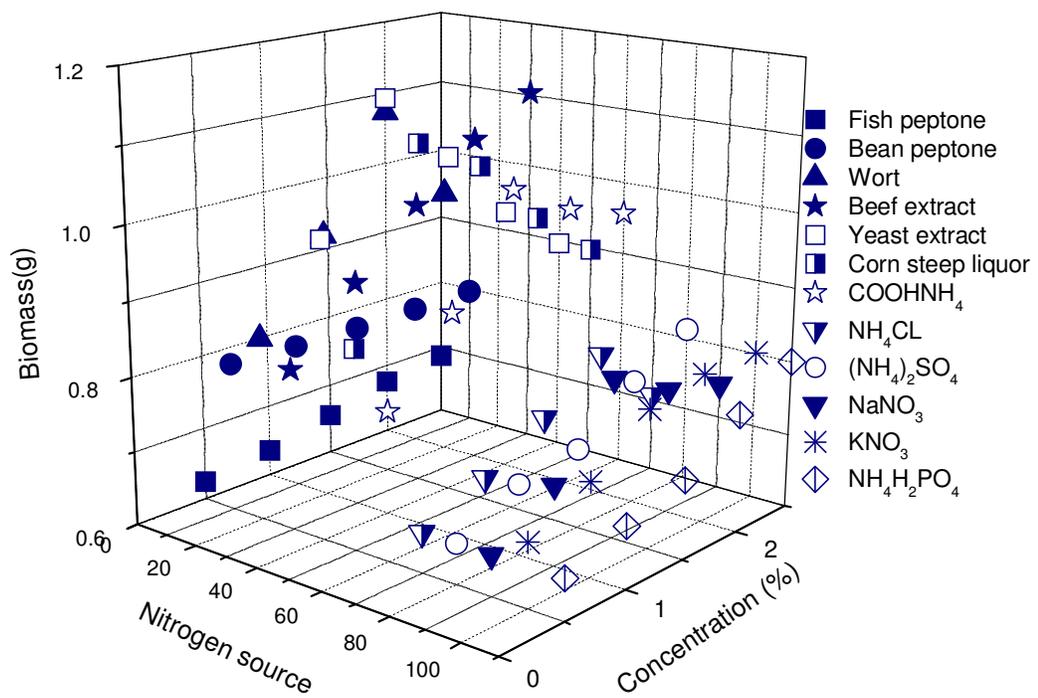
**Figure 2.** (a) The effect of carbon source on 4- $\alpha$ -GTase production. The minimal medium contained 10 g/l peptone, 5 g/l yeast extract, 10 g/l NaCl, 7.0 initial pH and incubation temperature of 37°C; (b) the effect of carbon source on biomass. The minimal medium contained 10 g/l peptone, 5 g/l yeast extract, 10 g/l NaCl, 7.0 initial pH and incubation temperature of 37°C

improves *E. coli* growth, but little is known about the mechanism of using glycerol as carbon source to enhance mass growth. Some other reports also found

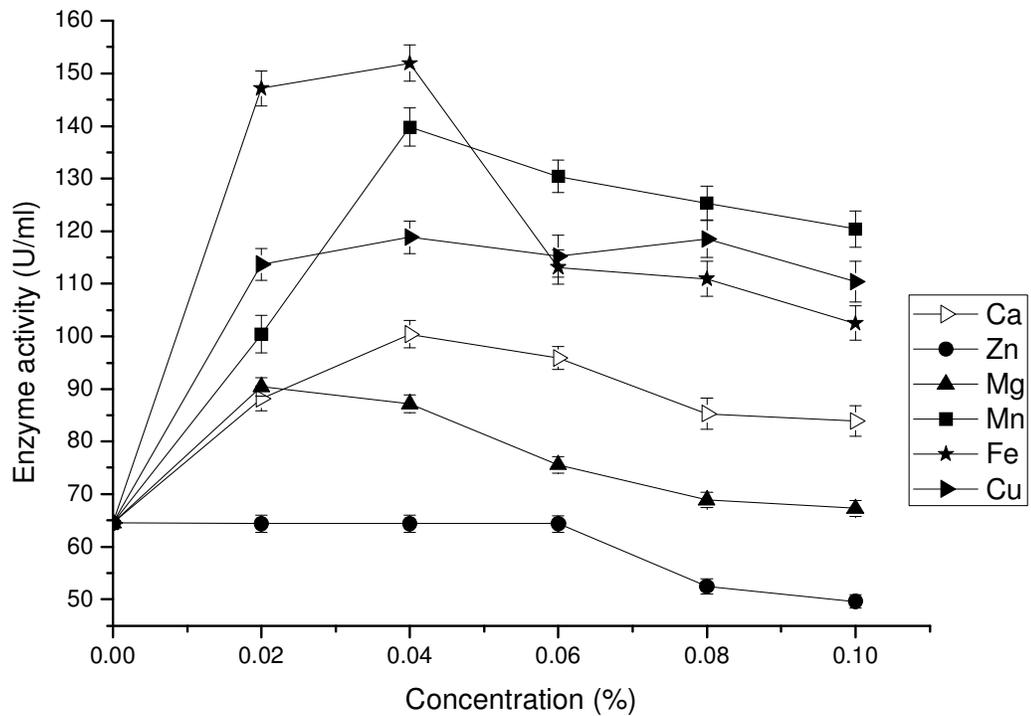
that glycerol as carbon source could improve recombinant protein expression (Luo et al., 2006). Interestingly, we observed that glucose caused a high



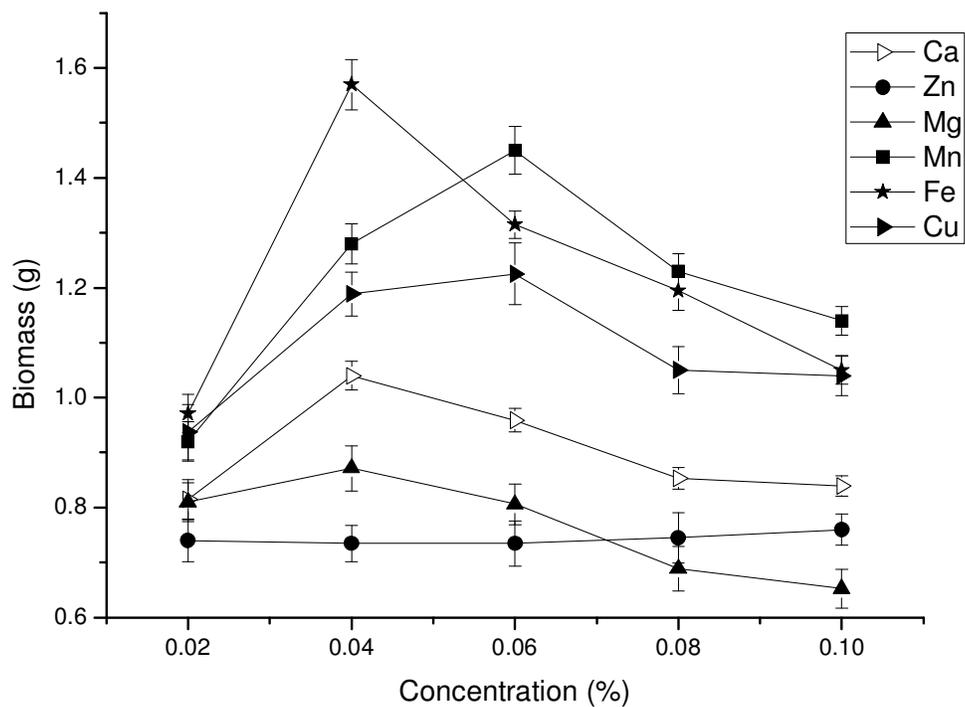
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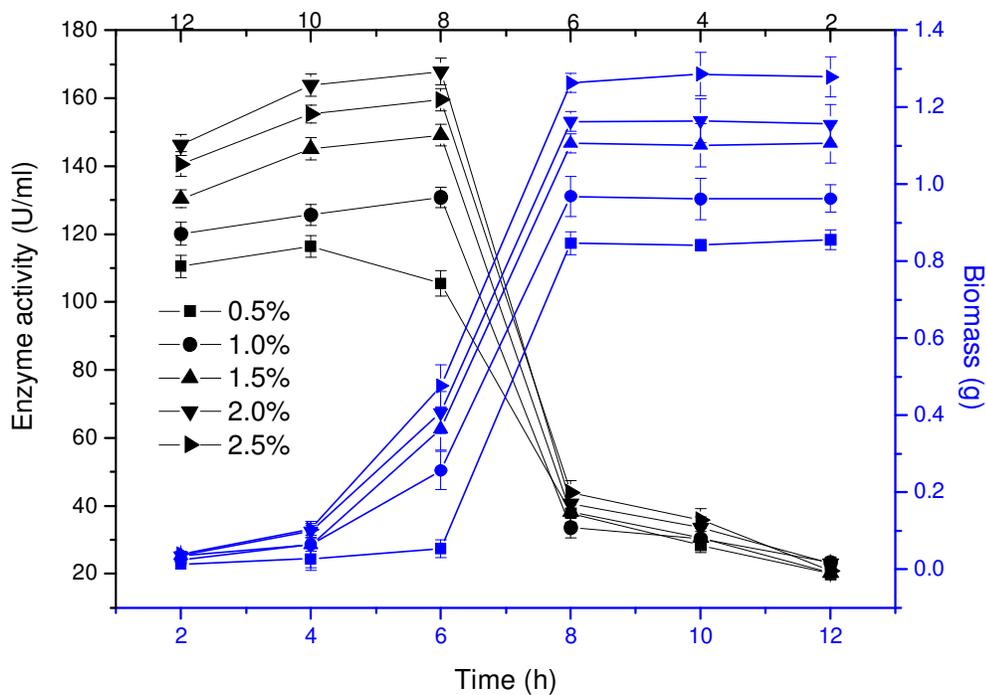
**Figure 3.** (a) The effect of nitrogen source on 4- $\alpha$ -GTase production. The medium contained 10 g/l peptone, 5 g/l yeast extract, 10 g/l NaCl, 20 g/l glycerol, 7.0 initial pH and incubation temperature of 37°C; (b) the effect of nitrogen source on biomass. The medium contained 10 g/l peptone, 5 g/l yeast extract, 10 g/l NaCl, 20 g/l glycerol, 7.0 initial pH and incubation temperature of 37°C.



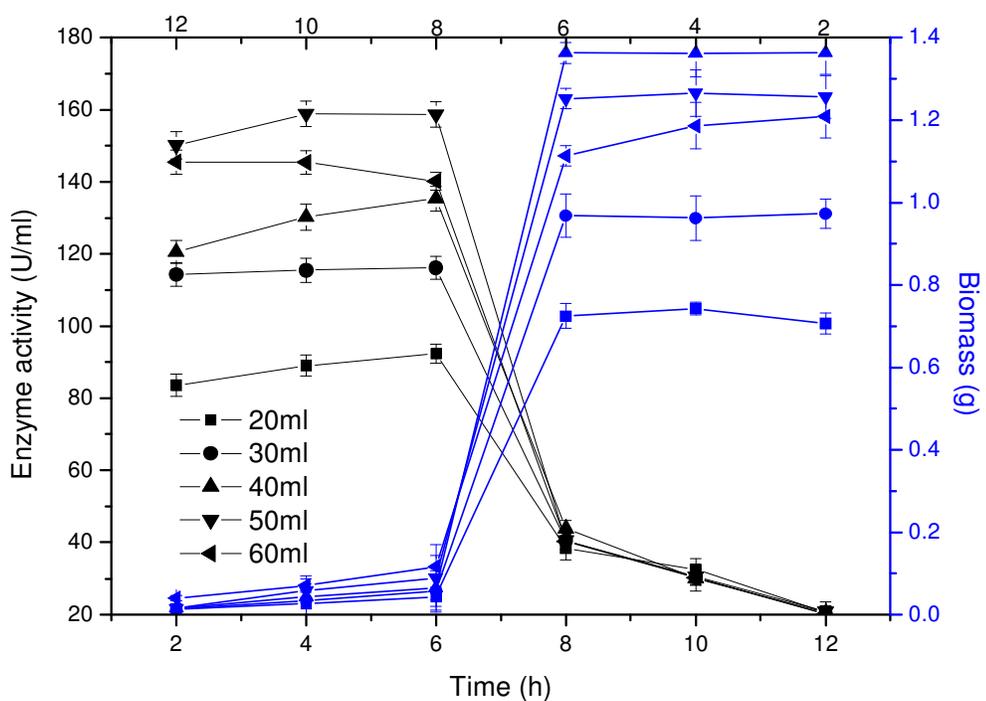
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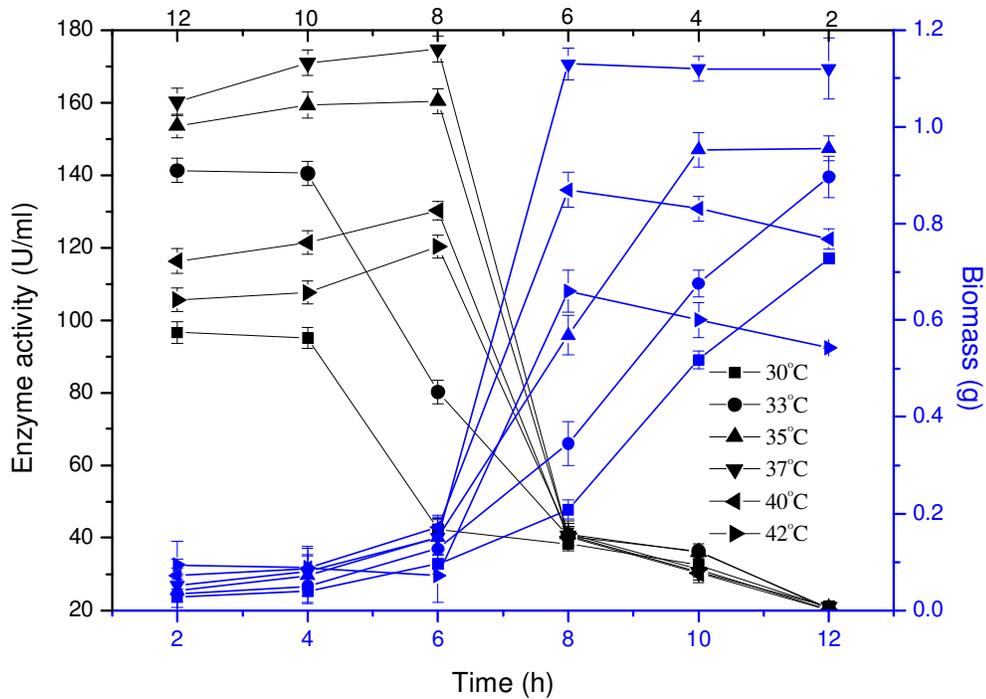
**Figure 4.** (a) The effect of trace elements on 4- $\alpha$ -GTase production. The medium contained 10 g/l peptone, 20 g/l beef extract, 10 g/l NaCl, 20 g/l glycerol, 7.0 initial pH and incubation temperature of 37°C; (b) the effect of trace elements on biomass. The medium contained 10 g/l peptone, 20 g/l beef extract, 10 g/l NaCl, 20 g/l glycerol, 7.0 initial pH and incubation temperature of 37°C.



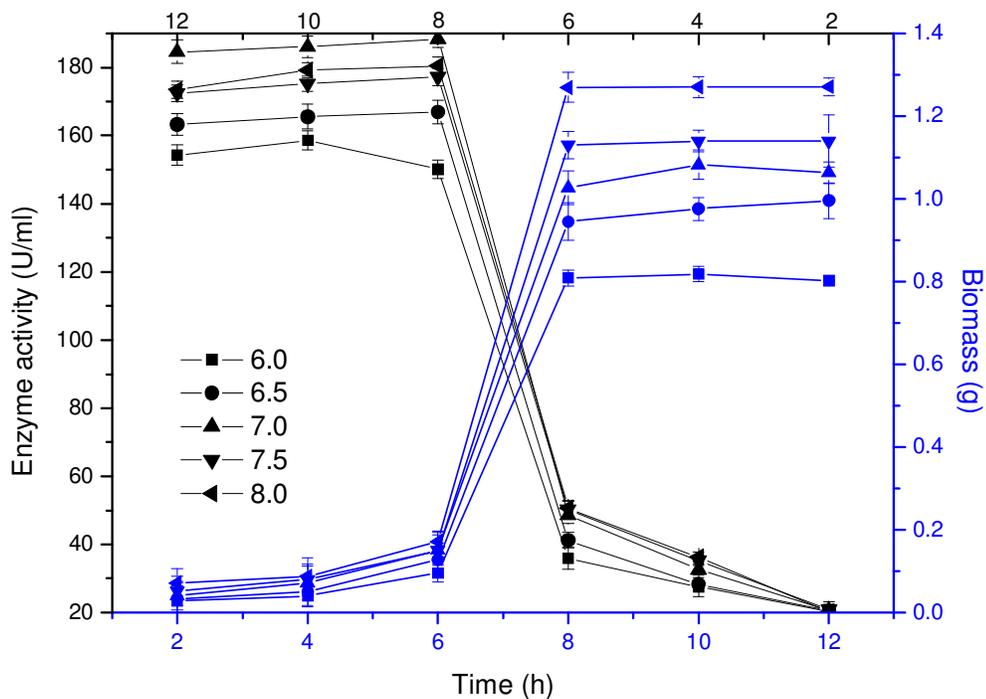
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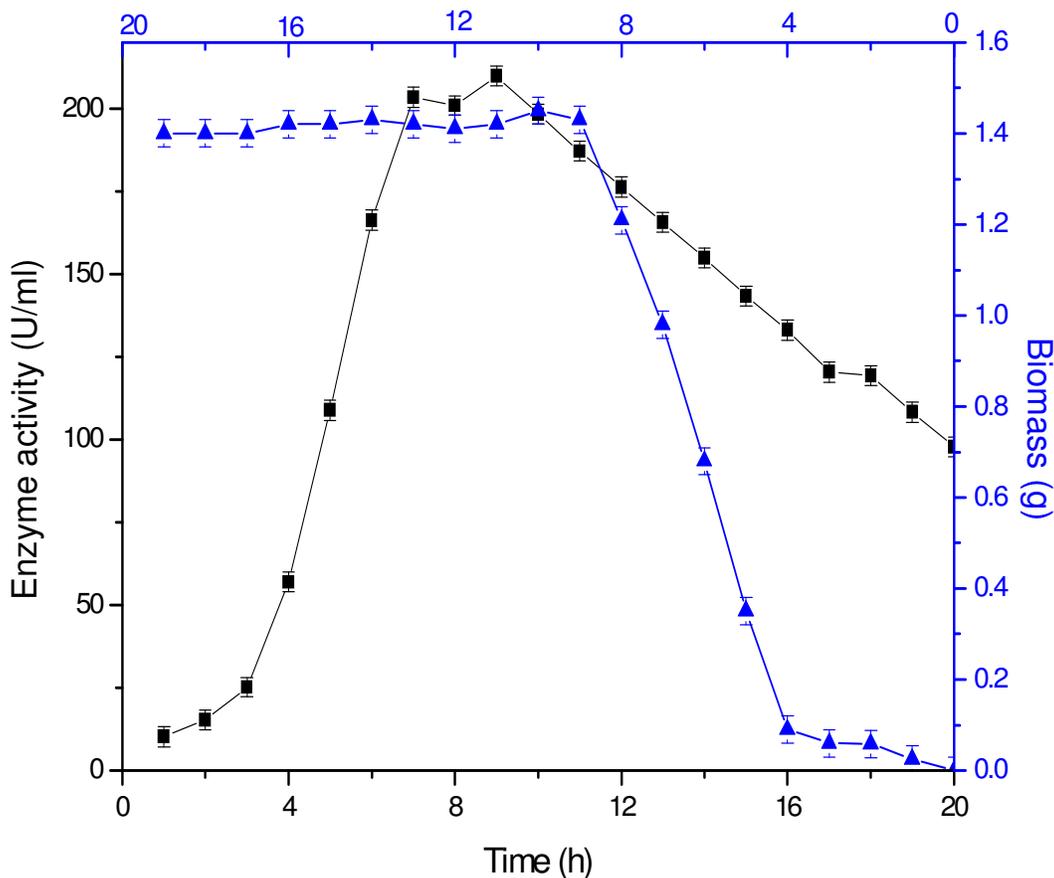
**Figure 5.** (a) Inoculum size on 4- $\alpha$ -GTase production and biomass. The medium contained 10 g/l peptone, 20 g/l beef extract, 10 g/l NaCl, 20 g/l glycerol, 0.4 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 7.0 initial pH and incubation temperature of 37°C; (b) liquid volume on 4- $\alpha$ -GTase production and biomass. The medium contained 10 g/l peptone, 20 g/l beef extract, 10 g/l NaCl, 20 g/l glycerol, 0.4 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 7.0 initial pH and incubation temperature of 37°C.



A



**Figure 6.** (a) Temperature on 4- $\alpha$ -GTase production and biomass. The medium contained 10 g/l peptone, 20 g/l beef extract, 10 g/l NaCl, 20 g/l glycerol, 0.4 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 7.0 initial pH; (b) pH on 4- $\alpha$ -GTase production and biomass. The medium contained 10 g/l peptone, 20 g/l beef extract, 10 g/l NaCl, 20 g/l glycerol, 0.4 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 7.0 initial pH and incubation temperature 37°C.



**Figure 7.** Time course of 4- $\alpha$ -GTase production and biomass in 5 L fermentation. The medium contained 10 g/l peptone, 20 g/l beef extract, 10 g/l NaCl, 20 g/l glycerol, 0.4 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , an incubation temperature of 37°C and initial pH of 7.0.

increase of biomass but with a low level of 4- $\alpha$ -GTase, we deduced that different stages existed between 4- $\alpha$ -GTase production and *E. coli* growth and the collection time (10 h) was probably not the maximum 4- $\alpha$ -GTase production time when glucose was the carbon source. Further, the metabolite acetate from glucose interferes with strain growth and inhibits protein formation (Rozkov, 2001). In the case of starches, higher biomass was obtained with the addition of more starch concentration. This biomass was probably not accurate because of the starches' insolubility and high viscosity properties that make final cell mixed together with starches. The medium containing insoluble starches was oyster white suspension, which was not good for *E. coli* growth. It is presumed probably that starch degrading products could be used as carbon source for cell growth (Tonkova, 1998).

Organic nitrogen source promoted both growth of *E. coli* and 4- $\alpha$ -GTase production, however, down trends

were observed with excessive organic nitrogen source addition, which was attributed to the excessive metabolite of amino acid from organic nitrogen. Although, much lower biomass when grew in inorganic nitrogen source than in organic source were found, but increasing *E. coli* biomass and 4- $\alpha$ -GTase production with increasing inorganic nitrogen source concentration were found. The most effective nitrogen source for *E. coli* growth and 4- $\alpha$ -GTase production was beef extract, rather than yeast extract, but no significantly differences were found. From the analysis of carbon source and nitrogen source, it was clear that the 4- $\alpha$ -GTase production was not only determined by *E. coli* growth, but also by metabolic process. Trace element has been known to change the metabolism of strains (Yang et al., 2003). In this study,  $\text{Fe}^{2+}$  provided a significantly positive effect for enzyme production and biomass increase. It is speculated that  $\text{Fe}^{2+}$  as the prosthetic group of dehydrogenase of nico-

tinamide adenine dinucleotide (NADH) plays an important role in electron transport for the respiratory chain and the addition of  $\text{Fe}^{2+}$  results in improved ATP for the strain growth, but an iron overload would generate highly toxic oxygenated radicals which inhibit strain growth. This speculation was supported by Rainnie and Bragg (1973).

### Fermentation conditions on the 4- $\alpha$ -GTase production

Fermentation conditions including temperature, pH, inoculum size and liquid volume were of important influences for recombinant protein production in *E. coli*. The growth of *E. coli* under various conditions was out of sync with the recombinant protein production. It was speculated that these fermentation conditions were to be regulatory factors for *E. coli* metabolism.

For example, much inoculum size resulted in shortened lag phase, but too much inoculum size was a burden for cell metabolism because of the competition with a synthesis of normal proteins for ribosomes, charged tRNAs and precursors (Sørensen and Kusk 2005). Also, more medium provided much nutrition, but too much medium in the Erlenmeyer decreased the soluble oxygen and increased the metabolite to decrease the *E. coli* growth and so do the 4- $\alpha$ -GTase production. Temperature can influence the recombinant protein synthesis rate and folded form in inclusion bodies, but no temperature differences for *E. coli* growth and 4- $\alpha$ -GTase production were found in this study. Slight pH differences between *E. coli* growth (pH 8.0) and 4- $\alpha$ -GTase production (pH 7.0) were found. Periplasm is permeable to relatively small molecules and therefore, must have pH equal or at least close to extracellular (Baneux et al., 1991). We also noticed the decrease in medium pH during the whole fermentation (data not shown), which could be the reason for low enzyme production as well. Thus, a proper alkaline pH was beneficial for biomass accumulation. It would be valuable and interesting to find the mechanism of relationships between recombinant protein and *E. coli* metabolites for further study.

### 4- $\alpha$ -GTase production in fermenter

Startline for 4- $\alpha$ -GTase production in 5 L fermenter gave satisfactory 4- $\alpha$ -GTase activity (198.7 U/ml). Compared with the results from flask shake scale, the yield of enzyme production was higher and the time of lag phase was shorter than the incubation time in the shake flasks, which were attributed to the difference of fermentation parameters in a scale-up of the fermentation process, such as dissolved oxygen, cell viability, metabolite and biomass. Nevertheless, selection of nutrition and fermentation conditions in flask shake scale provided valuable information for future cell growth kinetics and protein expression kinetics study in bioreactor, which would help to enhance cell density and to generate fed batch

cultivation model.

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