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Fed-Batch Cultivation and Adding Supplements to Increase Yield of β-1,3-1,4-Glucanase by Genetically Engineered *Escherichia coli*

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Abstract: The aim of this study was to analyze the major influence factors of culture medium on the expression level of β-1,3-1,4-glucanase, and to further develop an optimized process for the extracellular production of β-glucanase at a bioreactor scale (7 L) with a genetically engineered *Escherichia coli* (*E. coli*) JM109-pLF3. In this study, batch cultivation and fed-batch cultivation including the constant rate feeding strategy and the DO-stat (DO: Dissolved Oxygen) feeding strategy were conducted. At a 7 L bioreactor scale for batch cultivation, biomass reached 3.14 g/L and the maximum β-glucanase activity was 506.94 U/mL. Compared with batch cultivation, the addition of glycerol, complex nitrogen and complete medium during fed-batch cultivation increased the production of biomass and β-1,3-1,4-glucanase. The maximum biomass and β-glucanase activity, which were 7.67 g/L and 1680 U/mL, respectively, that is, 2.45 and 3.31 times higher than those obtained with batch cultivation, were obtained by feeding a complex nitrogen source at a constant rate of 1.11 mL/min. Therefore, these nutritional supplements and strategies can be used as a reference to enhance the production of other bioproducts from *E. coli*.

Keywords: β-1,3-1,4-glucanase; genetically engineered *Escherichia coli*; bioreactor; fed-batch cultivation; batch cultivation

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1. Introduction

β-1,3-1,4-glucans are non-starch polysaccharides formed by D-glucose polysaccharides linked by β-(1,3) and β-(1,4) glycosidic bonds. They are abundant in plant cell walls, such as barley and wheat, and significantly affect the efficiency of brewing, animal feed and related industries [1–3]. During brewing, the extraction and filtration rates will be limited by a large amount of soluble β-glucans, mainly β-1,3-1,4-glucans, due to the increased viscosity of solubilization. Moreover, the existing β-glucans will lead to gelatinous precipitates in the finished beer, thus affecting the taste and color of the beer [3,4]. Meanwhile, in animal feed and related industries, the increase in the digesta viscosity of animal food by β-glucans will influence the animal's digestion and absorption of the nutriment, because the increased viscosity will affect the digestion of targeted substrates by endogenous digestive enzymes [5]. Therefore, β-1,3-1,4-glucanases, which are able to hydrolyze the β-1,3-1,4-glucans, play an important role in reducing the negative effects caused by β-1,3-1,4-glucans, and are widely used in many applications [6–8]. For example, β-1,3-1,4-glucanases are often added to the malt during brewing to obtain good quality brewers' malt [4,6]. In addition, in animal feedstuff, especially for broiler chickens and piglets, β-1,3-1,4-glucanases help to reduce the diet viscosity, and thus improve nutrient digestibility and feed intake [8]. The application of β-glucanases also stimulates the development of certain probiotics [9] and enhances the efficiency of paper production [10] and biofuel production [11].

β-1,3-1,4-glucanases are usually obtained in plants, bacteria and fungi [8,12]. Among them, the fermentation of cellulolytic fungi, such as *Paecilomyces thermophile* [13], *Penicillium occitanis* [14], and *Penicillum pinophilum* [15], has been widely employed to produce exogenous β-1,3-1,4-glucanases, because fungi are able to secrete extracellular proteins and therefore easier and milder purification processes are required than with cytoplasmic proteins. However, compared with bacteria, the fermentation of fungi takes a much longer time, and the processes are more cumbersome, which limits its industrial application. Developing a facile way that enables bacteria to secrete periplasmic proteins would provide a solution to these problems. As reported by Miksch et al. [16], a novel secretion cassette named *kil*-km was used to overexpress extracellularly the recombinant *Bacillus*-derived β-glucanase. The recombinant *Bacillus* was transfected with a plasmid, which contained a *kil*-km secretion cassette and a gene encoding hybrid glucanase. As the bacterioncin release protein (BP) was produced by the expressed kil gene in the secretion cassette, β-glucanase would be released into the culture. The *kil*-km secretion cassette was employed to produce β-1,3-1,4-glucanases in *E. coli* by Miksch et al. as well, with the secretion cassette and β-1,3-1,4-glucanases from *Bacillus* being located in a recombinant plasmid pLF3 [16]. Later on, Lu et al. optimized the production culture medium for the genetically engineered *E. coli* constructed by Miksch as mentioned above, and the activity of β-1,3-1,4-glucanases in the optimized culture medium was 297.71 U/mL, which was a 14-fold increase when compared with the enzyme activity obtained in the original medium [17]. However, the investigation of Lu et al. on the culture medium components affecting the activity of β-1,3-1,4-glucanases was performed in 250 mL flasks, which were unsuitable for industrial applications; as such, studies on optimizing culture in scale-up bioreactors before industrialization will be necessary.

Therefore, the main purpose of this study was to analyze the major influence factors of culture medium on the expression level of β-glucanase, and to further develop an optimized process for the extracellular production of β-glucanase in bioreactors with the recombinant *E. coli* as the host organism.

2. Results and Discussion

2.1. Batch Cultivation in Bioreactor

Lu et al. optimized the medium and conditions for JM109-(pLF3) to produce β-1, 3-1, 4-glucanase in the previous shake flask scale study [17]. In this study, batch cultivation experiments were conducted using the optimized medium by Lu et al., and under conditions of 39 °C and pH 7.0. Moreover, the stirrer speed was controlled at 400 r/min, and the airflow was kept constantly at 5 L/min (1 vvm). The DO (dissolved oxygen), biomass, β-glucanase activity, glycerol concentration, and pH were monitored, and the results are shown as Figure 1. As can be observed, it took 36 h for the cell density to reach the maximum value of 3.14 g/L, and 51 h for maximal β-glucanase activity and relative β-glucanase activity to reach the maximum values of 506.94 U/mL and 181.38 U/mg, respectively. The curves of the biomass and DO indicated that JM109-(pLF3) underwent diauxic growth during fermentation, and the first and second logarithmic growths were started at after about 3 h and 27 h of fermentation, respectively. The diauxic lag period between the two logarithmic growths was started at around 12 h of fermentation and terminated as the second logarithmic growth started. The reason for this phenomenon may be due to the different kinds of carbon and nitrogen sources present in the medium, with *E. coli* growing preferentially on some kinds of carbon and nitrogen sources until they were exhausted, resulting in arrested growth from 12 h to 27 h of fermentation while the cells adjusted to growth on different carbon and nitrogen sources [18,19]. During the diauxic lag period, the oxygen consumption for cell growth and maintenance decreased, thus resulting in the incremental DO concentration. Afterwards, the DO decreased obviously as the second period of logarithmic growth started from 27 h of fermentation, and then increased with the arrestment of cell growth and finally reached saturation values. Furthermore, Figure 1 also reveals that the β-1,3-1,4-glucanase activity increased significantly during the second

logarithmic growth phase and reached the maximum value of 506.94 U/mL after about 51 h of fermentation. In the optimized enzyme production medium, glycerol was one of the carbon sources. According to Figure 1, glycerol decreased quickly from 6 g/L to about 1.35 g/L from the beginning to about 35 h of fermentation, indicating a quick consumption of nutrients during this period. In addition, the glycerol was almost used up at about 45 h of fermentation, which was consistent with the slowdown of cell growth, implying nutrient limitation in the later stage of fermentation, and that glycerol was an important carbon resource. Meanwhile, during the fermentation, the medium was under free pH conditions and was kept stable at about 6.8–7.3. Therefore, the pH was also freely controlled for the follow-up experiments in this study.

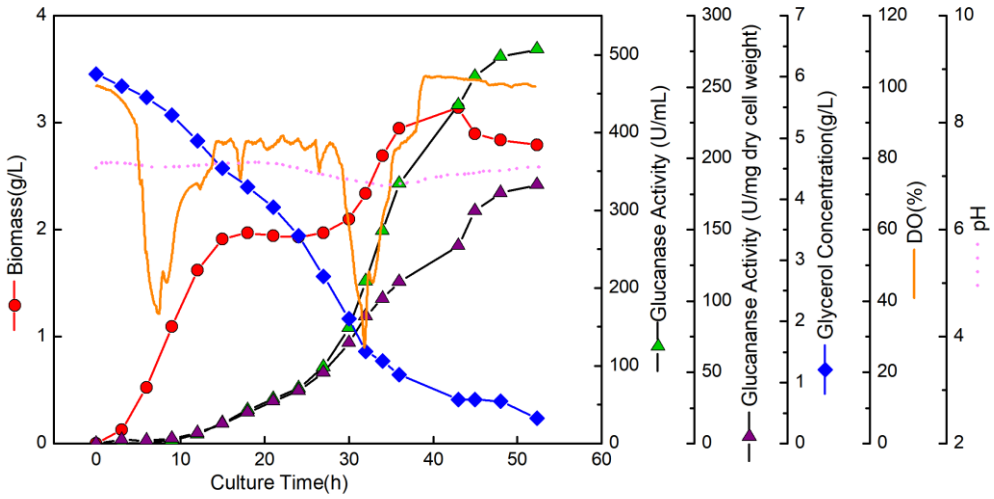

Figure 1. Time profile showing biomass, DO (dissolved oxygen), pH, and β-glucanase activity towards culture supernatant volume and dry cell weight, respectively, of batch fermentation of recombinant *E. coli* JM109-(pLF3) in a 7 L Braun bioreactor in an optimized enzyme production medium.

2.2. Fed-Batch Cultivation

Fed-batch cultivation is a useful strategy for increasing biomass and product concentration during fermentation and is widely applied in industry. Based on the batch cultivation results, during the diauxic lag period between the two logarithmic growths, two feeding strategies were applied as the constant feeding strategy and the DO-stat control feeding strategy, including the feeding of glycerol, total optimized medium and complex nitrogen source.

2.2.1. Constant Feeding Strategy

Constant feeding strategy was performed by feeding 12 g/L glycerol solution, a complex nitrogen source, with 24 g/L yeast extracts and 14.42 g/L $NaNO_3$, or twice concentrated complete medium, into the bioreactor, respectively, with a constant feeding rate of 1.11 mL/min after 12 h of fermentation. The total volume of all the nutritional supplements was 1 L.

Figure 2a–c shows biomass and β-glucanase activity in the culture medium during cultivation constantly fed with glycerol solution, complex nitrogen source or twice-concentrated complete medium, respectively. The results indicated that compared with batch cultivation, the diauxic lag period of JM109-(pLF3) would be shortened by constantly feeding with any one of the three nutritional supplements. Moreover, the biomass and β-glucanase activity were enhanced, especially when feeding with a complex nitrogen source. The maximum biomasses as a result of the constant feeding of glycerol solution, complex nitrogen source and twice-concentrated complete medium were 4.87 g/L, 7.67 g/L and 4.06 g/L, respectively. The maximum β-glucanase activities and relative β-glucanase

activities were reached at the end of fermentation and were 552.18 U/mL and 117.51 U/mg of glycerol feeding; 1680 U/mL and 221.07 U/mg of complex nitrogen source feeding; and 673.79 U/mL and 179.25 U/mg of twice-concentrated complete medium feeding. Correspondingly, the β-glucanase activities of the three nutritional feedings were increased by 8.92%, 231.40% and 32.91%, respectively, when compared with that of batch cultivation, which was 506.94 U/mL. The highest biomass and β-glucanase activity were obtained by complex nitrogen source feeding, which may be attributed to the ratio of C (carbon source)/N (nitrogen source) in the culture decreasing when a complex nitrogen source was fed into the culture, as a lower ratio of C/N would promote the expression of foreign proteins in recombinant *E. coli*. Furthermore, the results indicate that the nitrogen source was an important limitation factor during the cultivation of JM109-(pLF3) for producing β-glucanase as well. As reported previously [19–21], bacteria typically undergo intermittent periods between nitrogen starvation and adaptation to a different nitrogen source. Atkinson et al. found that the nitrogen consumption of *E. coli* was controlled by nitrogen-regulated genes and operons of the nitrogen regulated (Ntr) regulon. Transient growth arrest during the change to a a different nitrogen source was thus attributed to the activation of different regulated systems [19,21]. In this study, there are two kinds of nitrogen sources in the culture medium. *E. coli* showed diauxic lag during batch cultivation as in Section 2.1, and this diauxic lag was shortened through constant feeding with complex nitrogen sources. Therefore, it could be inferred that the efficiency of using different nitrogen sources may be responsible for the diauxic growth of JM109-(pLF3) during batch cultivation, and that increasing the cell density would be an efficient way to raise the yield of β-glucanase.

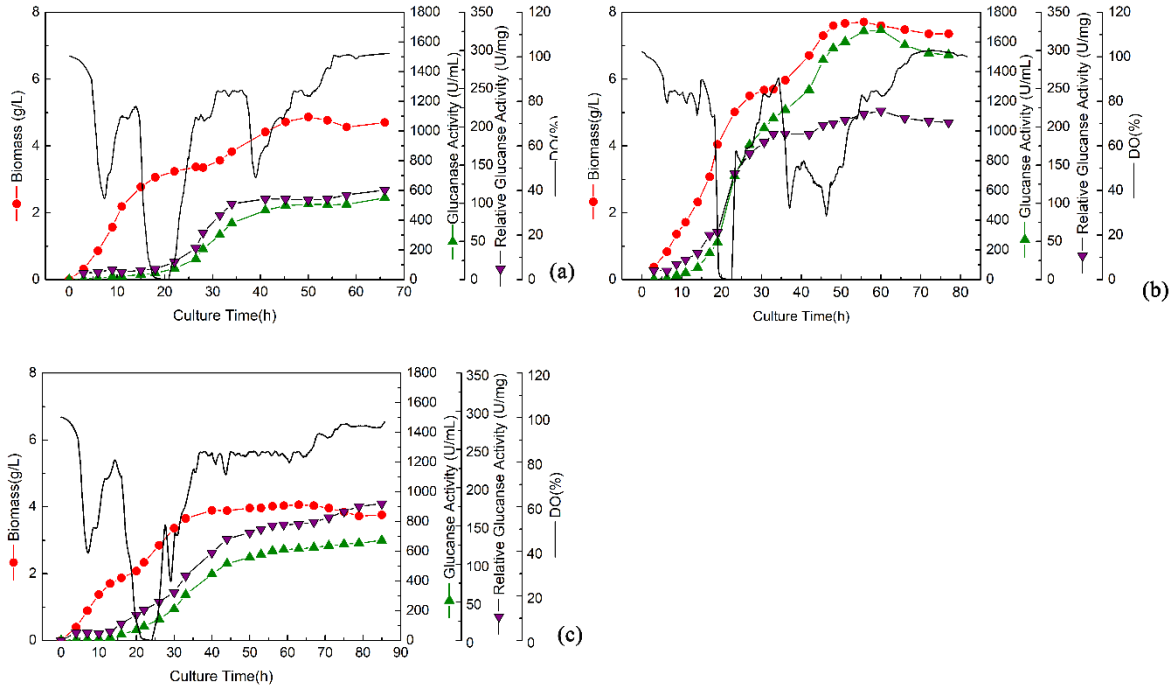

Figure 2. Time profile showing the biomass, DO and β-glucanase activity of recombinant *E. coli* JM109-(pLF3) in a 7 L Braun bioreactor by constantly feeding glycerol (**a**), complex nitrogen source (**b**) and complete medium (**c**), respectively.

2.2.2. DO-stat Feeding Strategy

The biomass and glucanase activities were enhanced by the constant feeding strategy, while the diauxic lag phase was shortened as well. However, during feeding with these three kinds of nutritional supplements, DO shortage appeared as a limiting factor. In order to solve the limitation, a DO-stat feeding strategy was applied from 12 h to 30 h of

fermentation, and the DO was controlled at between approximately 30 and 50% in this period. When the DO was above 50%, the nutritional supplements were fed separately at a rate of 1 mL/min, and when the DO was below 30%, the stirring speed was increased from 400 r/min to 600 r/min.

The time courses of biomass, β-glucanase activity and relative β-glucanase activity in the culture medium, keeping the balanced DO-stat by controlling stirring speed and feeding 12 g/L glycerol, a complex nitrogen source with 24 g/L yeast extracts and 14.42 g/L $NaNO_3$, or twice-concentrated complete medium, are shown in Figure 3a–c respectively. As shown in Figure 3a, when the DO of the culture medium was kept by feeding glycerol and controlling stirring speed, a reduced diauxic lag phase was observed and the maximum values of biomass, β-glucanase activity and relative β-glucanase activity were 3.84 g/L, 685.66 U/mL and 182.46 U/mg, respectively. The maximum values of biomass, β-glucanase activity and relative β-glucanase activity were 7.30 g/L, 1538.98 U/mL and 192.21 U/mg, respectively, from complex nitrogen source feeding (Figure 3b), and 5.45 g/L, 709.61 U/mL and 179.12 U/mg, respectively, from twice-concentrated complete medium feeding (Figure 3c). It can be noted that the maximum values of biomass and β-glucanase activity of the DO-stat control feeding strategy were obtained through complex nitrogen source feeding, which were the same as in feed batch cultivation. Therefore, it could be deduced that for both feeding strategies in this study, complex nitrogen source feeding was the most efficient way to enhance the biomass and enzyme activity. Compared with the constant feeding strategy when feeding with glycerol or complete medium, higher β-glucanase activities were achieved through the DO-stat control feeding strategy. On the contrary, the maximum values of biomass and β-glucanase activity of complex nitrogen source feeding with a balanced DO-stat were lower than those of the constant feeding strategy. The reason for this phenomenon may be due to the fact that the metabolism of the carbon source would be limited by insufficient oxygen in the culture, while the metabolism of the nitrogen source would not, and the DO-stat control feeding strategy provides sufficient oxygen, benefiting the growth of cell density. Meanwhile, as reported [22,23], the cell density of *E. coli* would be enhanced by using a feeding medium with a relatively high ratio of C towards N, as cell wall synthesis requires a sufficient carbon source. However, in feeding a nitrogen source in the DO-stat control feeding strategy, a large amount of the nitrogen source accumulated in the medium; therefore, the ratio of C towards N decreased and the growth of biomass was consequently limited.

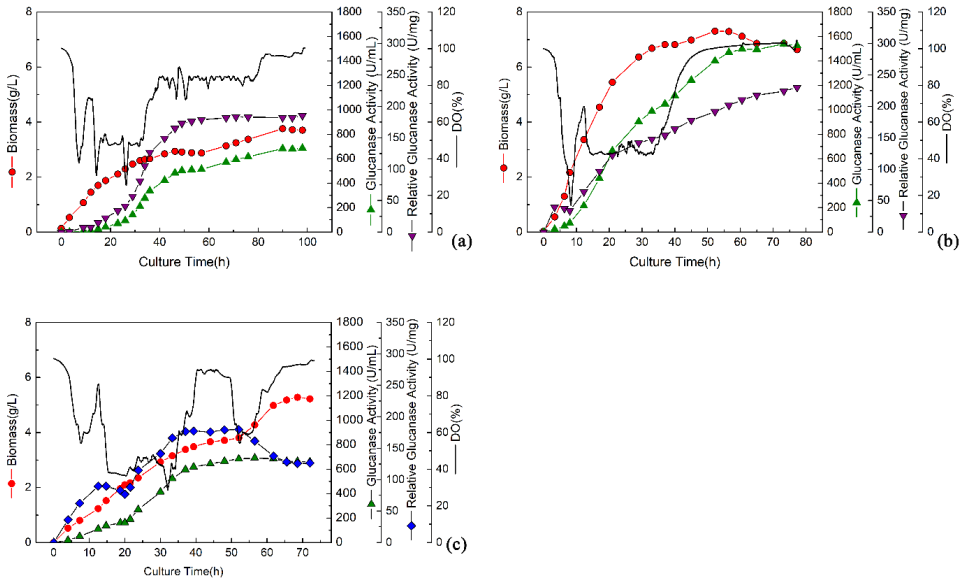

Figure 3. Time profiles showing biomass, DO and β-glucanase activity of recombinant *E. coli* JM109-(pLF3) in a 7 L Braun bioreactor by feeding glycerol (**a**), a complex nitrogen source (**b**) and a complete medium (**c**), respectively, at the balanced DO-stat.

3. Materials and Methods

3.1. Bacterial Strain and Plasmid

Genetically engineered *E. coli* JM109 (JM109- pLF3) with plasmid pLF3, as mentioned above, was donated by the research group of Professor Flaschel, Department of Fermentation Engineering, Faculty of Technology, University of Bielefeld, Bielefeld, Germany. The construction of the pLF3 plasmid was described elsewhere [24]; briefly, pLF3 contained the gene encoding a hybrid *Bacillus* β-1,3-1,4-β-glucanase (*bgl*), and the *kil* gene from the *E. coli*. The *bgl gene* contained the gene encoding 107 amino-terminal residues of the *Bacillus amyloliquefaciens* and 107 carboxy-terminal amino acid residues of the *B. macerans* β-glucanase, and the gene would be expressed by the natural promoter of the *B. amyloliquefaciens* β-glucanase. The extracellular hybrid β-glucanase produced by this genetically engineered *E. coli* JM109 exhibits improved thermostability, especially in acidic environments.

3.2. Reagents and Medium

Yeast extract and bacteriological peptone were obtained from Oxoid (Basingstoke, UK). All other reagents were of analytical grade and purchased from Sigma (St. Louis, MI, USA) or Merck (Darmstadt, Germany) unless specified otherwise.

For general purpose, bacterial cells were grown at 37 °C in LB medium, which contains 5 g/L yeast extract, 10 g/L peptone, 10 g/L NaCl. The culture medium optimized by Lu et al. was used for enzyme production studies as a complete medium [17], the optimized enzyme production medium contains 12 g/L yeast extract, 10 g/L NaCl, 7.21 g/L $NaNO_3$, 16 g/L glycerol, 2.4 g/L KH_2PO_4 and 12.5 g/L K_2HPO_4, and the pH was adjusted to 7.0 with NaOH and H_3PO_4. In both media, kanamycin (50 μg/mL) was added for bacterial selection as the pLF3 contains the kanamycin resistance gene.

When required, the media were solidified with 1.5% agar.

3.3. Batch and Fed-Batch Cultivation of JM109-(pLF3)

Batch and fed-batch cultivation were conducted in a 5 L working volume in a 7 L Braun stirred bioreactor (Biostat® B5, B. Braun Biotech International, Berlin, Germany) with temperature and pH control and dissolved oxygen monitoring.

JM109-(pLF3) kept in a glycerol tube at −80 °C was inoculated in a 100 mL optimized medium in shake flasks (500 mL) at 39 °C with a rotational speed of 150 min^{-1} for 16 h. Then, the inoculum with JM109-(pLF3) was added to the reactor containing sterilized optimized medium at a ratio of 1:100 for batch and fed-batch cultivation. The values for the speed of the agitator and airflow were 400 r/min and 5 L/min, respectively, and the temperature was kept at 39 °C for batch cultivation. Fed-batch cultivation was conducted with two different feeding strategies: the constant feeding strategy and the DO-stat control feeding strategy (DO: dissolved oxygen). For both feeding strategies, the cultivation medium and temperature were the same as for batch cultivation. For the constant feeding strategy, 12 g/L of glycerol solution, a complex nitrogen source (12 g/L yeast extract and 7.21 g/L $NaNO_3$) or twice-concentrated complete medium, were fed to the bioreactor with a constant feeding rate of 1.11 mL/min after 12 h fermentation, and the total volume for all of the nutritional supplements was 1 L. For the DO-stat control feeding strategy, the DO was controlled at between approximately 30 and 50% by the feeding of nutritional supplements with a rate of 1 mL/min when the DO was above 50%, or increasing the stirring speed from 400 r/min to 600 r/min when the DO was below 30%.

3.4. Analysis Methods

3.4.1. Determination of Biomass Concentration

Optical density measurement (UV310 UV/Visible, Shimadzu, Kyoto, Japan) at 600 nm (OD_{600}) was employed to monitor the bacterial growth. The biomass' dry weight was estimated from the OD_{600} of culture according to the predetermined correlation between OD_{600} and dry weight. The OD_{600} of each sample was measured three times and the experiments were performed twice, thus the data presented are the means of the six values.

3.4.2. Determination of β-Glucanase Activity

β-Glucanase activity in culture medium was determined by Miller's method, with little modification [25]. The culture supernatant with β-1,3-1,4-glucanase was collected by centrifugation (4000× *g*, 5 min, Centrifuge 5810R, Eppendorf, Hamburg, Germany) and properly diluted with a sodium acetate buffer before testing the enzyme activity. The activity of β-1,3-1,4-glucanase was measured by dinitrosalicylic acid (DNS) method at 50 °C with 50 μL of diluted culture supernatant and 1 mL of 1% lichen polysaccharide reacting for 10 min. Then, 0.5 mL DNS was added and the mixture was boiled for 10 min, the absorbance was measured at 540 nm immediately, and glucose was used as the standard. Three parallel samples were taken for a scheduled time to test the enzyme activity and relative enzyme activity, and each experiment was performed at least twice. One unit (U) of β-Glucanase activity and one unit (U) of relative β-Glucanase activity were defined as the amount of enzyme culture supernatant and dry cell mass, respectively, that released 1 μmol of D-glucose per minute at the condition of 50 °C pH 6.0. The relative glucanase activities (U/mg DCW) were calculated by dividing the glucanase activity (U/mL) by the biomass (g/L).

3.4.3. Glycerol Concentration Measurement

In order to measure glycerol concentration in the culture medium, the culture samples were centrifuged at 4000× *g* for 5 min (Centrifuge 5810R, Eppendorf, Hamburg, Germany) and water was used as a control. Then, the supernatant was vortex mixed with 1.5 mL 0.02 mol/L KIO_4, followed by adding 1 mL 0.24 mol/L KI solution and 1 mL 0.24 mol/L HCl solution. 0.05 mol/L $Na_2S_2O_3$ solution was used for titration calibration and 400 μL starch solution was used as indicator when approaching the termination point. The concentration of glycerol was calculated by Equation (1). The data presented are the means of the three values.

$$\text{Glycerol content} = (V_1 - V_2) \times CE/4V_3 \quad (1)$$

V_1: Volume of $Na_2S_2O_3$ consumed by the control sample, mL
V_2: Volume of $Na_2S_2O_3$ consumed by the sample, mL
C: Concentration of $Na_2S_2O_3$, mol/L
V_3: Volume of the culture, mL
E: Relative molecular weight of glycerol

4. Conclusions

In this study, at the bioreactor scale (7 L), batch fermentation and fed-batch fermentation including the constant rate feeding strategy and the DO-stat feeding strategy were conducted to enhance β-glucanase production. Under batch fermentation, the maximum biomass achieved was 3.14 g/L and the maximum β-glucanase activity was 506.94 U/mL. The biomass and β-glucanase activity were enhanced by fed-batch fermentation. During the constant rate feeding strategy, the addition of glycerol, a complex nitrogen source and a complete medium at a constant rate increased biomass production to 4.87 g/L, 7.67 g/L and 4.06 g/L, respectively. Meanwhile, β-glucanase activity was increased to 552.18 U/mL, 1680 U/mL and 673.79 U/mL, respectively. During the DO-stat feeding strategy, the maximum biomass and β-glucanase activity obtained were respectively 3.84 g/L and 685.66 U/mL for feeding with glycerol, 7.30 g/L and 1538.98 U/mL for feeding with a complex nitrogen source, and 5.45 g/L and 709.61 U/mL for feeding with a complete medium. The highest biomass production and β-glucanase activity were obtained under fed-batch strategy by feeding a complex nitrogen source at a constant rate, indicating that the nitrogen source might be a limiting factor for biomass and β-glucanase activity in the medium, and the ratio of C to N would regulate cell growth and enzyme production. Therefore, the findings in this study may help to design the culture and processes for *E. coli* fermentation, and the nutritional supplements and strategies can be used as a reference to enhance the production of other bioproducts from *E. coli*.

Author Contributions: Conceptualization, L.Z., Z.L. and Y.L.; investigation, L.Z. and Z.L.; methodology, L.Z. and Z.L.; supervision, Y.L.; writing—original draft, L.Z. and Z.L.; writing—review and editing, Y.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data generated in this study are fully disclosed in this manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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